1953

Effect of protein materials upon the growth promoting activity of vitamin B12 for Lactobacillus leichmannii

Erma Mildred Schumacher Van der Zant
Iowa State College

NOTE TO USERS

EFFECT OF PROTEIN MATERIALS UPON THE GROWTH PROMOTING ACTIVITY OF VITAMIN B_{12} FOR *LACTOBACILLUS LEICHMANNII*

by

Erma Mildred Schumacher van der Zant

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
1953

TABLE OF CONTENTS

INTRODUCTION

In 1926, Minot and Murphy reported that liver could be used to treat persons suffering from pernicious anemia. Twenty-two years later the active curative agent, vitamin B_{12}, was isolated. This substance proved to be more important than had been expected and is one of the most potent biological substances ever discovered. It not only alleviated the symptoms of pernicious anemia but it proved to be the substance present in animal tissue, known as the "animal protein factor", which enhanced the growth promoting properties of vegetable feeds.

The discovery that vitamin B_{12} is a growth factor required for certain microorganisms resulted in the development of a very sensitive method of microbiological assay. However, two factors made the vitamin B_{12} problem more complex than studies on crystalline vitamin B_{12} would indicate. First, only parenterally administered liver extracts or vitamin B_{12} were effective in the treatment of pernicious anemia. When this vitamin was given orally, gastric juice or gastric mucosa had to be present. It is believed that there is some substance present in gastric juice which is necessary for the alleviation of pernicious anemia. Castle named this substance the "intrinsic factor", an unknown

heat-labile substance, perhaps a protein.

Secondly, although all animal tissues contain vitamin B_{12}, the assay of these protein materials is very difficult because drastic methods designed to destroy the structure of proteins must be applied to obtain solutions which can be accurately analyzed for vitamin B_{12} content. In fact, no entirely reliable method has yet been found. In bacterial assay proteins appear to bind vitamin B_{12} or interfere with its action in some way, as do substances extracted from stomach mucosa. It is not known whether there exists any relationship between the intrinsic factor and protein binding of vitamin B_{12}. However, it is to be expected that the binding of proteins by vitamin B_{12} will have significance in its metabolic functions. Vitamin B_{12} is so intimately associated with protein materials that it is doubtful that it would act as a single entity in metabolic systems. Some substance, protein in nature or closely allied to protein, may well be a part of an active vitamin B_{12} complex.

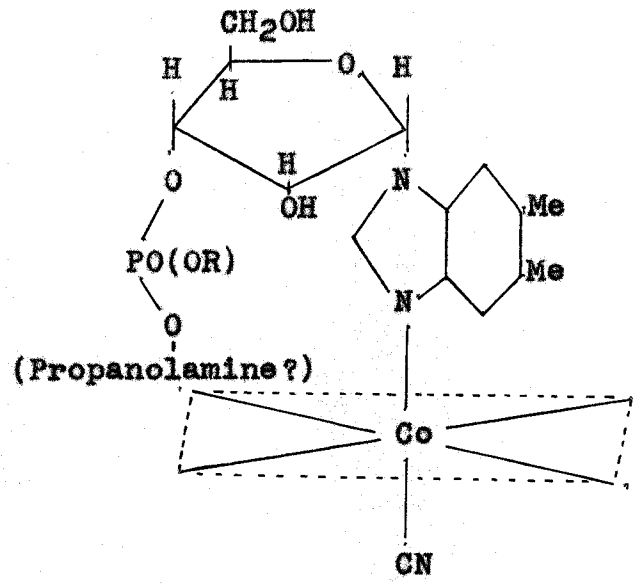
This study was initiated to gain a better understanding of the significance of vitamin B_{12} binding in tissue by studying the effects of protein materials upon the growth of Lactobacillus leichmannii in the presence of vitamin B_{12} with the hope that some information about the protein-vitamin B_{12} linkage might be obtained.

REVIEW OF LITERATURE

Chemistry of Vitamin B_{12}

The isolation of vitamin B_{12} from liver occurred simultaneously in the United States and England. In the Merck laboratories the method for determining the concentration of vitamin B_{12} in liver extracts was based upon the need of Lactobacillus lactis Dorner for the antipernicious anemia principle as a nutrient. The British scientists, who used the more difficult assay method, the treatment of pernicious anemia patients, isolated the same compound. Another aid to the isolation was the development of a characteristic red color in the solution during the purification process. The isolation and characterization of vitamin B_{12} have been discussed in several reviews (47, 66).

The structure of the vitamin B_{12} molecule has not yet been established but a structure has been proposed to explain the linkages between the products obtained by cleavage. Depending upon the conditions of acid hydrolysis there are obtained two residues of 1-amino-2-propanol, a ribotide or riboside of 5,6-dimethylbenzimidazole and 1-α-D-ribofuranose, or the free base, and a cobalt compound. These fragments may be joined together according to the following diagram (4).



Brink, Kuehl, and Folkers (16), who showed that vitamin B_{12} was a cyanide complex, suggested that the name cobalamin be given to the part of the molecule which is attached to the cyanide group. Vitamin B_{12} would then be called cyanocobalamin. The cyanide group was replaced by a hydroxyl group in acid hydrolysis (16), catalytic hydrogenation (68), or photochemical reaction (110) to give hydroxocobalamin, vitamin B_{12a}.

Vitamin B_{12b} was isolated from the fermentation liquors of Streptomyces aureofaciens and from liver (84, 85). This vitamin has now been proved to be identical with vitamin B_{12a} (67, 116). The aquocobalamin cation $[\text{cobalamin } OH_2]^{+}$ probably exists in solution since the spectral shifts observed for vitamin B_{12b} and its basic properties fail to account for a formula having only hydroxyl in place of cyanide ion (16, 17). An equilibrium between aquocobalamin

cation and hydroxocobalamin in solution depends upon the pH. The aquocobalamin cation reacts with the anions cyanide, nitrite, and thiocyanate to form non-dissociable neutral compounds. Basic ionic compounds, salts of aquocobalamin cation, are formed with sulfate and chloride. Acidic compounds contain sulfite or two cyanide ions. The latter compounds are unstable (35, 36, 69, 101, 113).

Cooley and co-workers (35) have replaced the non-ionic hydroxyl group of aquocobalamin cation by basic ligands such as ammonia or histidine. They call these compounds cobalichromes. In this type of compound may lie the answer to the combined form of vitamin B_{12} in natural materials (91, 116). In the assay of ammonia cobalichrome its growth activity varied widely with the species used. It may be of interest to mention the preparation of histidine cobalichromes (35). One mg. of DL-histidine was added to a solution containing 1 mg. vitamin B_{12b} in 250 µl. The mixture was submitted to paper chromatography using secondary butyl alcohol saturated with water as the developing solvent. Aqueous eluates of histidine cobalichrome were obtained which had a characteristic absorption spectrum and appeared to have a microbiological activity towards E. coli of the same order as vitamin B_{12}.

Compounds of vitamin B_{12} with more than two groups are known. Alicino (1) prepared an orange salt of the unchanged vitamin containing six perchlorate groups. This revealed

at least six weakly basic groups in the vitamin B_{12} molecule.

None of the degradation products of vitamin B_{12} are as active biologically as the vitamin itself. Schlindler (93) obtained a crystalline acid containing cobalt and phosphorus from vitamin B_{12} by hydrogen peroxide treatment in weakly basic solution. Its absorption spectrum was identical to that of vitamin B_{12} and it had 20 to 40 per cent of the activity of vitamin B_{12}. Beiler, Moss, and Martin (10), however, obtained a substance having inhibitory action upon the growth of L. leichmannii and which seemed to be specifically antagonistic to vitamin B_{12}. Hydrogen peroxide treatment in acid solution was used.

Dion, Calkins, and Pfiffner (46) have isolated a compound, pseudovitamin B_{12}, from cultures of a bovine rumen organism. This molecule contains adenine in place of 5,6-dimethylbenzimidazole. It is a growth factor for bacteria but not for rats or chicks (41). The compound, vitamin B_{12f} reported by Lewis, Tappan, and Elvehjem (75) may be a similar compound.

It can be seen that the chemistry of vitamin B_{12} is very complex but a solution of its structure will probably help to reveal the nature of its biological activity.

The Intrinsic Factor

The concept of the intrinsic factor and extrinsic factor (vitamin B_{12}) was postulated as a result of the experiments of Castle and his co-workers with the gastric contents of normal humans and those afflicted with pernicious anemia (23, 25, 27, 28). Liver therapy created a more normal blood composition and alleviated the neurological symptoms, but it failed to restore hydrochloric acid and pepsin to the stomach. In addition, a deficiency of hydrochloric acid occurring in other diseases sometimes caused pernicious anemia to appear.

Castle (23) found that the contents of a normal stomach would cause remission of the symptoms of pernicious anemia. A healthy subject was fed 300 grams of beef muscle. The digest was regurgitated in an hour, the pH adjusted to approximately 3 with hydrochloric acid, and the material incubated for various periods of time up to 30 hours. It was then put through a fine sieve, adjusted to pH 5.0 or higher, and fed to a fasting patient. The results of feeding this mixture were similar to those obtained when liver was fed (28, 78). Neither beef muscle nor gastric juice was effective when fed alone. The substance found in beef and other foods was called extrinsic factor and the heat labile substance found in gastric juice and gastric mucosa

of swine was termed the intrinsic factor (25). These factors were effective even when fed 6 hours apart. Mixtures were ineffective unless the pH was brought to 5 to 7 before feeding. Furthermore, the hematopoietic principle produced in the incubated mixtures could not withstand heat (26). However, Klein and Wilkinson (72) found that an alcohol precipitate of the press juice of hog stomach incubated with beef muscle was active after it was heated to 60 to 65° C. After vitamin B_{12} was isolated from liver, it was established that it was the anti-anemia factor and the food extrinsic factor (11, 12).

Evidence that the intrinsic factor is a substance that is necessary for the absorption of vitamin B_{12} has been accumulated (112). Since liver extracts and crystalline vitamin B_{12} were effective therapeutic agents when injected, but vitamin B_{12} in the diet was not, the intrinsic factor had to be responsible for the absorption of vitamin B_{12}. The tissues of untreated pernicious anemia patients do not contain vitamin B_{12} (51) and excessive amounts of parenterally administered vitamin B_{12} are excreted in the urine. Continuous feeding of vitamin B_{12} to humans or a single massive dose results in vitamin B_{12} being excreted in the feces (29). This was confirmed by feeding vitamin B_{12} containing radioactive cobalt-60 to rats. Almost all of the radioactivity was present in the feces (30). It is believed, therefore, that the intrinsic factor is responsible

for passage of the vitamin through the cells of the intestinal mucosa (92).

All claims made for the identity of the intrinsic factor have been disproved. Gastric aminopolypeptidase has no activity; its concentrates presumably contained intrinsic factor as an impurity (92). Lysozyme from egg white was reported to cause the remission of the symptoms of pernicious anemia (77). However Hall (55) showed that a complex of egg lysozyme and vitamin B_{12} had no greater activity than an equivalent amount of the free vitamin. Glass and associates (52) have isolated a crystalline gastric mucoprotein from gastric juice by alcohol, acetone, and isoelectric precipitation. Daily oral doses of 50 to 100 mg. had to be given to patients with pernicious anemia in relapse in order to induce remission. Welch and Heinle (112) pointed out that they, in collaboration with Castle and his associates, have obtained hematologic responses with as little as 0.6 mg. daily of a fraction obtained from gastric juice. The role of gastric mucoprotein in pernicious anemia remains uncertain.

Concentration of intrinsic factor activity from powdered hog stomach (Ventriculin, Parke Davis and Co.) has been accomplished. Extraction with a 2 per cent solution of sodium chloride, adjustment to pH 9.0, removal of inert material at pH 1.5, and fractional precipitation with ammonium sulfate at pH 4.5 separated the activity from large

amounts of inert material. From approximately 50 g. of powdered stomach fraction A (210 mg.) was precipitated by 35 per cent saturation, fraction B (15 mg.) by 55 per cent saturation and fraction C (570 mg.) by full saturation. Fraction B was the most active hematopoietically (87). Intrinsic factor activity also can be precipitated from gastric juice by half saturation with ammonium sulfate (59, 112).

Recently evidence has been presented for an extragastric intrinsic factor. Lajtha (73), using bone marrow cultures, found that vitamin B_{12} did not affect the maturation of megaloblasts unless a small amount of sterile filtered gastric juice was present. An inhibitory factor was found in pernicious anemia serum and cerebrospinal fluid which reversed the reaction. The inhibitor could be counteracted by a mixture of vitamin B_{12} and gastric juice (40, 108). Horrigan, Jarrold, and Vilter (64) instilled vitamin B_{12} directly into the marrow cavity and found that it was effective in pernicious anemia contrary to the findings of Lajtha. It should be mentioned that folic acid and citrovorum factor also were included in the tests. These vitamins are associated with vitamin B_{12} in many of its metabolic reactions, but they will not be discussed here. The findings of Lajtha (73) suggest that the intrinsic factor converts vitamin B_{12} into an active form. Ross (90) has shown that vitamin B_{12} injected into the

blood appears to circulate in a form which is unavailable for microorganisms until the vitamin B_{12} is released from the diluted serum by heating at 100° C. for 1 hour. Whether or not this represents a combination with an intrinsic factor like substance has not yet been determined.

Experiments on the vitamin B_{12}-intrinsic factor complex have yielded confusing results. Progress is slow because humans are used for the assay. Hall and associates (56) found that extracts from hog mucosa incubated with vitamin B_{12} and then heated to 100° C. were still hematopoietically active. Bethell and associates (14) later found that they could treat the mixtures with trypsin at pH 8.0 and heat the digest to 100° C. for 10 minutes without loss of activity. They also confirmed the report of Castle and Ham (24) that intrinsic factor extracts and vitamin B_{12} could be given either simultaneously or from two to six hours apart. Berkefeld filtered gastric juice was also found to be a potent source of intrinsic factor (57). Wolf and associates (119) studied the absorption spectrum of vitamin B_{12} added to gastric juice from pH 2.0 to 7.0 and found no change in the absorption spectrum of vitamin B_{12}. All studies on the gastric intrinsic factor now indicate that vitamin B_{12} cannot pass through the intestinal wall until its physical or chemical nature has been changed by the intrinsic factor. In this respect Castle's original theory has not been changed.

Welch and associates (114) have developed an assay for intrinsic factor concentrates based on the absorption of vitamin B_{12} in the intestine. Patients are given radioactive vitamin B_{12} labeled with radioactive cobalt-60. When no intrinsic factor is present, 70 to 95 per cent of the radioactivity is excreted in the feces. When gastric juice or hog stomach extracts are given, only 5 to 30 per cent of the radioactivity occurs in the feces.

Absorption of vitamin B_{12} by bacteria in the upper gastro-intestinal tract was once believed to be a cause of pernicious anemia. In the absence of hydrochloric acid there is a copious growth of bacteria. Lichtman, Ginsberg, and Watson (76) fed aureomycin along with vitamin B_{12} and noticed an improvement of the patients. However, Ungley (109) who removed organisms from the intestine by washing segments of it found that vitamin B_{12} was not absorbed afterward. It appears that bacterial absorption may only augment the severity of the disease.

It is of interest to note that in diseases which cause degeneration of the hydrochloric acid-producing cells of the stomach, pernicious anemia may sometimes occur. Also a megaloblastic anemia appears in individuals infected with the fish tapeworm, Diphyllobothrium latum, presumably because either vitamin B_{12} or intrinsic factor is destroyed by the worm since the worm must be expelled before the anemia responds to vitamin B_{12} and the intrinsic factor (111).

Vitamin B_{12} Binding

The reaction of hematopoietic factors with microorganisms has become the basis for two methods of assaying vitamin B_{12} and/or vitamin B_{12}-binding substances. Growth of microorganisms which require vitamin B_{12} among their nutrients may be measured. The organisms being used are Lactobacillus lactis Dorner (98), Lactobacillus leichmanii, (99), Euglena gracilis (65), and a mutant strain of E. coli requiring methionine and vitamin B_{12} (13, 42).

The resting cells of several types of bacteria will absorb vitamin B_{12} and it can be recovered by washing the cells under certain conditions. Oginsky (80) has studied the uptake of vitamin B_{12} by E. coli. The wild type and a mutant strain both rapidly absorbed vitamin B_{12} labeled with cobalt-60 and the binding resisted simple elution. Cells had a finite capacity proportional to the cell weight. Davis and co-workers (43, 44, 45) have studied the uptake of vitamin B_{12}-Co^{60} by resting cells of L. leichmannii.

There are many facets to the problem of assaying materials for vitamin B_{12} due to the presence of inhibitors and other growth producing substances which substitute for vitamin B_{12}. As indicated above absorption on cells or other protein materials makes assaying of vitamin B_{12} difficult. A discussion of some of these growth factors is presented under microbiological assay and covered in

several reviews (47, 113).

Ternberg and Eakin (107) were the first to propose that there is a substance in normal gastric juice which unites stoichiometrically with vitamin B_{12} so that the latter is unavailable for the growth of the mutant of E. coli that requires vitamin B_{12}. This substance rendered the vitamin non-dialyzable. The complex could be destroyed by heating so that vitamin B_{12} was available for bacterial growth. They claimed that the vitamin B_{12}-binding substance was the intrinsic factor. This claim has aroused a controversy which has not yet been resolved. Prusoff and associates did not find bacterial growth inhibiting substances in their most hematopoietically active substances (112).

E. Hoff-Jørgensen (61) showed that cells of E. coli, isolated from a patient with pernicious anemia failed to absorb vitamin B_{12} when an extract of desiccated hog stomach was added to the medium. Shaw (96) prepared an extract of the entire small intestine of swine which inhibited the microbiological activity of vitamin B_{12}. In addition to the inhibitor this material contained non-dialyzable growth factors and a complex of vitamin B_{12}-like material which was dissociated by boiling. Added vitamin B_{12} was not completely recovered by heating.

Experiments designed to correlate bacterial inhibition with intrinsic factor activity have been carried out by

Burkholder (21, 22). Bacteria isolated from jejunal juice of pernicious anemia patients were tested for their ability to absorb vitamin B_{12}. Those with the most absorptive power were grown on a medium containing inorganic salts, glucose, and asparagine at pH 6.8. The cells were collected and resuspended in a medium containing vitamin B_{12} and gastric juice or extracts of hog mucosa. These latter substances prevented the absorption of vitamin B_{12} by the bacteria. Unabsorbed vitamin B_{12} was measured microbiologically in the following manner with a mutant of E. coli requiring vitamin B_{12}. The cells were removed and the medium was heated to destroy the vitamin B_{12}-binding substance after which the vitamin B_{12} could be assayed. The intrinsic factor concentrates were prepared by extracting dried hog stomach and fresh pyloric mucosa. Zinc-ethanol reagent was used to precipitate the proteins according to the method of Cohn and associates (33). An inhibitory activity of 190 mμg. vitamin B_{12} per mg. protein was obtained upon analysis. Enzymatic digestion of mucosal extracts was also performed to determine whether proteolysis would assist the concentration of vitamin B_{12}-binding substances. Papain and trypsin were without effect whereas pepsin caused a slight increase in binding activity. Hematopoietic activity of the extracts has not yet been reported.

Spray (104) found a vitamin B_{12} binding factor in gastric juice which was only partially released from

vitamin B_{12} by heating to make it available to bacteria. These heated extracts however, did not potentiate the action of orally administered vitamin B_{12} in a patient with untreated pernicious anemia. He concluded that the binding factor was not identical with Castle's intrinsic factor. The factor was more stable in neutral than in acid solutions.

A heat labile vitamin B_{12} complex was found in rat feces. Warm water extracts of feces were heated to 100° C. in order to obtain maximum vitamin B_{12} activity for Lactobacillus lactis Dorner (83).

Gregory, Ford, and Kon (53) found that sow's milk contained a vitamin B_{12}-binding component which survived heating at 100° C. for 30 minutes. It was destroyed by autoclaving at pH 12.0, but was stable at pH 7.0 unless a trace of cyanide was present. Vitamin B_{12} was not dialyzable. The binding substance was not ultrafiltrable and was inactivated by papain digestion. Fractionation of whey proteins was done according to the method used by Prusoff and associates (87) for intrinsic factor. Vitamin B_{12} could be dialyzed from cow's milk but not from sow's milk.

Beerstecher and co-workers (5, 6, 7, 8, 9) have published several papers on a vitamin B_{12} binding substance in saliva and substances related to or identical with the intrinsic factor of the stomach. This work has been poorly defined and cannot be evaluated here.

Bird and Hoevet (15) studied the vitamin B_{12}-binding ability of a wide variety of proteins which included intrinsic factor concentrates, lysozyme, soy bean globulin, egg albumin, urease, and blood globulin. Inhibition of growth of L. leichmannii in the presence of vitamin B_{12} was used as the method of analysis. Intrinsic factor concentrates and lysozyme were the most potent; the other proteins were practically without effect. In dialysis experiments vitamin B_{12} could not be separated from intrinsic factor concentrates, but could be easily dialyzed from lysozyme.

The vitamin B_{12} content of blood has received a considerable amount of study since the constitution of blood generally indicates the nutritional status of the animal. The report by Ross (90) of vitamin B_{12}-binding by serum which is released upon heating has already been mentioned. A paper-electrophoresis technique is being applied to serum in order to separate the vitamin B_{12}-binding component but no results of these experiments have yet been reported (74). Some workers claim that vitamin B_{12} can be dialyzed from plasma (34, 121). Rosenthal and Sarett (89) made studies upon the release of vitamin B_{12} by heating. A one to ten dilution of serum heated at 100° C. for 30 minutes with one per cent acetate buffer at pH 5.1 gave consistent results with L. leichmannii. Heating for periods of 5 to 120 minutes did not alter this value. Recovery of crystalline vitamin B_{12} by this method was possible. Dialysis did not

release vitamin B_{12} from the serum and it was found that additional amounts of the vitamin also could be bound. In whole blood the concentration of vitamin B_{12} appears to be greater in the cells than in the serum.

Several investigators (3, 39) have found that only enzymatic digestion would release all of the vitamin B_{12} for bacterial assay. Enzymatic digestion of liver extracts was one of the steps used in the isolation of vitamin B_{12} from liver. No process has yet been described for the isolation of vitamin B_{12} from liver in which no proteolytic enzyme is used. Wijmenga, Veer, and Lens (116) found that potassium cyanide would replace proteolysis as it apparently removes a protein or peptide group originally linked to the vitamin B_{12} molecule. Conjugated forms of vitamin B_{12} with histidine, ammonia, and peptides have been reported (35).

There are a large number of papers on the preparation and assaying of protein materials for vitamin B_{12}. A variety of enzymes have been used. One difficulty with enzyme preparations is that they contain vitamin B_{12} or growth factors substituting for vitamin B_{12}. Papain, pancreatin, and trypsin have been commonly used. Cyanide ion has been shown to have an enhancing effect upon vitamin B_{12} and its derivatives assayed with Lactobacillus lactis (37). Cyanide has been used in the extraction of vitamin B_{12} from tissues (31, 38, 58), the advantage over enzymatic proteolysis being that no contaminating substances are added.

Heat treatment of tissues has been a satisfactory method in some cases. At the present time the wide range of properties ascribed to vitamin B_{12}-binding substances and the methods necessary for their destruction indicate that many vitamin B_{12} conjugates exist.

Microbiological Assay of Vitamin B_{12} with L. leichmannii

Since the microbiological assay of vitamin B_{12} plays an important part in this study, some of the problems involved should be discussed. The difficulties encountered with this organism are legion due to the fact that it as well as other ones used for the assay of vitamin B_{12} do not have a specific requirement for this vitamin. The environmental conditions, therefore, must be carefully controlled so that growth occurs only when vitamin B_{12} is present. No attempt will be made to present a review of the microbiological assay of vitamin B_{12}; only the pertinent facts will be discussed here. A review of microbiological assay methods for vitamin B_{12} is presented in Vitamin Methods (54).

Skeggs, Huff, Wright, and Bosshart (99) suggested the use of L. leichmannii 4797 for the assay of vitamin B_{12}. Hoffmann, Stokstad, Franklin, and Jukes (62) used L. leichmannii 7830, another strain which had a similar requirement for vitamin B_{12}.

Snell, Kitay, and McNutt (103) found that thymidine was

a growth factor for L. leichmannii, but others (62) found that it could be replaced by vitamin B_{12}. It was soon discovered that a large number of purine and pyrimidine derivatives could replace vitamin B_{12}. Kitay, McNutt, and Snell (70) showed that thymine, adenine, hypoxanthine, and cytosine desoxyribosides and desoxyribonucleic acids were equally effective in promoting growth. Using paper chromatography, Winsten and Eigen (117, 118) found six entities in vitamin B_{12} concentrates capable of supporting growth, and this was confirmed later by Smith and Cuthbertson (102). Shive, Sibley, and Rogers (97) found that nucleotides could be used to replace vitamin B_{12}. Skeggs and associates (100) claimed that the effect of thymidine was enhanced when ascorbic acid was added to the medium or when air was removed by autoclaving. Kitay, McNutt, and Snell (71) suggested that the growth enhancing effect of ascorbic acid was due to hydrolytic products of nucleic acids present in casein digests. This was confirmed by Welch and Wilson (115) who isolated some nucleosides from the hydrolyzed casein. Elimination of these growth factors from the medium was finally brought about by substituting amino acids for the casein hydrolysates. Since nucleic acids are present in all cells, the assaying of vitamin B_{12} is complicated by the presence of other growth factors in the tissues being analyzed. Vitamin B_{12} is destroyed by alkali with the

result that analyses performed before and after heating the samples with sodium hydroxide will give the amount of vitamin B_{12} by difference (63).

The assay is further complicated by the presence of different forms of vitamin B_{12} in natural materials. The protein conjugates have already been discussed. Vitamin B_{12b} is present in some tissue extracts. There have been many theories about the comparative activity of vitamin B_{12} and B_{12b} in microbiological assay. Autoclaving, reducing agents, aseptic additions to the medium, and other physical agents are claimed to have influence on these molecules. Broquist, Stokstad, and Jukes (18) found that a reaction product more potent than vitamin B_{12} was formed when vitamin B_{12} was heated with thiomalic acid. This product moved between vitamin B_{12} and B_{12b} on paper strips. The activity of vitamin B_{12b} was not increased by autoclaving it with the medium. The problem of interference of vitamin B_{12b} can be resolved by treating with cyanide which converts vitamin B_{12b} to vitamin B_{12} (37, 116).

The presence in samples of vitamin B_{12}-like compounds such as pseudovitamin B_{12} produced by certain microorganisms, adds more difficulties to an already complex problem (32, 46, 48, 83).

MATERIALS

Microorganisms

Lactobacillus leichmannii 4797 and Lactobacillus leichmannii 7830, obtained from the American Type Culture Collection, Washington, D. C., were the microorganisms used in this investigation.

Chemicals

Nutrients used for media

The amino acids used in the basal medium were purchased from Merck and Co., Rahway, New Jersey, Nutritional Biochemicals Co., Cleveland Ohio, Pfanstiehl Chemical Company, Waukegan, Illinois, and Eastman Kodak Company, Rochester, New York. Guanine hydrochloride and uracil were purchased from the Eastman Kodak Company. Vitamins were obtained from Merck and Company and Nutritional Biochemicals Corporation. Libby's tomato juice was purchased on the open market. Glucose (Pfanstiehl) and the inorganic salts were obtained from Chemistry Stores. Tween 80 was obtained from Hill Top Laboratories, Incorporated, Cincinnati, Ohio. Bacto Yeast Extract, Bacto Peptonized Milk, and Bacto Agar were purchased from Difco Laboratories, Detroit, Michigan.

Vitamin B_{12}

Vitamin B_{12} (Cobione, Merck and Company) was purchased from Merck and Company. This is a sterile solution of 15 micrograms crystalline vitamin B_{12} per one ml. ampule.

Powdered hog stomach

Dried defatted powdered hog stomach (Ventriculin, Parke, Davis, and Company, Detroit, Michigan) was purchased from a local drug store.

Purified proteins

Several protein fractions from blood plasma were obtained through the courtesy of Dr. E. J. Cohn, Harvard Medical School, Boston, Massachusetts.

Enzymes

Trypsin (I-110) was purchased from Pfanstiehl Chemical Company.

Adsorbents

Celite (diatomaceous earth), Norit A (decolorizing charcoal), Alphacel (powdered cellulose, Nutritional Biochemicals Corporation) and Whatman no. 1 filter paper were obtained from Chemistry Stores. Amberlite IR-120, a nuclear sulfonic-acid type cation exchange resin, manufactured by

Rohm and Haas Company, was obtained by courtesy of the General Filter Company, Ames, Iowa.

Apparatus

A Beckman pH meter Model G. was used for all pH determinations. Turbidity measurements were made with a KWSZ photometer using filter number 7 (650 millimicrons). This photometer has a balanced circuit and reads directly in per cent transmission. It is distributed by Wilkens-Anderson Company, Chicago, Illinois. Basal medium and vitamin B_{12} solution were dispensed with a Cannon Automatic Dispenser-Titrator, purchased from International Instrument Company, Los Angeles, California. Chromatographed fractions were collected with a Technicon automatic time/flow fraction collector, purchased from the Technicon Chromatography Corporation, New York.

METHODS

Stock Cultures

Cultures of Lactobacillus leichmannii 4797 and Lactobacillus leichmannii 7830 were carried throughout this investigation in a peptonized milk agar. This medium had the following composition: 15 g. agar, 10 g. glucose, 10 g. peptonized milk, 10 g. yeast extract, 2 g. Tween 80, and distilled water to make 1 liter. This mixture was heated for 20 minutes in flowing steam and then dispensed into test tubes, 10 ml. per tube. The medium was sterilized by autoclaving for 15 minutes at 15 lb. steam pressure. The cultures were transferred weekly from one stab to another, incubated at 37° C. for 40 to 48 hours, and then stored in a refrigerator.

Preparation of Inoculum

An inoculum was prepared by transferring organisms from a stab to a sterile broth (about 5 ml.) of the same composition except for the agar. The broth was incubated for 16 hours at 37° C. The cells were centrifuged, washed with distilled water, again collected by centrifugation, and suspended in distilled water to make a heavy suspension.

One drop of this suspension was used to inoculate the assay tubes.

Solutions for Basal Medium

All solutions used in the preparation of the basal medium were stored in a refrigerator.

Amino acids

The following amino acids were dissolved in 200 ml. warm water: 850 mg. DL-alanine, 850 mg. L-arginine hydrochloride, 850 mg. DL-aspartic acid, 850 mg. glycine, 850 mg. L-histidine hydrochloride, 850 mg. DL-isoleucine, 425 mg. L-leucine, 425 mg. L-lysine hydrochloride, 850 mg. DL-methionine, 200 mg. DL-phenylalanine, 850 mg. L-proline, 100 mg. DL-serine, 100 mg. DL-threonine, 850 mg. DL-valine, 300 mg. L-asparagine and 400 mg. L-glutamine. This solution was stored under toluene.

L-tyrosine, 800 mg., and 2.425 g. glutamic acid were dissolved in water with just enough hydrochloric acid to dissolve them and diluted to 100 ml.

L-tryptophan, 100 mg., was dissolved in water with the aid of dilute sodium hydroxide and diluted to 100 ml.

Vitamins

Twenty mg. each of nicotinic acid, riboflavin,

thiamine, and calcium pantothenate were diluted to 100 ml. with distilled water.

Twenty mg. each of pyridoxine and pyridoxal were diluted to 100 ml. with distilled water.

A folic acid solution was made to contain 1.1 mg. in 100 ml. of distilled water.

A solution of p-aminobenzoic acid contained 0.8 mg. in 100 ml. distilled water.

A biotin solution was made by diluting 25 micrograms, contained in 1 ml. ampules, to 25 ml. with distilled water.

Purines and pyrimidine bases

Fifty mg. each of adenine sulfate, guanine hydrochloride, and uracil and 75 mg. xanthine were dissolved in 50 ml. of distilled water with the addition of sodium hydroxide.

Inorganic salts

Two g. magnesium sulfate heptahydrate, 0.7 g. ferrous sulfate heptahydrate and 0.068 g. manganous sulfate were contained in 250 ml. of distilled water, containing a little hydrochloric acid. Twenty g. of potassium dihydrogen phosphate and 26.2 g. dipotassium hydrogen phosphate trihydrate were dissolved and diluted to 250 ml. with distilled water.

Tween 80

Two g. of Tween 80 were diluted to 40 ml. with water.

Tomato juice filtrate

The contents of a 46 ounce can of tomato juice were centrifuged to remove pulp and filtered with the aid of Celite and suction. The filtrate was stirred for 30 minutes with 20 g. of Norit A per liter and again filtered with the aid of Celite. The filtrate was divided into 50 and 100 ml. portions and stored in a freezer.

Preparation of Assay Tubes

To prepare 100 ml. of basal medium the following amounts of nutrient solutions were used: 10 ml. amino acid solution, 5 ml. each of tyrosine-glutamic acid, tryptophan, and the two salt solutions, 1 ml. each of the bases and the five vitamin solutions, 4 ml. Tween 80 solution and 50 ml. tomato juice filtrate. To these were added 4 g. glucose, 1.2 g. sodium citrate, 1.2 g. sodium acetate and 0.2 g. cysteine hydrochloride. The pH was adjusted to 5.5 with 5 M sodium hydroxide and the volume brought to 100 ml. The constituents of the double strength basal medium are shown in Table 1.

To make up the assay tubes, aliquots of vitamin B_{12} solutions, water, or test solutions were pipetted first into

Table 1.

Constituents for 1 Liter of Double Strength Basal Medium[a]

Compound	Amount	
DL-alanine	425	mg.
L-arginine hydrochloride	425	mg.
DL-aspartic acid	425	mg.
Glycine	425	mg.
L-histidine hydrochloride	425	mg.
DL-isoleucine	425	mg.
L-leucine	213	mg.
L-lysine hydrochloride	213	mg.
DL-methionine	425	mg.
DL-phenylalanine	100	mg.
L-proline	425	mg.
DL-serine	50	mg.
DL-threonine	50	mg.
DL-valine	425	mg.
L-asparagine	150	mg.
L-glutamine	200	mg.
L-tyrosine	400	mg.
L-glutamic acid	1213	mg.
L-tryptophan	50	mg.
Adenine sulfate	10	mg.

(Continued on next page)

Table 1. (Continued)

Compound	Amount	
Guanine hydrochloride	10	mg.
Xanthine	15	mg.
Uracil	10	mg.
$MgSO_4 \cdot 7\ H_2O$	2.0	g.
$FeSO_4 \cdot 7\ H_2O$	0.1	g.
$MnSO_4$	0.068	g.
KH_2PO_4	4.0	g.
$K_2HPO_4 \cdot 3\ H_2O$	5.24	g.
Pyridoxine	2.0	mg.
Pyridoxal	2.0	mg.
Nicotinic acid	2.0	mg.
Riboflavin	2.0	mg.
Calcium pantothenate	2.0	mg.
Thiamine	2.0	mg.
Biotin	0.01	mg.
Folic acid	0.11	mg.
p-aminobenzoic acid	0.08	mg.
L-cysteine hydrochloride	2.0	g.
Sodium acetate	12.0	g.
Sodium citrate	12.0	g.
Tween 80	2.0	g.

(Concluded on next page)

Table 1. (Concluded)

Compound	Amount
Glucose	40.0 g.
Tomato juice	500 ml.

[a] The pH of this medium was adjusted to 5.5

the assay tubes. Five ml. of the basal medium were then added to each tube with a Cannon dispenser. When the same concentration of vitamin B_{12} was desired in a large number of tubes, 4 ml. of a vitamin B_{12} solution also was added in this manner. The assay tubes were covered with aluminum caps and autoclaved for 5 minutes at 15 lb. pressure. The tubes were cooled as rapidly as possible. Unheated or specially treated test samples were added at this time, the total volume of the medium finally being 10 ml. Each tube was inoculated with one drop of a bacterial suspension and placed in an incubator at 37° C. for 20 to 22 hours.

Measurement of Growth

In early experiments the cells were incubated for 48 hours and the amount of growth was determined by titrating the lactic acid with 0.1 N sodium hydroxide. The contents

of a tube were transferred to a 150 ml. beaker with approximately 70 ml. distilled water. A stirrer and the electrodes were immersed in the solution and the acid titrated to the pH of the control tube, about 5.5, with 0.1 N sodium hydroxide using a pH meter. The amount of growth was plotted as ml. 0.1 N sodium hydroxide against concentration in mμg. vitamin B_{12}. Turbidity was measured in later experiments, density of suspensions being proportional to growth. The tubes were shaken to resuspend the cells, transferred to a colorimeter tube and the per cent transmission read, a low reading indicating copious growth. Typical standard curves for both methods of determining growth are shown in Figures 1 and 2.

Protein Determinations

The amount of protein was determined using the biuret test as described by Baudet and Giddey (2). A standard curve was prepared using known amounts of egg albumin. (See Figure 3.)

Chromatography

Chromatography of extracts on paper was accomplished in the following manner. A pencil line was drawn 2 cm. from the bottom of a sheet of filter paper (E. and D. no. 613) 20 cm. wide and 22 cm. long. The solutions were applied

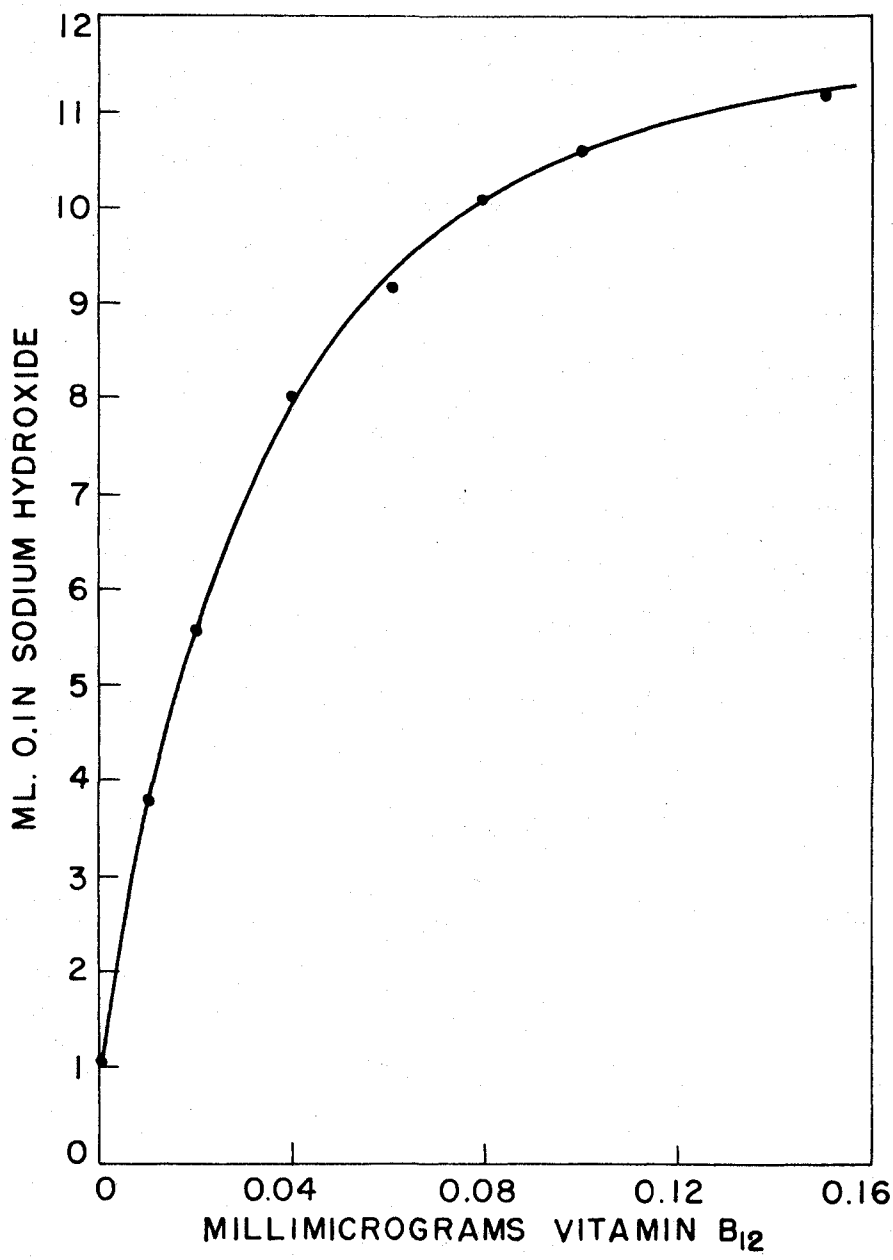


Figure 1. Standard Growth Curve for 48 Hours Incubation.

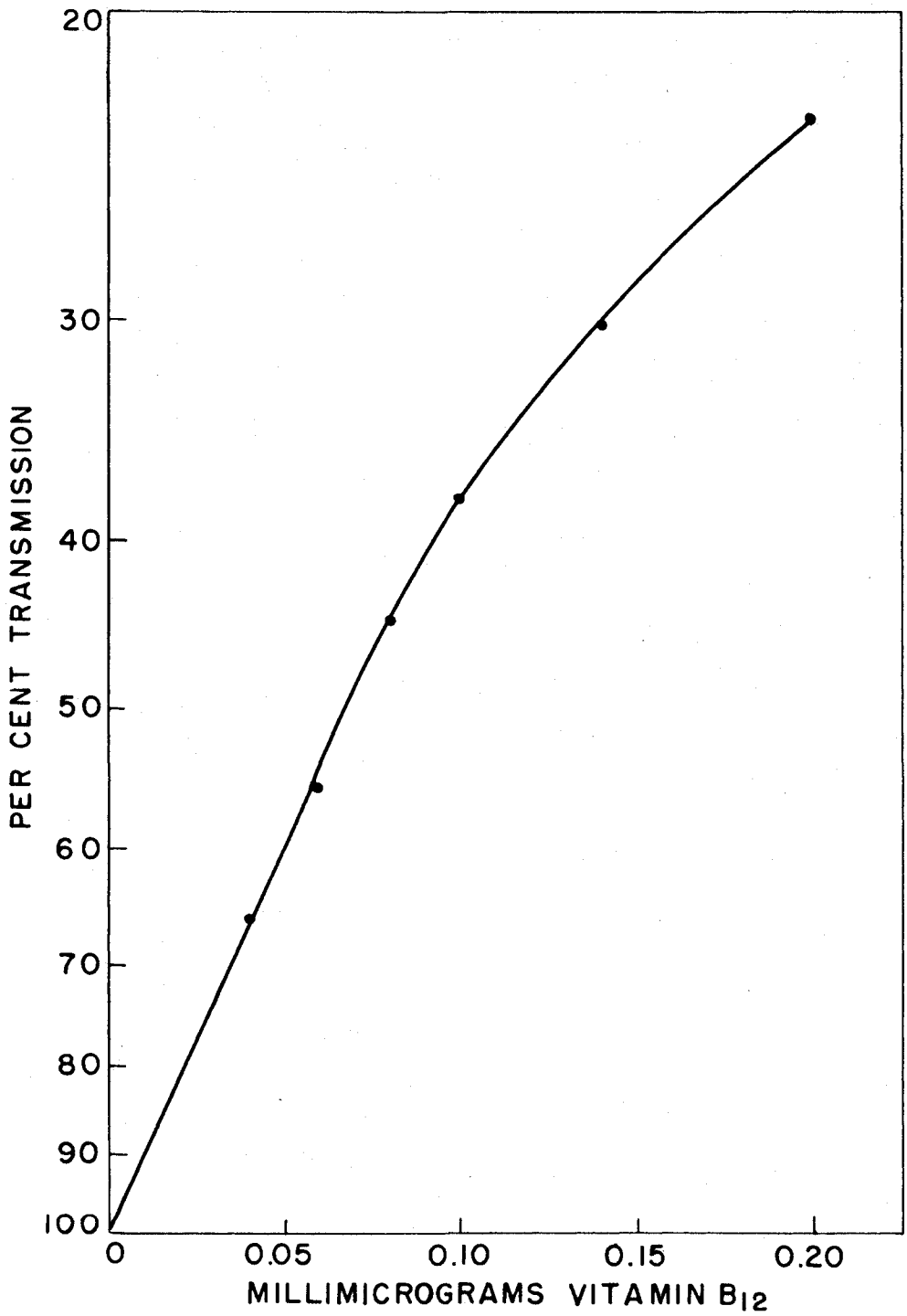


Figure 2. Standard Growth Curve for 22 Hours Incubation.

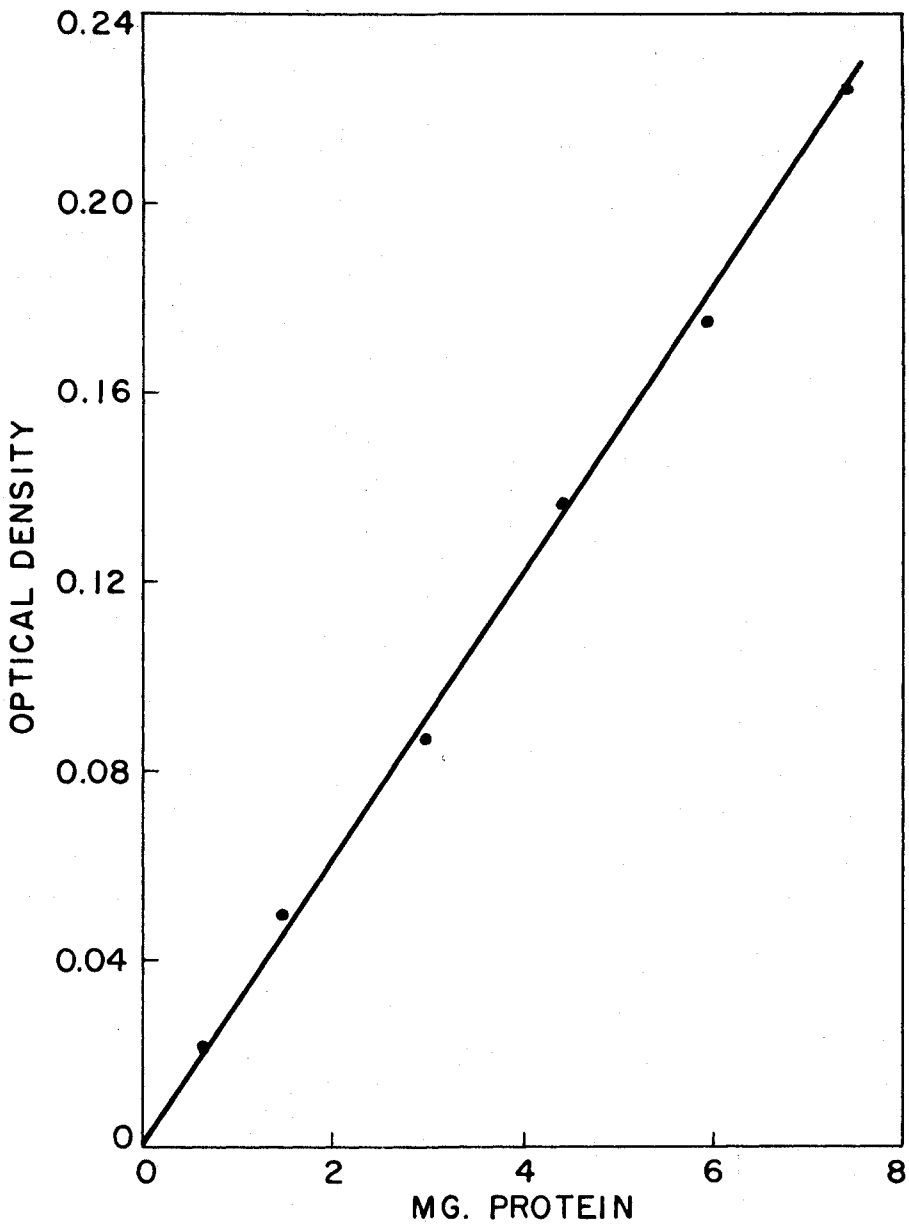


Figure 3. Standard Curve for Protein Determination by the Biuret Method.

with capillary pipettes as spots not more than 0.5 cm. in diameter along the top of the line and allowed to dry in the air. Additional applications were made in the same manner. After the applied solutions had thoroughly dried, the paper was turned lengthwise into a cylinder and stapled in such a manner that the edges of the paper did not touch each other. The solvents used were put in the bottom of large glass jars. After the paper cylinder had been placed in the solvent, the lid was tightly screwed on the jar. The solvent was allowed to reach the top of the paper, after which the paper was dried in the air. This procedure was carried out at room temperature.

EXPERIMENTAL RESULTS

Vitamin B_{12} occurs in such low concentrations in biological materials that chemical methods of analysis for this vitamin are not practical. The tissues must first be broken down, then extracted and the extracts concentrated. A sensitive biological method, if carefully controlled, will afford a more direct determination of the vitamin B_{12} concentration. The most rapid biological assays are those in which microorganisms are used. In addition, the conditions in the medium can easily be changed so that metabolic relationships can be studied. When a metabolic function is discovered in one species, the investigator may be able to explain a similar one in another species. The development of a satisfactory assay method for vitamin B_{12} was the first problem to be solved. Lactobacillus leichmannii was chosen as the organism for the assay of vitamin B_{12} in this laboratory. The cultures of this organism were stable in their characteristics.

Modification of a Microbiological Assay for Vitamin B_{12}

The assay medium of Skeggs, Huff, Wright, and Bosshart (99) was used for L. leichmannii 4797. This medium was composed of amino acids present in an acid casein

hydrolysate and enzymatically hydrolyzed casein, vitamins, purines, pyrimidines, salts, glucose, and Tween 80. A tomato juice filtrate also was added. This medium was made up each day from stock solutions of constituents similar to those listed in the section on methods. The medium was dispensed into test tubes (5 ml. per tube) containing 5 ml. aliquots of vitamin B_{12} of varying concentration, and autoclaved for 15 minutes at 15 lb. pressure. The tubes were inoculated and after a two day incubation period at 37° C., the lactic acid formed was titrated with 0.1 N sodium hydroxide. A typical assay for crystalline vitamin B_{12} is presented in Table 2.

Table 2.

Assay of Vitamin B_{12} Using Skeggs's Method[a]

Amount of vitamin B_{12} mμg.	Titration values ml. 0.1 N NaOH
0	0.6
0.1	1.8
0.2	4.0
0.4	6.8
0.6	8.5
1.0	10.5
2.0	12.3
6.0	13.2

[a] L. leichmannii 4797 was used in the assay.

Over a period of time, the growth of the organism decreased in the presence of the same concentrations of vitamin B_{12}. It was reported (63, 71, 100) that reducing agents added to the medium would increase the response to vitamin B_{12}. Therefore, experiments were carried out to determine the effect of some of these compounds. The effect of adding ascorbic acid and cysteine was studied and the concentration of tomato juice which contains ascorbic acid and unknown growth factors was varied. The results of this experiment are shown in Table 3. Ascorbic acid was the most effective in enhancing the amount of growth. Tomato juice was effective if freshly prepared and added in high

Table 3.

Effect of Reducing Agents upon the Assay of Vitamin B_{12} with L. leichmannii 4797[a]

Substance added per tube	Titration values[b] ml. 0.1 N NaOH
1 mg. ascorbic acid	10.2
1 mg. cysteine	7.3
1 mg. ascorbic acid and 1 mg. cysteine	11.1
5 mg. ascorbic acid and 5 mg. cysteine	12.8
1 ml. tomato juice	8.2
3 ml. tomato juice	9.6

[a] Skeggs's medium was used.

[b] 2 mμg. vitamin B_{12} = 5.5 ml. 0.1 N NaOH.

concentration. Although the growth was increased by adding reducing agents, growth in the controls also was increased so that low concentrations of vitamin B_{12} could not be evaluated accurately.

The medium contained enzymatically hydrolyzed casein which contained the substances responsible for the high controls when reducing agents were used. But when enzymatically hydrolyzed casein was omitted the sensitivity to vitamin B_{12} was decreased. Other hydrolyzed proteins and protein filtrates were tried but they had effects similar to the enzymatically hydrolyzed casein.

A method suggested by Peeler, Yacowitz, and Norris (82) was attractive because it contained pure amino acids instead of casein hydrolysates. A well defined medium of known composition could then be prepared. However, a tomato juice filtrate was included. This improved growth by accelerating it in the initial stages (81). It has been observed that the use of this filtrate of unknown composition over a long period of time has not interfered in any way with the substances tested. For periods of incubation longer than 24 hours it was found that tomato juice filtrate could be replaced with distilled water.

Since Peeler's assay method failed to give reproducible results, several modifications were studied. Changes in the concentrations of the vitamins, within the limits studied, did not seem to have a significant effect upon growth.

In Table 4 the results obtained by adding additional amounts of vitamins to the medium of Peeler with and without vitamin B_{12} and heating by autoclaving for 15 minutes (Skeggs's method) and steaming for 5 minutes (Peeler's method) are compared.

The five minute steaming period did not affect the growth response even when widely varying concentrations of different vitamins were used. However, when the medium was autoclaved for 15 minutes at 15 lb. pressure the control (without vitamin B_{12} and the usual amounts of other vitamins) showed more growth, but the B_6 vitamins promoted growth more than any of the substances tested, even without vitamin B_{12}. It was concluded that no changes had to be made in any of the vitamin concentrations when the medium was steamed for 5 minutes.

Since the concentration of the B_6 vitamins in Peeler's medium was twice that present in Skeggs's medium the effect upon growth of each form was determined so that the amount could be decreased, thus eliminating variations in growth, which might be caused by heating. Table 5 shows that L. leichmannii can use pyridoxine, pyridoxal, and pyridoxamine equally as well for growth singly or together. There seemed to be no advantage in using all three forms. In a later experiment it was found that when the concentration of the B_6 vitamins was cut in half no decrease in growth occurred.

Table 4.

The Effect of Autoclaving and Steaming Added Vitamins with the Basal Medium upon the Growth of *L. leichmannii*[a]

Vitamins added	Titration values, ml. 0.1 N NaOH	
	Autoclaved 15 min.	Steamed 5 min.
None (blank)	3.8	0.8
Vitamin B_6 soln. (5xP.)[b]	5.0	1.9
Folic acid (5xP.)	1.0	1.5
p-Aminobenzoic acid (5xP.)	1.0	1.2
Vitamin B_6 soln. (5xP.) + 0.0075 mμg. B_{12}	3.4	2.3
Folic acid (5xP.) + 0.0075 mμg. B_{12}	2.3	1.4
p-Aminobenzoic acid (5xP.) + 0.0075 mμg. B_{12}	2.9	1.6
Peeler's vitamin soln. + 0.0075 mμg. B_{12}	1.6	2.0
Skeggs' vitamin soln. (5xS.)[c]	0.8	1.5

[a] Peeler's medium without tomato juice (pH 5.5) was employed.

[b] Five times the amount used by Peeler was employed.

[c] Five times the amount used by Skeggs was employed.

Table 5.

Effect of B_6 Vitamins upon the Growth of *L. leichmannii*[a]

Substance tested[b]	Amount mg.	Titration values ml. 0.1 N NaOH
Pyridoxal	0.04	7.0
Pyridoxamine	0.04	7.7
Pyridoxine	0.04	7.3
Pyridoxine,	0.02	
Pyridoxal, and	0.02	7.4
Pyridoxamine	0.004	

[a] Peeler's medium was used.

[b] 0.30 mμg. vitamin B_{12} was present in each tube.

The concentrations of magnesium, ferrous, and manganous salts were decreased since the concentrations recommended by Peeler exceeded the amounts needed by the organism for growth. Data showing the effect of varying concentrations of salts are presented in Table 6. A concentration representing five times the amount recommended by Skeggs was finally adopted since it gave the most consistent results. The amount of ferrous sulfate now being used is one-eighth of the amount in Peeler's medium.

Cystine, hydroxyproline, and norleucine were omitted after it was found that no decrease in growth occurred

Table 6.

Effect of Varying Concentrations of Certain Salts upon the Growth of L. leichmannii

Amount of vitamin B_{12} mμg.	Titration values, ml. 0.1 N NaOH		
	Skeggs' salts[a]	Skeggs' salts (5x)	Peeler's salts[b]
None	1.4	1.4	1.0
0.0075	1.7	4.6	---
0.0150	3.2	5.7	5.4
0.0225	3.9	6.6	---
0.030	7.0	7.0	5.6
0.075	11.7	8.8	6.5
0.15	---	10.4	9.4
3.00	11.9	11.4	11.2

[a] 0.02 g. $FeSO_4 \cdot 7H_2O$, 0.014 g. $MnSO_4$, and 0.4 g. $MgSO_4 \cdot 7H_2O$ per liter.

[b] 0.8 g. $FeSO_4 \cdot 7H_2O$, 0.406 g. $MnSO_4$, and 2.8 g. $MgSO_4 \cdot 7H_2O$ per liter.

when they were left out of the medium. Cysteine, which serves as the reducing agent, can be used by this organism for the synthesis of cystine. Norleucine and hydroxyproline are not commonly found in proteins. It can be seen by comparing the vitamin B_{12} concentrations in Table 2 with those in Table 6 that the sensitivity to low concentrations

of vitamin B_{12} has been greatly increased by changing to the modified Peeler medium.

Acid production as a measure of growth was studied for L. leichmannii 4797 at the end of a 48 and 72 hour incubation period at 37° C. (See Table 7.) The increased length of time did not cause any significant increase in the amount of acid produced, so a two day incubation period was used.

Table 7.

Comparison of Growth of L. leichmannii after 48 and 72 Hour Incubation Periods[a]

Amount of vitamin B_{12} mμg.	Titration values, ml. 0.1 N NaOH	
	48 hours	72 hours
0.02	3.8	4.2
0.04	5.6	5.8
0.10	7.1	7.8
0.30	8.2	8.5

[a] Peeler's medium was used.

When L. leichmannii 7830 was used for the assay, growth was determined turbidimetrically after 24 hour incubation at 37° C. Both strains of L. leichmannii responded similarly to the same concentrations of vitamin B_{12}. L. leichmannii 7830 was the faster growing strain. The acid production of

the two strains for a 24 hour incubation period is compared in Table 8. L. leichmannii 7830 produced more acid, particularly at high concentrations of vitamin B_{12}. The latter organism has been used in all experiments in which growth was determined turbidimetrically.

Table 8.

Comparison of the Growth of L. leichmannii 4797 and L. leichmannii 7830 after 24 Hours Incubation[a]

Amount of vitamin B_{12} mμg.	Titration values, ml. 0.1 N NaOH	
	L.L 4797	L.L. 7830
None	0.9	2.1
0.0075	1.4	2.9
0.015	2.0	2.4
0.045	4.2	4.3
0.075	5.5	6.3
0.15	6.1	7.9
0.60	7.3	9.0
1.50	8.5	11.4

[a] Peeler's medium, steamed for 5 minutes, was used.

The basal medium was freshly made up each time before use from the solutions and solids already described. Some constituents of the medium are oxidized when they remain in contact with the air. It is particularly important that the oxidation of cysteine and ferrous ion be prevented. Cysteine hydrochloride is added just before the pH is adjusted.

In early experiments the medium was dispensed into assay tubes and steamed in an autoclave with the outlet valve open. It was difficult to control the heating in this manner due to variations in the steam supply. A short heating period resulted in good sensitivity and decreased the pyridoxine effect. In recent experiments the tubes were autoclaved for 5 minutes at 15 lb. pressure because the heating could be more easily controlled and the results were more reproducible. Although the length of time of heating will not completely sterilize the medium, no difficulties have ever been encountered due to contamination.

In the assaying of samples, aliquots were pipetted into the cooled tubes, inoculated, and then shaken in order to mix them thoroughly. The mixing as well as the heating seemed to be the most critical manipulations since they had a greater effect upon reproducibility of results than any other minor variation in technique. These solutions were not sterile but some were heated for special experiments. In each set of tubes two controls, one containing vitamin B_{12} but not inoculated, and the other containing no vitamin B_{12} but inoculated, were used to validate the results of the assay. Lack of growth in these tubes excludes the interference of contaminating organisms.

Inhibition of vitamin B_{12} was measured in the following manner. A known amount of vitamin B_{12} solution was put in each tube. In this investigation either 0.1 mμg. or 0.15

mμg. vitamin B_{12} was used in most experiments. This amount of vitamin B_{12} produced less than maximum growth which made it possible to detect growth substances which substitute for vitamin B_{12}.

As a result of the foregoing experiments a modified Peeler method was obtained which gave reproducible results in the assay of vitamin B_{12} using L. leichmannii. It should be mentioned that two other investigators in this laboratory have already independently made modifications of the Peeler medium (3, 50).

Fractionation of Vitamin B_{12} Inhibitory Principle from Hog Stomach Mucosa

Ternberg and Eakin (107) found that there was a substance in gastric juice which they called apoerythein that combined stoichiometrically with vitamin B_{12} (erythein) making the latter unavailable for the growth of bacteria. In this combination vitamin B_{12} was non-dialyzable but it could be released by heating the complex. These workers claimed that apoerythein was identical with the intrinsic factor. If this was true, an assay method for intrinsic factor had been discovered. Furthermore, the discovery of this compound stimulated the study of other vitamin B_{12} protein complexes involved in metabolic processes.

Fractionation of hog stomach mucosa extract with ammonium sulfate

The intrinsic factor is found in gastric juice and stomach mucosa. Ventriculin, a dried granular protein material prepared from the stomach mucosa of hogs, is used medicinally as a source of intrinsic factor. A daily dose of 40 g. is needed for the absorption of ingested vitamin B_{12}. Extraction of this material was begun to test the theory of Ternberg and Eakin.

Two g. of Ventriculin were mixed with approximately 20 ml. of distilled water and allowed to stand for one hour. This mixture was transferred to a Waring blender and mixed until the granules were broken. The insoluble material was removed by centrifugation and the supernatant filtered by suction through Whatman No. 1 filter paper. The filtrate was diluted to 50 ml. The pH of the extract was 5.7 and it had a brown color.

To test vitamin B_{12} combining power of the extract, 5 ml. were mixed with approximately 0.1 mg. of crystalline vitamin B_{12}. After 15 minutes 5 ml. of saturated ammonium sulfate were added. A solution 50 per cent saturated with respect to ammonium sulfate was obtained and no protein precipitate appeared. An additional volume of 15 ml. of saturated ammonium sulfate was added giving a solution 80 per cent saturated with ammonium sulfate. In this instance

a protein precipitate was formed. The precipitate was removed by centrifugation, and the supernatant decanted. No more precipitate was obtained by additional amounts of saturated ammonium sulfate. About 100 mg. of protein material were collected. The precipitate was washed twice with 80 per cent ammonium sulfate solution, recovered by centrifugation, then transferred to a cellophane bag and dialyzed against distilled water for 18 hours to remove ammonium sulfate and excess vitamin B_{12}.

One-half of the precipitate was diluted for analysis of vitamin B_{12}; the remainder was diluted and autoclaved for 15 minutes at 15 lb. steam pressure before being analyzed. The autoclaved sample contained more vitamin B_{12} than the unheated sample as determined microbiologically. Although quantitative data were not available, it was concluded that some substance present in the extract had bound vitamin B_{12} so that it was non-dialyzable and that heating was necessary to release most of the vitamin B_{12}.

In a quantitative experiment 150 mμg. vitamin B_{12} dissolved in 1 ml. of distilled water were added to 4 ml. of the Ventriculin extract and treated as described above to precipitate the protein. The supernatant, washings and dialysate were diluted for analysis of vitamin B_{12}. The dialyzed precipitate was diluted, autoclaved, and analyzed for vitamin B_{12}. Appropriate dilutions were made for comparison with a standard growth curve for vitamin B_{12}.

The results showed that only 20 mμg. of vitamin B_{12}-activity were left in the supernatant solution, the remainder had been precipitated with the protein. The total vitamin B_{12}-activity, however, was greater than 150 mμg. This may be explained by the presence of other growth factors in the Ventriculin extract. This will be shown in a later experiment. Throughout this study it must be kept in mind that vitamin B_{12} is not a specific growth factor for L. leichmannii. Purines, pyrimidines, and their compounds may be substituted for vitamin B_{12}.

About this time Prusoff and associates (87) proposed a method for extracting and concentrating the intrinsic factor from Ventriculin. They prepared three fractions by ammonium sulfate precipitation. One was active hematopoietically, all were active as bacterial growth inhibitors, but the fraction showing the most growth inhibition was not active hematopoietically.

Prusoff (86) prepared concentrates in the following way: five hundred grams of ventriculin were suspended in 2 per cent saline, stirred one hour, and filtered with the aid of Celite. The pH of the extract was 5.7 and 15 per cent of the solids were extracted. The pH was then adjusted to 9.0 and the solution was allowed to stand for 30 minutes to destroy pepsin activity. Then the pH was adjusted to 1.5 and the precipitate which formed was removed. The supernatant was brought to pH 4.5 and ammonium sulfate was

added to give 38,55, and 100 per cent saturation in order to precipitate protein. These three precipitates were dialyzed against distilled water or acetate buffer and stored frozen until analyzed.

An attempt was made to duplicate this work in this laboratory; however, Prusoff's results could not be duplicated. In the first place no precipitate was formed at pH 1.5. Secondly, the inhibitory activity was spread throughout the fractions. The procedure given above has not been published and it is probable that some important detail of the procedure is missing.

A new procedure was tried in which 5 ammonium sulfate fractions were prepared by direct precipitation upon the Ventriculin extract. Twenty g. of Ventriculin were suspended in approximately 80 ml. of distilled water and allowed to stand for one hour. Ventriculin swells up to about double its volume when water is absorbed. The solid material was removed by centrifugation and extracted two more times with distilled water. The supernatant solutions were combined making the final volume of the extract 100 ml. Five precipitations with ammonium sulfate were made on the combined extracts. The concentration of ammonium sulfate was increased by 15.76 g. each time to give fractions which were 20, 40, 60, 80, and 100 per cent saturated with this salt. After each addition of ammonium sulfate, the pH was adjusted to 4.5 and the solution allowed to

stand 30 to 40 minutes for precipitation to take place. The precipitate was then centrifuged and the supernatant decanted. The precipitates were air dried until time for analysis. No precipitate was obtained from the 100 per cent saturated solution.

For bacterial assay the precipitates were dissolved in distilled water and made up to 100 ml. Dilutions 1:1,000, 1:5,000, 1:10,000, 1:50,000, and 1:100,000 were also made. In the assay 1 ml. of solution was added per tube, before autoclaving, to one set of tubes containing 0.15 mμg. of vitamin B_{12} and to another set of tubes without vitamin B_{12}. The solutions were also added to a set of sterilized tubes, each containing 0.15 mμg. vitamin B_{12} and to another set of sterile tubes without vitamin B_{12}.

The results of these assays are presented in Table 9. The data for the 80 per cent fraction are plotted in Figure 4. The residual solution and the 20 per cent fraction contained no inhibitory activity. The 40, 60, and 80 per cent fractions were all active, but the 80 per cent fraction was most active. The results indicate that there is a factor in the Ventriculin extract which is inhibitory to the growth of L. leichmannii with vitamin B_{12}. This is shown by the minimum in the curve in Figure 4 where no growth occurs. There is a factor stimulatory to growth in high concentrations of the extract. All fractions at a

Table 9.

Inhibitory action of Ventriculin Extracts Obtained by Ammonium Sulfate Precipitation at pH 4.5

Dilution	Titration values, ml. 0.1 N NaOH[a]											
	0.15 mμg. B_{12}			0.15 mμg. B_{12} and heated			No B_{12}			No B_{12} and heated		
	(20-40)	(40-60)	(60-80)	(20-40)	(40-60)	(60-80)	(20-40)	(40-60)	(60-80)	(20-40)	(40-60)	(60-80)
1:100	9.0	6.0	10.6	11.2	10.8	11.9	8.9	5.9	10.6	10.8	9.8	11.6
1:1,000	2.6	1.8	4.3	10.4	10.7	11.0	2.8	1.7	4.2	4.9	4.8	10.4
1:5,000	1.7	2.8	1.5	10.6	--	10.9	1.4	0.8	1.4	3.9	3.6	9.4
1:10,000	9.2	10.6	5.7	10.6	--	10.9	0.6	0.5	0.6	0.8	1.6	4.0
1:50,000	10.2	11.1	10.2									
1:100,000	10.3	11.2	10.4									

[a] 0.15 mμg. vitamin B_{12} = 10.5 ml.

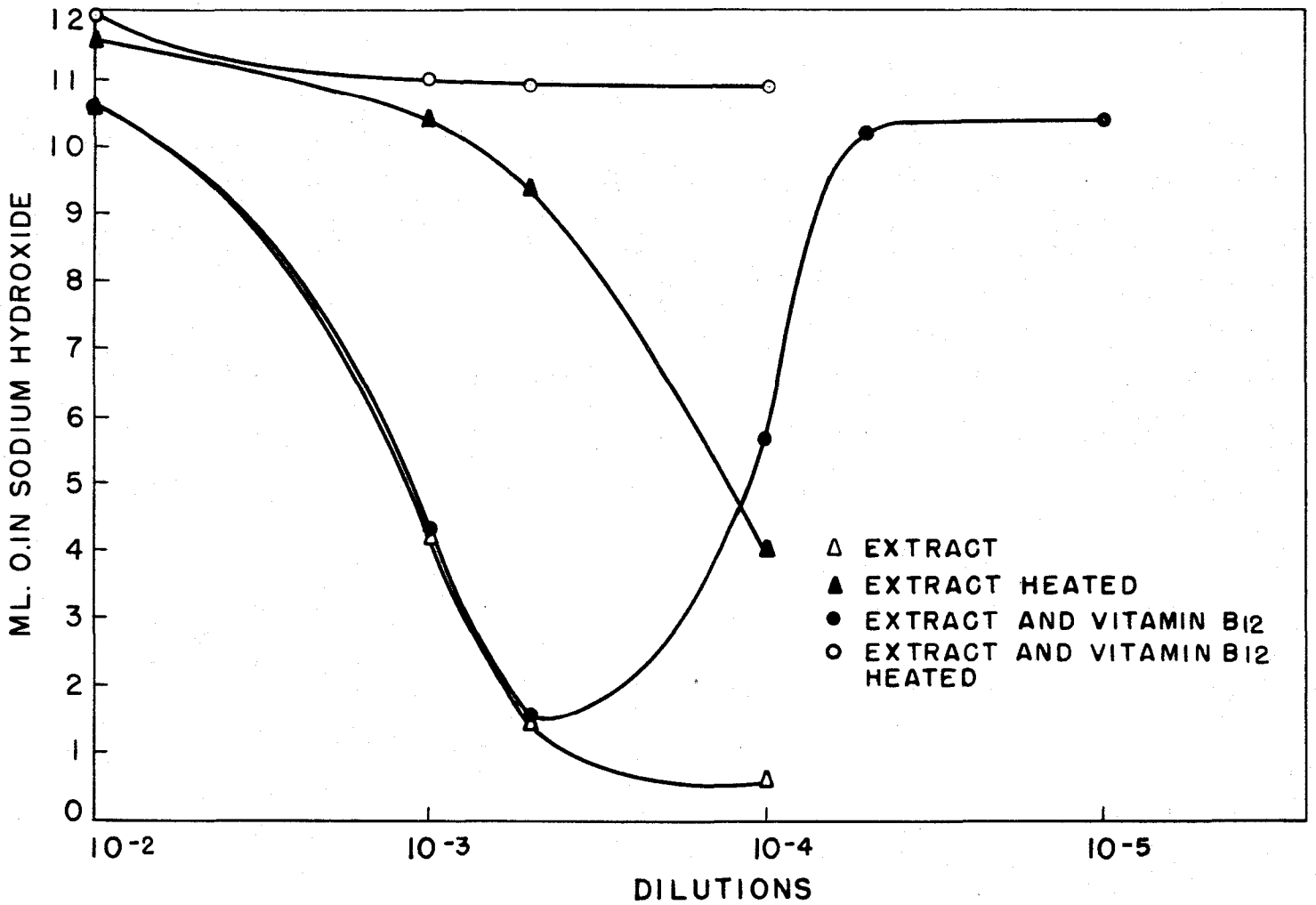


Figure 4. Growth of L. leichmannii in the Presence of 80 Per Cent Saturated Ammonium Sulfate Fraction.

dilution of 1:100 supported abundant growth with and without vitamin B_{12}. When the fractions were heated in the tubes with the medium, growth in excess of the amount caused by 0.15 mμg. of vitamin B_{12} was observed. The stimulatory factor could be diluted out with retention of the inhibitory activity as shown by the V-shaped curve. Heating destroyed the effect of the vitamin B_{12}-inhibitor but did not destroy the growth factor.

Ammonium sulfate in the concentration at which it appeared in the extracts did not interfere in the assay. One ml. of a 10 per cent saturated ammonium sulfate solution was added to an assay tube containing vitamin B_{12} and the amount of growth did not differ from that in one which contained no ammonium sulfate.

Extracts were tested after they had stood in the refrigerator for one week and no loss in activity was observed.

The presence of a growth factor which replaced vitamin B_{12} was undesirable because it complicated the assay of the inhibitor, although in high concentrations of the inhibitor the growth factor could be diluted out. In this experiment the separation of the inhibitory fraction from the inactive material was not complete because the inhibitory activity was spread through all the fractions. Therefore, the fractionation was performed at a different pH to see if the inhibitory activity could be separated from the growth activity and the inactive protein.

Extraction of Ventriculin was performed as described earlier. The pH of the extract was adjusted to 6.5. Ammonium sulfate was added in increments to make solutions 30, 60, and 80 per cent saturated with respect to ammonium sulfate. The fractions were dialyzed against distilled water and stored frozen. Appropriate dilutions of the fractions were made with distilled water. One ml. aliquots of the dilutions were tested for inhibitory activity. The results of this experiment are shown in Table 10.

Table 10.

Inhibitory Activity of Ventriculin Fractions Obtained by Ammonium Sulfate Precipitation at pH 6.5

Dilution	Titration values, ml. 0.1 N NaOH[a]		
	Fraction (30)	Fraction (60)	Fraction (80)
1:100	0.5	2.0	7.4
1:1,000	5.2	0.8	1.8
1:5,000	---	0.4	0.2
1:10,000	6.5	6.5	6.1
1:100,000	---	6.8	7.1

[a] Each tube contained 0.15 mμg. vitamin B_{12} = 7.0 ml.

It can be seen that only a trace of activity is present in the first fraction (30). The 1:100 dilution only gives inhibition of growth. The second (60) and third (80) per cent saturated fractions have approximately equal

amounts of inhibitory activity and the third fraction contains the stimulatory factor also, as can be seen by comparing the second and third columns in Table 10. In the third column, for example, the 1:100 dilution promotes growth in excess of the 7 ml. of 0.1 N NaOH due to the crystalline vitamin B_{12} added. In the 1:5,000 dilution, the growth factor was diluted out and only the inhibitor was present. However, in the 1:10,000 dilution, the inhibitor is practically diluted out and in the 1:100,000 dilution, growth was caused only by the added vitamin B_{12}.

Fractionation of hog stomach mucosa extracts with ethanol

Twenty g. of Ventriculin were extracted with distilled water as described previously. The final volume of the extract was 120 ml. and the pH 5.35. Ten ml. of the extract were put in a 50 ml. centrifuge tube and chilled in an ice salt bath kept at 0 to -4.0° C. Absolute ethanol was added dropwise with stirring. The tubes were allowed to stand 15 to 20 minutes and were then centrifuged for 2 minutes, using centrifuge cups previously chilled in the ice bath. The precipitates were diluted immediately to 100 ml. with distilled water. The following amounts of ethanol were added consecutively: 2.5, 2.5, 2.5, 5.0, 7.5, 10, and 10 ml. making a final volume of 40 ml. of ethanol, or approximately 80 per cent. Seven fractions were obtained in this way. Appropriate dilutions were made and 1 ml. of each dilution

was tested for inhibitory activity. The results of this experiment are presented in Table 11.

The first three fractions were cloudy, the remainder clear. Those containing the most inhibitory activity were yellow-brown in color. The most active inhibitory fractions were numbers 3 and 4; the growth factor was still present, however. The results obtained with ethanol fractionation are similar to those obtained with ammonium sulfate. The original extract contained such a high concentration of growth substances that no estimate of its inhibitory activity could be made, since the inhibitory principle was diluted out along with the growth substances. Some values are presented in Table 12.

The original extract was analyzed for nitrogen by the Kjeldahl method and contained 3.20 mg. of nitrogen per ml.

The alcohol precipitation was repeated at pH 4.5 using an extract prepared from 20 g. of Ventriculin and diluted to 80 ml. Absolute ethanol was added dropwise in the following aliquots: 5, 5,5, 10, and 15 ml. After each addition of alcohol the precipitate was removed by centrifugation, dissolved in 50 ml. of distilled water, and 1 ml. samples of appropriate dilutions were tested for inhibitory activity. The results of this experiment are presented in Table 13. A comparison of these results with those presented in Table 11 shows that they are similar in many respects. However, at pH 5.35 a better separation from inert material was accomplished.

Table 11.

Inhibitory Activity of Ventriculin Fractions Obtained by Ethanol Precipitation at pH 5.35

Dilution	Titration values, ml. 0.1 N NaOH[a]						
	F1	F2	F3	F4	F5	F6	F7
1:100	5.7	4.0	7.0	5.8	5.1	8.1	8.8
1:1,000	6.8	7.8	3.6	3.0	7.2	8.7	8.7
1:10,000	8.0	8.9	8.1	7.3	8.5	---	---
1:100,000	8.0	8.7	8.7	8.6	---	---	---

[a] 0.1 mμg. vitamin B_{12} = 8.7 ml.

Table 12.

Assay of Ventriculin Extract for Inhibitory Activity

Dilution	Titration values, ml. 0.1 N NaOH[a]	
	With 0.1 mμg. vitamin B_{12}	No vitamin B_{12}
1:10	6.1	5.8
1:100	7.7	4.6
1:1,000	6.6	2.2
1:10,000	8.4	

[a] 0.1 mμg. vitamin B_{12} = 8.7 ml.

Table 13.

Inhibitory Activity of Ventriculin Fractions Obtained by Ethanol Precipitation at pH 4.5

Dilution	Titration values, ml. 0.1 N NaOH[a]				
	F1	F2	F3	F4	F5
1:50	5.0	4.2	2.2	2.8	5.6
1:5,000	2.8	2.2	0.6	3.0	6.3
1:50,000	5.0	5.5	5.8	5.6	6.5

[a] 0.1 mμg. vitamin B_{12} = 6.5 ml.

Kjeldahl analyses for nitrogen and biuret determinations for protein were run on the original extract and the fractions obtained. The results of these determinations are presented in Table 14. The original Ventriculin

Table 14.

Protein Values for Ventriculin Precipitates Obtained by Kjeldahl and Biuret Analyses

Sample tested	Protein by Kjeldahl mg.	Protein by biuret mg.
Original extract	281	200
Fraction 1	50.5	51.0
Fraction 2	14.8	15.0
Fraction 3	8.6	9.0
Fraction 4	14.5	13.0
Fraction 5	7.7	7.5
Supernatant	187.5	102.5
Total protein recovered	283.6	198.0

extract contained 4.5 mg. of nitrogen per ml. or 28.1 mg. of protein. Almost one-third of the nitrogen extracted was non-protein nitrogen, according to the values found for the biuret and Kjeldahl analyses of the original extract. The third fraction which is inhibitory for 0.1 mμg. of vitamin B_{12} at a 1:5,000 dilution contained 0.0018 mg. of protein in 1 ml. of this dilution. There is good agreement between the results obtained with the biuret and the Kjeldahl methods in the protein values for the precipitates.

Fractionation with ethanol at pH 6.85, following the procedure as outlined above, gave the same results. No separation of either growth factors or inactive proteins was obtained.

Fractionation of hog stomach mucosa extract with ammonium sulfate and ethanol

A combination of the ammonium sulfate and ethanol fractionation was tried next. It was thought that the ammonium sulfate could be used first to separate out the soluble protein materials from the non-protein materials in the extract. With the protein thus separated other substances could not be carried down in the ethanol precipitation. The procedure developed for this method was as follows.

Two hundred g. of Ventriculin were extracted with distilled water and made up to 1 liter. To the extract at

pH 5.2 was added 788 g. of ammonium sulfate, stirred until dissolved, and allowed to stand for 30 minutes. The precipitate was removed by centrifugation and diluted with distilled water to 250 ml. One hundred ml. of this thick suspension were dialyzed in a cellophane tube against running tap water for 6 hours and then dialyzed against distilled water for 24 hours in a refrigerator. The solution was diluted to 200 ml. and the pH adjusted to 5.2. The precipitate which formed was removed by centrifugation (ppt. 1). The supernatant was cooled to 0° C. in an ice bath and ethanol was added dropwise with mechanical stirring. Three portions of absolute ethanol were added giving approximately 30, 60, and 80 per cent by volume alcohol. The precipitate obtained after the addition of alcohol was recovered by centrifugation and the supernatant decanted and cooled in an ice bath before the next portion of ethanol was added. No precipitate was obtained above 60 per cent alcohol. One ml. of the appropriate dilutions of the precipitates in distilled water was tested for inhibitory activity. The results of this experiment are presented in Table 15. The second alcohol precipitate was still active at a dilution of 1:50,000. A trace of inhibitory activity remained in the other precipitates. The growth factor was also more concentrated in the second precipitate.

This method did not seem to have any advantages over the previous salt or ethanol precipitations. Furthermore,

Table 15.

Inhibitory Activity of Ventriculin Fractions Obtained by Ammonium Sulfate--Ethanol Precipitation at pH 5.2

Dilution	Per cent transmission[a]		
	Precipitate (1)	Precipitate (0-30)	Precipitate (30-60)
1:100	---	46	18
1:10,000	100	74	100
1:50,000	---	--	100
1:100,000	53	50	77
1:1,000,000	---	--	53

[a] 0.1 mμg. vitamin B_{12} = 48.

it was carried out under conditions which could not be fully controlled, such as variations in the temperature during precipitation. Ethanol precipitations should be carried out at controlled temperatures in a cold room to obtain good separations without causing protein denaturation.

Fractionation of hog stomach mucosa extract by acetone-isoelectric precipitation

An extraction procedure for the substance in Ventriculin which makes vitamin B_{12} unavailable to bacteria was adapted from a report by Glass and associates (52) for the isolation from gastric juice of a mucoprotein with intrinsic factor activity.

Two hundred g. of Ventriculin were stirred for 1 hour with 700 ml. of distilled water. The precipitate was removed by centrifugation and 300 ml. of supernatant solution were collected. Two more extractions with 300 ml. of water each time were carried out and a total volume of 925 ml. of extract was obtained. This was diluted to 1 liter before proceeding. The pH was adjusted to 2.0 by the addition of concentrated hydrochloric acid. A white precipitate was formed. Then 500 ml. of a 10 per cent solution of trichloroacetic acid were added and the solution was allowed to stand for 20 minutes. The precipitate was removed by centrifugation and 1430 ml. of supernatant were collected. To this brown solution were added 1.5 volumes of acetone (2145 ml.) and the mixture was allowed to stand at room temperature for 2 hours. Upon centrifugation a white precipitate was obtained which was then dissolved in 50 ml. of 0.1 N sodium hydroxide giving a brown solution. To this solution was added 0.1 N hydrochloric acid until the pH reached 1.85. The solution was allowed to stand for 2 hours to permit formation and coagulation of the precipitate. After centrifugation the precipitate was dissolved in 20 ml. of 0.1 N sodium hydroxide. The solution was then acidified to pH 1.9 with 0.1 N hydrochloric acid. This solution was allowed to stand for 1 hour, centrifuged, and the precipitate washed once with a mixture of equal parts of acetone and water and then washed twice with acetone

alone. The precipitate was allowed to dry at room temperature. The yield was 532 mg. of a brown amorphous material.

For inhibition analysis 100 mg. of this material were dissolved in distilled water with the addition of a few ml. of 0.1 N sodium hydroxide (when the material was found to be insoluble at acid or neutral pH) and diluted to 50 ml. The pH was 11.45 and the solution had a yellow-brown color. Several dilutions were made and one ml. of each used per tube containing 0.1 mμg. of vitamin B_{12}. These solutions were added to the tubes after autoclaving and the pH was not adjusted on the solutions since the medium had enough buffering power to neutralize the excess base. The tubes were titrated 22 hours after inoculation. The results of this experiment are presented in Table 16.

Table 16.

Inhibitory Activity of a Ventriculin Fraction Obtained by Acetone-Isoelectric Precipitation

Amount of substance tested mg.	Vitamin B_{12} added mμg.	Titration values, ml. 0.1 N NaOH
---	0.1	4.9
2	0.1	2.2
0.2	0.1	0.6
0.1	0.1	1.4
0.02	0.1	3.9
0.01	0.1	4.4
0.004	0.1	4.8
---	---	0.5

Although the isolated material contained vitamin B_{12}-inhibitory activity, it was not as active as the precipitate obtained by ethanol fractionation which had complete inhibitory activity for 0.1 mμg. vitamin B_{12} in 0.0018 mg. of protein per ml. of fraction compared with 0.2 mg. of protein per ml. here for 0.10 mμg. vitamin B_{12}. Since most of the active material was lost by this procedure either by precipitation in some step or by denaturation, it did not seem profitable to pursue this method any further.

Fractionation of hog stomach mucosa extract with ethanol and salt with controlled ionic strength

In order to permit a maximum separation of protein with a minimum increase in volume of solution a procedure developed by Cohn and co-workers (33) was adapted to the separation of components of Ventriculin extract. In this method, alcohol concentration and pH remain practically constant and the salt concentration is changed. A combination of change in ionic strength and the addition of heavy metals causes the protein to precipitate. The heavy metals can be removed from the protein by adding citrate which forms complexes with them.

Ten g. of Ventriculin were extracted with 2 per cent sodium citrate adjusted to pH 6.4 by the addition of 1 M acetic acid. The mixture was allowed to stand one hour

then mixed in a blender, centrifuged, and the residue extracted again with sodium citrate. Citrate was used for extraction in order to duplicate conditions under which blood is collected. Twenty ml. of extract collected after the first extraction were combined with 10 ml. obtained after the second extraction. Twenty-five ml. of extract were used in the procedure of Cohn. All work was conducted in a cold room at 2° C., the solutions having been brought to that temperature before use. One hundred ml. of a solution containing 250 ml. of 95 per cent ethanol and 2.5 ml. of 0.8 Γ/2 pH 4.0 sodium acetate per liter were added at a rate of 20 ml. per minute with stirring. The precipitate was removed by centrifugation for 15 minutes at 4000 r.p.m. The supernatant was decanted and the precipitate dissolved in 20 per cent sodium citrate solution (pH 6.4). Then 10 ml. of a freshly prepared solution containing 200 ml. of 95 per cent ethanol and 54.8 g. of zinc acetate dihydrate per liter were added to the supernatant, allowed to stand 15 minutes and centrifuged for 15 minutes at 4000 r.p.m. The precipitate was dissolved in sodium citrate solution (pH 6.4). The supernatant solution had a volume of 125 ml. and a pH of 5.55. For analysis the two protein precipitates were diluted to 25 ml. each with sodium citrate.

The results of the nitrogen determinations on extract and precipitates by the Kjeldahl method are shown in Table 17.

Table 17.

Nitrogen Values of Extract and Precipitates Obtained by Ethanol-Salt Precipitation

Sample	Nitrogen per ml. sample mg.	Nitrogen of total sample mg.
Original extract	5.71	142.8
Precipitate 1	0.35	8.8
Precipitate 2	0.97	24.3

Only 23 per cent of the nitrogen in the original extract was precipitated.

One ml. samples of appropriate dilutions of the extract and precipitates were tested for inhibitory activity. The results of this experiment are presented in Table 18.

Table 18.

Inhibitory Activity of Ventriculin Fractions Obtained by Ethanol-Salt Precipitation

Dilution	Per cent transmission[a]			
	Original extract	Precipitate 1	Precipitate 2	Supernatant
1:25	28	48	9	--
1:2,500	80	52	100	--
1:12,500	--	--	---	86
1:250,000	--	--	50	--

[a] 0.1 mμg. vitamin B_{12} = 50.

Precipitate 1 was not inhibitory to bacterial growth, while precipitate 2 contained the growth factors as well as the inhibitory principle. This active precipitate gave a brown solution characteristic of previously isolated active materials. The supernatant, however, still contained inhibitory factors.

The procedure was repeated with another 25 ml. portion of extract prepared in the same manner with the purpose of purifying the inhibitory factor contained in the second precipitate. After the separation of precipitates 1 and 2, further fractionation was performed on the latter. Extraction was carried out at 2° C. for 1.5 hours with 175 ml. of a reagent containing 160 ml. of 95 per cent ethanol, 2.6 g. of barium acetate, 20 ml. of 1 M sodium acetate, and 7.3 ml. of 1 M acetic acid per liter. The mixture was centrifuged for 20 minutes at 4000 r.p.m. The supernatant was decanted and extracted with 25 ml. of a reagent containing 160 ml. of 95 per cent ethanol, 50 ml. of 1 M sodium acetate, and 0.1 g. of zinc acetate dihydrate per liter. The mixture was centrifuged for 20 minutes at 4000 r.p.m. The supernatant was decanted and the precipitate dissolved in 2 per cent sodium citrate. The nitrogen values of all fractions as determined by the Kjeldahl method are presented in Table 19.

One ml. samples of appropriate dilutions of the

Table 19.

Nitrogen Values for Ventriculin Fractions Obtained by Controlled Alcohol-Salt Precipitation

Fraction tested	Amount available ml.	Total nitrogen mg.
Original extract	25	140.5
Precipitate 1	30	8.2
Precipitate 2	30	30.7
Solution 1	250	95.5
Extract 1	175	15.8
Extract 2	50	5.0
Precipitate 3	25	15.0

fractions were tested for inhibitory activity. The results of this experiment are presented in Table 20. The data (Table 20) show that separation of vitamin B_{12}-inhibitor was not achieved by further fractionation according to the Cohn scheme. The activity is spread throughout the fractions.

Table 20.

Inhibitory Activity of Ventriculin Fractions Obtained by Ethanol-Salt Precipitation and Further Fractionation

Dilution	Per cent transmission[a]					
	1	2	3	4	5	6
1:1	24	8	11	29	82	14
1:10	52	57	41	94	100	100[b]
1:100	57	99	90	48	53	92
1:1,000	42	56	--	--	--	45

[a] 0.1 mμg. vitamin B_{12} = 40.

[b] Dilution 1:25.

Chromatography and Ion Exchange

There are many reports in the literature of resolution of proteins and enzymes by chromatography on paper. Buffers and other salt solutions have been used as developing agents. Dilute salt solutions have generally been used for ascending chromatography and the proteins have been detected by reagents which color them.

Franklin and Quastel (49) suggested the use of 0.05 M and 0.1 M acetate buffers and 1 to 2 per cent salt solutions as developing agents. According to their procedure 50 ml. of 0.2 M sodium acetate were adjusted to the desired pH with 2 N hydrochloric acid and the volume was made up to 200 ml. Sodium acetate buffers of the following concentrations were used: 0.05 M at pH 5.0, 0.05 M at pH 8.5, and 1 M at pH 6.0. Two per cent sodium citrate at pH 7.4 was also tried.

Solutions of inhibitory factor were applied to the paper, allowed to dry, then placed in the jar with the developing agent. At room temperature the solvent required about 1.5 hours to reach the top of the paper. The paper was removed and allowed to dry in the air. In order to locate the substances on the paper it was cut lengthwise in strips and two strips were sprayed with reagents which would react with as many components as possible. One strip was sprayed with an alcoholic solution of silver nitrate and the other with ninhydrin, 0.1 per cent in butanol. The strips

were dried at 110° C. to develop the spots. Ninhydrin gave a violet color with proteins, peptides, and amino acids. Silver nitrate produced gray colored spots of reduced silver. When dilute salt solutions were used, all of the substances colored by these reagents moved to the top of the paper. Ninhydrin showed one large spot; silver nitrate showed a spot in the same place and one just below it.

Inhibitory activity for vitamin B_{12} was determined in the following way. The paper strips were cut into equal segments and placed in assay tubes containing 10 ml. of medium and 0.1 mμg. of vitamin B_{12}. The tubes were shaken gently to aid extraction of substances from the paper, then inoculated and incubated. Growth appeared in all tubes except in the one containing the top segment of the paper. All inhibitory material had moved to the top of the paper as shown in Table 21. Different pH values did not effect

Table 21.

Assay of Vitamin B_{12}-Inhibitor in Paper Strip Chromatogram

Segment Tested	Per Cent Transmission[a]
Bottom	40
Middle	39
Top	100

[a] 0.1 mμg. vitamin B_{12} = 38.

the separation of the inhibitory factor from other substances present in the extract because all chromatograms were the same.

Since dilute salt solutions failed to make a separation, concentrated solutions were tried. Developing solutions of sodium citrate containing 10, 30, 40, 50 and 60 g. of sodium citrate each added to 100 ml. portions of distilled water were prepared. Sodium citrate was chosen because it is not inhibitory to the growth of the microorganisms used in the assay. Preparation of the chromatograms was performed as described earlier. The strips were sprayed with ninhydrin and spots appeared at the top as in the previous experiments. However another violet streak appeared at the bottom, arising from the point of application. With the use of more concentrated solutions of sodium citrate, the streak diminished and most of the inhibitory activity remained at the point of application.

Using 60 g. of sodium citrate in 100 ml. of distilled water no growth was observed in the tube containing the bottom segment (No. 1) of the paper as is shown in Table 22. There was very little spreading of inhibitor up the strip; the second strip gave a reading of only 60. The top segment which showed a deep violet spot with ninhydrin contained no inhibitory principle.

The previous experiments with paper chromatography indicated that absorption of inhibitor on cellulose

Table 22.

Assay of Vitamin B_{12}-Inhibitor in Chromatogram Developed in 60 g. Citrate and 100 ml. Water

Segments of paper arranged in ascending order	Per cent transmission[a]
1	100
2	60
3	48
4	41
5	40
6	40

[a] 0.1 mμg. vitamin B_{12} = 38.

materials could be used as a method of purification of the vitamin B_{12}-inhibitor from the Ventriculin extract. The filter paper chromatopile suggested by Mitchell, Gordon, and Haskins (79) or a column of powdered cellulose were considered as possible methods of purification of large quantities of extract. Both methods employ a liquid flowing downward through the column. Since ascending technique had been used on filter paper the effect of change in direction of flow upon the separation was unpredictable.

A filter paper pile was constructed in the following way. Seven disks of 9 cm. Whatman No. 1 filter paper were used to absorb 5 ml. of the Ventriculin extract. These disks were dried and placed in position 40 sheets from the bottom of a pile of 300 disks. The pile was clamped

together, placed in a glass jar (35 cm. diameter) with a solution containing 60 g. of sodium citrate in 100 ml. of distilled water so that the liquid was below the level of the impregnated disks. The solvent was allowed to rise to the top of the filter paper pile. The impregnated disks were removed and extracted with water, as were the other disks. The extracts were tested for inhibitory activity. The results of this experiment are presented in Table 23.

Table 23.

Assay of Vitamin B_{12}-Inhibitor in Fractions Extracted from Chromatopile

Dilution	Per cent transmission[a]		
	Original extract	Extract 2	Extract 3
1:20	62	100	39
1:200	85	100	37
1:2,000	43	40	36
1:20,000	34	38	36

[a] 0.1 mμg. vitamin B_{12} = 37.

The data show that most of the interfering substances had been removed in the chromatography. This may provide a better way of purifying larger quantities of materials than by single sheets of filter paper.

A cellulose column was prepared as follows. Alphacel (powdered cellulose) was suspended in 0.5 M sodium citrate solution and poured into a tube 2 cm. in diameter. The

column was 50 cm. long. A Ventriculin extract (50 ml., prepared from 10 g. of Ventriculin and designated extract No. 1) was added at the top of the column. Elution was performed with 0.5 M sodium citrate. Samples approximately 10 ml. in volume were collected at 15 minute intervals. One ml. of eluate and appropriate dilutions were used to test for inhibitory activity.

In a second sample of extract (No. 2) the proteins were precipitated in 80 per cent saturated ammonium sulfate solution. The ammonium sulfate was removed by dialysis against distilled water; then the solution was dialyzed against 0.5 M sodium citrate so that the sample would have approximately the same salt concentration as the eluting agent. Elution and analysis were performed as described for sample No. 1. The results of these two experiments are shown in Figure 5.

Using extract No. 1, the greatest amount of growth activity was collected in tubes 13 to 21. The growth factor was still associated with the inhibitor because eluates which permitted growth had inhibitory power after they were diluted. All of the inhibitory factor had passed through the column before tube 40 was reached. The data are plotted in curve A of Figure 5.

The results obtained for extract No. 2 were similar. A little of the growth factor had been removed in the precipitation and dialysis because the maximum growth obtained

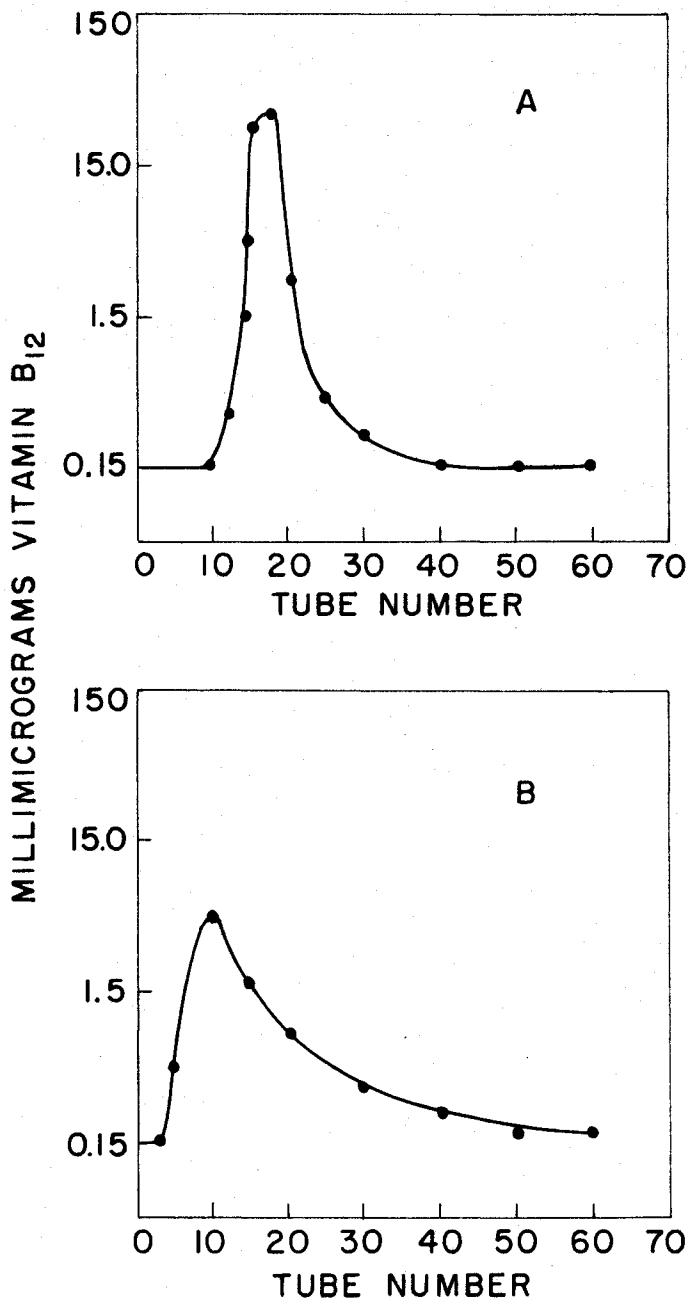


Figure 5. Inhibitory Activity of Ventriculin Fractions Obtained by Chromatography on Cellulose Columns.

gave a transmission of 15 in contrast to 5 per cent for the first extract. The vitamin B_{12} inhibitor was spread over a wide range, tubes 6 to 30, over twice the range encountered with extract No. 1. There was still a trace of activity in tube 60. The data are plotted in curve B of Figure 5. The fact that water was the solvent in the first fraction and 0.5 M sodium citrate in the second may account for this difference. There was no evidence that a separation of the inhibitory factor from the growth factors could be achieved by using chromatography on cellulose columns, so this method was abandoned.

Several reports have appeared on the separation of proteins by ion exchange methods. Hirs, Stein, and Moore (60) were able to prepare crystalline ribonuclease by passing it through resin IRC-50 and eluting with 0.2 M sodium phosphate buffer of pH 6.45. Tallen and Stein (106) developed a method for lysozyme and were able to separate it from diluted egg white using the same resin and a phosphate buffer at pH 7.2.

A column 44 by 1.5 cm. was prepared using the sulfonic cation exchange resin IR-120. The column was brought into the hydrogen cycle by passing one bed volume of 5 per cent hydrochloric acid solution through the column. Distilled water then was passed through the column until the eluate was no longer acidic. Twenty ml. of Ventriculin extract were brought to pH 8.0 with sodium hydroxide and poured on

the column. Elution was performed with a sodium phosphate buffer at pH 5.5. The flow rate was 2 to 3 ml. per hour. About 150 ml. of eluent were collected. All samples were inactive.

Another column (IR-120 resin) 36 by 1 cm. was prepared as described before. Thirty-eight ml. of the pooled fractions 14 through 17 of the first separation on cellulose (pages 76-77) were poured on the column. The material was eluted with distilled water at a rate of 5 ml. per minute. The eluate contained both the inhibitor and growth factors and had a characteristic brown color. The original Ventriculin extract (50 ml.) contained 5.0 mg. of nitrogen per ml., after passage through the cellulose and the ion exchange column, the sample (41 ml.) contained 1.0 mg. of nitrogen per ml. One ml. of a 1:100 dilution of this sample (0.01 mg. of nitrogen per ml.) gave complete inhibition of growth with 0.15 mμg. of vitamin B_{12}.

The use of ion exchange resins deserves further study. Although IR-120 resin was not satisfactory under the conditions used, other resins may be found which will separate the two substances.

Spectrophotometric Studies

Assuming that vitamin B_{12} forms a chemical compound with the unknown factor, it seemed that it might be possible to detect a change in the spectrum of vitamin B_{12} after it had reacted with this factor.

A solution containing approximately 55 µg. of vitamin B_{12} and 5 ml. of a solution containing the inhibitor (pH 7.0) were made up to 10 ml., incubated for 1 hour at 37° C., then read along with controls containing vitamin B_{12} alone and the protein solution alone in a Beckman ultraviolet spectrophotometer at wave lengths from 328 to 425 mµ. A peak appeared at 362 mµ. for vitamin B_{12} and at 409 mµ. for the protein. When the two were mixed, the spectra were additive and no effect of the constituents upon each other could be discerned (Figure 6). Since the concentration of the inhibitor was not sufficient to react with all the vitamin B_{12} present, it is probable that if any compound was formed it could not have been present in sufficient amount to affect the spectrum. The peak at 409 mµ. may not be due to any part of the active principle.

Wolf and associates (119) failed to find any change in the spectrum of vitamin B_{12} when gastric juice was added. It is possible that they did not have a high enough concentration of the intrinsic factor to react with all the vitamin B_{12} present as may have also been the case in this experiment.

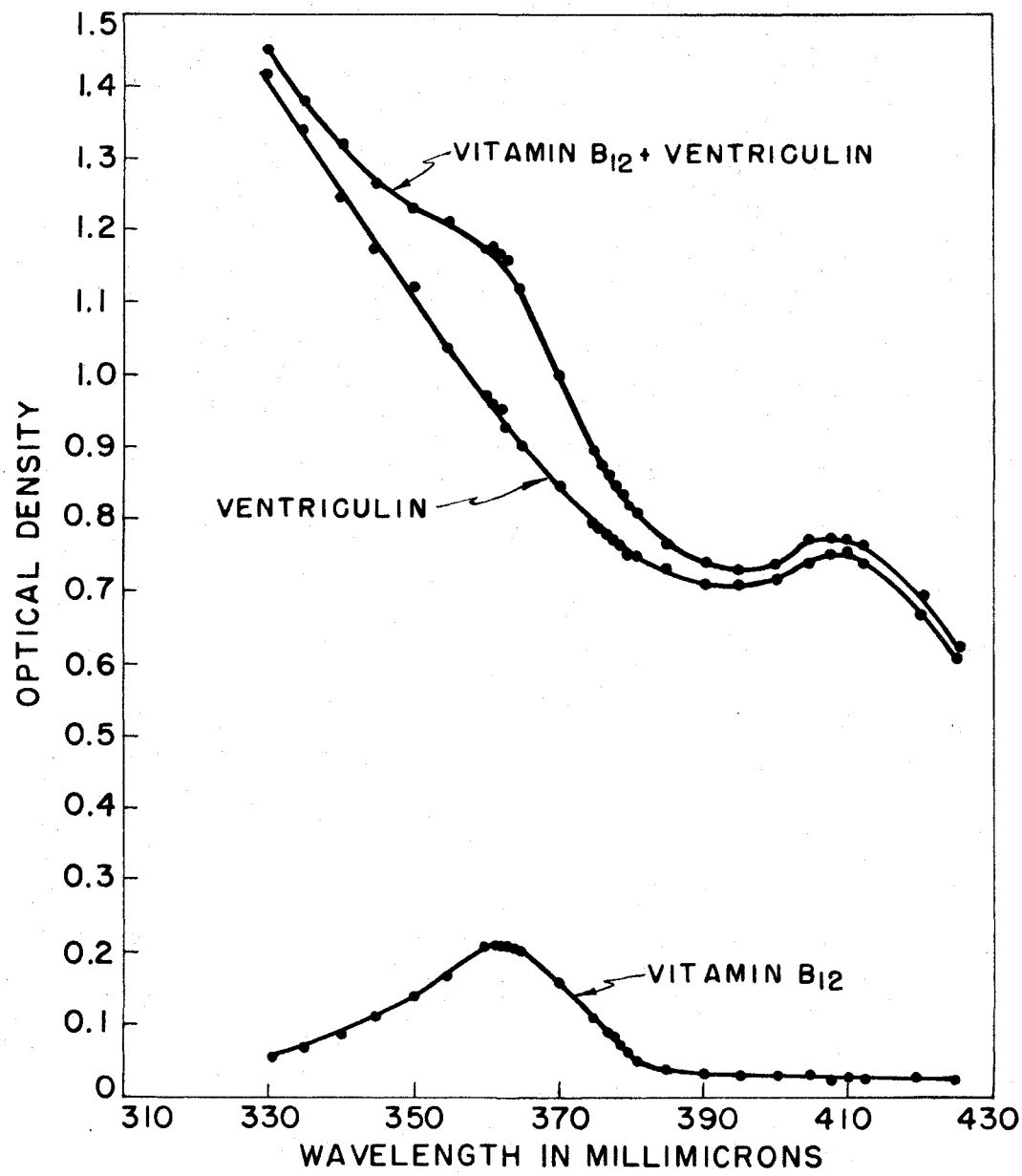


Figure 6. Absorption Spectrum of Vitamin B_{12} and Ventriculin Extract at pH 7.0.

Effect of Enzymatic Digestion of Ventriculin Extracts on the Vitamin B_{12} Inhibitory Principle

The effect of proteolysis on the vitamin B_{12}-inhibitory activity of the Ventriculin extracts was studied for two reasons. First to determine whether vitamin B_{12}-binding substances would be destroyed, secondly as a method of removing inert protein. Conflicting reports have appeared in the literature about the result of enzymatic treatment of vitamin B_{12}-binding substances.

Bethell and coworkers (14) treated intrinsic factor and vitamin B_{12} at pH 8.0 with trypsin with no loss of activity. Burkholder (22) found that papain and trypsin had no effect upon the vitamin B_{12}-binding substances in mucosal extracts, but pepsin increased the activity. Gregory, Ford, and Kon (53) found that a vitamin B_{12}-binding substance in sow's milk was inactivated by pepsin.

The effect of proteolytic enzymes on the vitamin B_{12}-binding principle of mucosal extracts was studied in this laboratory as follows. Twenty-five ml. of Ventriculin extract, prepared as described previously were added to a 25 ml. of trypsin solution prepared in the following manner. An amount of 0.025 g. of commercial trypsin was dissolved in 50 ml. of distilled water. This solution was brought to pH 7.0 with 0.05 M sodium carbonate. A solution of 0.53 g.

of sodium carbonate in 25 ml. of water was added to the mixture and the pH adjusted to 8.7. The mixture was covered with a layer of toluene and incubated at 37° C. for 24 hours. The pH at the end of the digestion was 8.95. The solution was brought to pH 7.0 and 325 ml. of saturated ammonium sulfate solution were added, giving 400 ml. of solution 80 per cent saturated with respect to ammonium sulfate. A trace of protein was precipitated and was removed by filtering with suction. The filter paper was extracted with 50 ml. distilled water. One ml. samples of appropriate dilutions of the precipitate and filtrate were tested for vitamin B_{12}-inhibitory activity. The results of this experiment are presented in Table 24.

Table 24.

Effect of Tryptic Digestion on Vitamin B_{12}-Inhibitor

Dilution	Per cent transmission[a]
Precipitate	
1:50	29
1:1,250	53
1:5,000	70
1:50,000	64
Filtrate	
1:400	30
1:2,000	70
1:20,000	48

[a] 0.1 mμg. vitamin B_{12} = 50.

It appears that most of the inhibitory activity was lost in tryptic digestion, since complete inhibition of 0.1 mμg. of vitamin B_{12} was not attained in any dilution. However, the filtrate contains inhibitory action also and it is possible that the separation with ammonium sulfate was not complete. The results are not conclusive.

Effect of Heating Ventriculin Extracts on the Vitamin B_{12} Inhibitory Principle

Several investigators have claimed that heating does not destroy the intrinsic factor. Hall and associates (56) heated extracts of hog mucosa at 100° C. for as long as 10 minutes without loss of hematopoietic activity. Klein and Wilkinson (72) had active material after heating it to 65° C. Both groups of workers heated the intrinsic factor in the presence of vitamin B_{12}. Ternberg and Eakin (107), Shaw (96), and Burkholder (22), all heated extracts of hog mucosa in order to make vitamin B_{12} available to bacteria. A vitamin B_{12}-binding substance in sow's milk was stable to heating at 100° C. for 30 minutes (53). Vitamin B_{12} is reported to be released from blood by heating (89, 90).

It already has been shown in previous assays that the vitamin B_{12}-inhibitory activity was lost when Ventriculin extracts were heated with the basal medium. The effect on the inhibitory activity when solutions containing the

vitamin B_{12}-inhibitor are heated separately had not yet been determined. Therefore, a sample of Ventriculin extract was heated in two different ways namely by autoclaving for 5 minutes at 15 lb. pressure and by boiling for 10 minutes. It was the purpose of this experiment to determine whether heat alone would inactivate the inhibitor in aqueous extracts of Ventriculin. The results of this experiment are given in Table 25.

Table 25.

Effect of Heating upon Vitamin B_{12}-Inhibitory Factor

Dilution	Per cent transmission[a]		
	Unheated	Autoclaved with medium 5 min. at 15 lb.	Heated 10 min. in boiling water
1:25	62	23	56
1:250	85	33	82
1:2,500	43	--	--
1:25,000	34	--	--

[a] 0.1 mμg. vitamin B_{12} = 37.

The results indicate that there was only a small loss in inhibitory activity (62 to 56 per cent transmission in the 1:25, and 85 to 82 in the 1:250 dilution) when heated 10 minutes in boiling water, whereas autoclaving with the medium released both vitamin B_{12} and the growth promoting substances, the transmission values being lower than those for 0.1 mμg. of vitamin B_{12}. It can be concluded that no

appreciable destruction of vitamin B_{12}-inhibitor occurs in dilute solutions when heated for 10 minutes in boiling water.

The effect upon the inhibitory power by autoclaving the extract separately and then adding it to the medium was next determined. The results of this experiment are shown in Table 26.

Table 26.

Comparison of Effect upon Inhibitory Factor of Autoclaving 5 Minutes at 15 Pounds Pressure with Boiling for 10 Minutes

Sample	Dilution	Per cent transmission[a]				
		Not heated	Autoclaved with medium		Autoclaved separately	Heated in boiling water
Extract 1	1:10	87	16	16[b]	21	21
Extract 1	1:100	100	54	--	70	86
Extract 2	1:10	75	35	15[b]	65	65
Extract 2	1:100	100	65	--	100	100

[a] 0.06 mμg. vitamin B_{12} = 56.

[b] No vitamin B_{12}.

It is apparent from the last two columns in Table 26 that autoclaving Ventriculin extracts for 5 minutes at 15 lb. steam pressure is equivalent to heating for 10 minutes in a boiling water bath. Again it is seen that only a slight loss of inhibitory activity occurs when dilute solutions are

heated as shown by the results obtained with the 1:100 dilutions of both extracts. Extract No. 1 at the 1:10 dilution was inactivated by each method of heating used. This does not agree with the results obtained with the other three dilutions. The inactivation might have been caused by some of the other substances in the extracts.

Effect of Certain Substances on the Vitamin B_{12} Inhibitory Principle

Previous experiments have shown that the vitamin B_{12} inhibitor is inactivated by heating in contact with the basal medium containing vitamin B_{12} and that the inhibitor when heated either in the autoclave for 5 minutes at 15 lbs. pressure or in a water bath for 10 minutes at 100° C. is not affected. The identification of constituent(s) in the medium responsible for the inactivation was the object of subsequent experiments. It was also believed that the study of the inactivation of the inhibitor would lead to some information about the nature of the vitamin B_{12} inhibitory principle.

Extracts of Ventriculin have been heated at pH values ranging from 5.5 to 7.0 and very little loss of inhibitory activity occurred, but it was lost when autoclaved in the medium at pH 5.5. The effect of heating at different pH values was determined in the following way. In this experiment (and all of the following) a Ventriculin extract was

diluted with distilled water so that it would give complete inhibition for 0.1 mμg. of vitamin B_{12} with a transmission of approximately 100 per cent. Ten ml. of the test solution were carefully adjusted to pH 2.5 with hydrochloric acid and another 10 ml. sample to pH 11.0 with sodium hydroxide so that no significant change in volume would occur. These two tubes and the one containing the diluted extract were heated for 10 minutes in a boiling water bath, then cooled and 1 ml. aliquots taken for the assay tubes. The results of this experiment (Table 27) show that the inhibitory power was destroyed by alkali, but not by acid; in fact it was increased slightly by heating in acid solution.

Table 27.

Effect of Heating Vitamin B_{12}-Inhibitory Principle in the Presence of Acid and Alkali

	Transmission[a]
Extract, not heated	90
Extract, heated	85
Extract and HCl at pH 2.5	94
Extract and NaOH at pH 11.0	46

[a] 0.1 mμg. vitamin B_{12} = 63.

Amino acids, salts, vitamins, and other constituents of the basal medium were heated with the diluted extract. One ml. of amino acid solution containing 10 mg. of the amino acid was heated with 4 ml. of the test solution. One

ml. containing 2 mg. of amino acid per tube in excess of the amount already present in the basal medium was used in the assay. Two-tenths ml. of 0.1 M salt solutions were used per 4 ml. of test solution and the volume made up to 5 ml. Other concentrations are given in Table 28. When both amino acids and salts were heated with the test solution, 1 ml., 0.2 ml., and 4 ml. were used respectively. The substances tested are presented in Table 28.

Only three significant reversals of vitamin B_{12}-inhibitory activity appear, caused by histidine with iron(II), cysteine with histidine, and purine and pyrimidine bases. The latter substances in high concentrations are growth factors which replace vitamin B_{12} for L. leichmannii; hence no effect upon the inhibitory factor is necessary. No reason for growth in the tube containing cysteine and histidine is apparent.

A theory for the reversal of the inhibition in the case of histidine and iron(II) ion may be postulated. The vitamin B_{12} molecule contains benzimidazole joined to cobalt. Histidine is an imidazole derivative and iron forms complexes similar to cobalt. In porphyrins iron is attached to nitrogen in pyrrole groups. Iron and histidine substitute for vitamin B_{12} by joining to the inhibitory factor in such a way that it can no longer react with vitamin B_{12}.

This hypothesis formed the basis for the next experiment in which histidine and benzimidazole with iron(II),

Table 28.

Effect of Heating Certain Substances with Vitamin B_{12}-Inhibitory Principle

Substance heated with extract	Transmission[a]
Extract alone	95
$MgSO_4 \cdot 7H_2O$	96
$FeSO_4 \cdot 7H_2O$	92
$MnSO_4$	96
L-arginine	99
DL-aspartic acid	97
L-cysteine	86
L-histidine	91
L-proline	95
L-tryptophan	98
L-tyrosine	98
DL-aspartic acid, $MnSO_4$	89
L-histidine, $MgSO_4 \cdot 7H_2O$	95
L-histidine, $FeSO_4 \cdot 7H_2O$	50
L-tryptophan, $FeSO_4 \cdot 7H_2O$	96
L-tryptophan, $MnSO_4$	91
L-proline, $FeSO_4 \cdot 7H_2O$	100

(Continued on next page)

[a] 0.1 mμg. vitamin B_{12} = 50.

Table 28. (Continued)

Substance heated with extract	Transmission[a]
L-cysteine, histidine	61
Sodium citrate (1 per cent)	92
Glucose (1 per cent)	81
Amino acid solution[b]	80
Purines and pyrimidine[b]	22
B_1, B_2, Ca-pantothenate, niacin[b]	99
Folic acid[b]	87
paraaminobenzoic acid[b]	86
B_6 solution[b]	98

[a] 0.1 mμg. B_{12} = 50.

[b] One ml. of solution used for preparing test solution.

manganese(II), and cobalt(II) ions were added to the test solutions containing the inhibitory factor, heated, and assayed. Salt solutions were used in the same concentrations as described previously. Histidine was used in 0.05 M and benzimidazole in 0.01 M solution. Portions of 2.5 ml. and 0.5 ml. of the salt solutions were mixed with 10 ml. of histidine solution and 10 ml. of benzimidazole solution, respectively, before they were added to the test solutions. All mixtures were adjusted to pH 5.5 to 6.0 before being heated.

The results for histidine in Table 28 do not check with those in Table 29. However, the results obtained with benzimidazole support the hypothesis. It should be mentioned that the mixture of inhibitory factor and substances

Table 29.

Effect of Heating Metal Ion-Imidazole Complexes with Vitamin B_{12}-Inhibitory Principle

Substances heated with extract	Transmission[a]
Extract alone	90
L-histidine + Fe^{II}	89
L-histidine + Mn^{II}	94
L-histidine + Co^{II}	91
Benzimidazole + Fe^{II}	59
Benzimidazole + Co^{II}	55

[a] 0.15 mμg. vitamin B_{12} = 50.

added has been prepared in two different ways. In the first experiment (Table 28), the test substances were added one at a time to the solution containing inhibitor and mixed between each addition. In the second experiment (Table 29), histidine was first mixed with iron(II) and cobalt(II) ions. The same amounts of the test substances were used in both experiments. Both techniques of mixing were repeated in a

subsequent experiment and similar anomalies occurred (Table 30).

Burk and associates (19, 20) have studied the reactions of histidine with metal ions. Histidine reacts instantaneously with cobalt(II) ion at 24° C. to form cobaltodihistidine. The complex is reversible. However, it slowly takes

Table 30.

Effect of Heating Metal Ions and Imidazoles with Vitamin B_{12}-Inhibitory Principle

Substances heated with extract	Transmission[a]
Extract alone	90
L-histidine and Fe^{II}	57
L-histidine and Fe^{II} [b]	83
L-histidine and Co^{II}	59
L-histidine and Co^{II} [b]	98
Benzimidazole and Fe^{II}	54
Benzimidazole and Co^{II}	41

[a] 0.15 mμg. vitamin B_{12} = 36.

[b] Substances were mixed before adding inhibitory principle.

up oxygen irreversibly. The reaction is 97 per cent complete at pH 7.5 and 74 per cent complete at pH 6.0 with 0.01 M histidine. Complexes of histidine with iron(II) and manganese(II) ions are not reversible. The iron(II) histidine compound is rapidly oxidized to the iron(III) compound.

In these experiments histidine and the metal ions were mixed in stoichiometric amounts and the conditions were favorable for the formation of their complexes.

It is apparent that the products formed from histidine and cobalt(II) or iron(II) cannot react with the vitamin B_{12}-inhibitor under the conditions described, but when they are added separately to the vitamin B_{12}-inhibitor reversal of inhibition is possible. It may be that a reaction between the inhibitor and histidine or cobalt is not possible because a bond required is being used in a cobalt histidine linkage.

Benzimidazole was found (120) to be a growth inhibitor for several strains of yeasts and bacteria. However, in this experiment benzimidazole with iron and cobalt neither promoted nor inhibited the growth of L. leichmannii (Table 31).

Table 31.

Effect of Iron, Cobalt, and Benzimidazole upon the Growth of L. leichmannii

Substance added	Transmission	
	0.15 mμg. Vitamin B_{12}	No Vitamin B_{12}
Benzimidazole and Fe^{II}	38	
Benzimidazole and Co^{II}	37	100
None	36	100

The inactivation of inhibitory factor in the presence of these compounds would be of physiological significance if the same reaction occurred at 37° C. Histidine and benzimidazole were incubated at 37° C. for 2 hours with iron(II) and cobalt(II) in the presence of inhibitory factor. The data in Table 32 indicate that no reaction with the inhibitor occurred at this temperature.

Table 32.

Effect of Incubating Metal Ions and Imidazoles with Vitamin B_{12}-Inhibitory Principle at 37° C. for 2 hours

Substance added	Transmission[a]
L-histidine and Fe^{II}	100
L-histidine and Co^{II}	100
Benzimidazole and Fe^{II}	100
Benzimidazole and Co^{II}	100

[a] 0.15 mμg. vitamin B_{12} = 36.

Table 33.

Effect of Heating Metal Ions and Vitamin B_{12}-Inhibitory Principle at pH 2.0

Substance added	Transmission[a]	
	37° C. for 2 hr.	100° C. for 10 min.
L-histidine and Fe^{II}	100	77
L-histidine and Co^{II}	100	47
Benzimidazole and Fe^{II}	100	63
Benzimidazole and Co^{II}	100	62

[a] 0.15 mμg. vitamin B_{12} = 36.

Heating at pH 2.0 was performed to test the effect of low pH upon the reversal of inhibition. It should be pointed out that intrinsic factor incubated with vitamin B_{12} at pH 2.0 is hematopoietically active. However, the mixture must be brought back to pH 5.0 or higher before being fed to a patient. The test mixtures were adjusted to pH 2.0, one set was incubated at 37° C. for 2 hours, the other set boiled at 100° C. for 10 minutes. The pH was then brought to 5.5 before the solutions were added to the assay tubes. The results (Table 33) do not differ significantly from those presented in Table 30.

Testing of Blood Plasma Protein Fractions for Vitamin B_{12} Inhibitory Principle

Blood is analyzed to determine nutritional status or changes in its composition caused by disease. When vitamin B_{12} was discovered, blood was one of the first tissues to be analyzed for this vitamin. Many investigators have found that dilution of the blood does not release all of the vitamin B_{12} for microbiological assay. Heating, enzymatic digestion and treatment with cyanide had to be used for preparing the samples. The nature of the binding substance was not known; however it appeared to be protein. Therefore, fractionation of blood into its components should assist in locating the binding factor. It was expected that

the substance(s) binding vitamin B_{12} in blood would react similarly with vitamin B_{12} in the bacterial assay causing an inhibitory effect analogous to that found for gastric mucosal extracts. Fractionation of blood serum was begun to separate, if possible, the vitamin B_{12}-binding proteins from other proteins.

Fractionation of blood plasma

Bovine plasma was separated into fractions by a method used by Cohn and his associates(33) for human plasma. This method has already been described under the section on fractionation of the Ventriculin extract (page 68), and will only be summarized briefly here.

Bovine blood was collected in a flask containing sodium citrate which removes calcium and prevents clotting. The cells were removed by centrifugation and a clear straw yellow plasma was obtained. The plasma and all reagents used were brought to 2° C. before the fractionation was begun. Ethanol-sodium acetate reagent was added to 25 ml. of plasma. The precipitate (Ppt. 1) was centrifuged and the supernatant (Soln. 1) decanted. All precipitates were dissolved immediately in 2 per cent sodium citrate solution to remove the metal ions from the protein as citrate complexes. The first precipitate is reported to contain the γ-globulin, β_1-lipoprotein, β_1-lipid-poor euglobulins, isoagglutinins, prothrombin, plasminogen, and fibrinogen. The

zinc-ethanol reagent was added to the supernatant to precipitate the albumins, β_1-metal-combining protein, glycoproteins, α_2-mucoprotein, choline esterase, and α_1-lipoprotein. The serum albumins were extracted with the ethanol-barium acetate-sodium acetate reagent (Ext. 1). The precipitate remaining after the extraction of the serum albumins was extracted with ethanol-sodium acetate-zinc acetate reagent to remove β_1-metal-combining protein and α_1-lipoprotein (Ext. 2).

Table 34.

Nitrogen Values for Blood Plasma Fractions Obtained by Cohn's Method

Fraction	Volume ml.	Total nitrogen mg.
Plasma	25	276.5
Precipitate 1	50	55.7
Solution 1	100	14.6
Extract 1	175	67.1
Extract 2	25	9.4
Precipitate 2R	50	127.1

The values given by Cohn (33) for per cent of each constituent in blood do not agree with the proportion of nitrogen obtained here (Table 34). For example, the serum albumin was too low and the precipitate (ppt 2R) remaining after extraction still contained more nitrogen than would

be found in the α_1-lipoprotein. The discrepancy may have been caused by the temperature difference, Cohn used -5° C., or the different kind of blood used. It could not be due to loss of nitrogen since 99 per cent was recovered.

Testing of the fractions for inhibitory activity

The results of the analysis for inhibitory activity are shown in Table 35. None of the fractions inhibited vitamin

Table 35.

Effect of Blood Plasma Fractions upon the Growth of L. leichmannii

Fraction	Per cent transmission[a]				
	Not diluted		Dilutions		
			1:10		
	Not dialyzed	Dialyzed	Not heated	heated	1:100
Plasma	--	--	39	--	--
Precipitate 1	30	54	37	29	38
Solution 1	40	--	36	34	37
Extract 1	29	64	41	34	38
Extract 2	36	68	40	34	36
Precipitate 2R	46	56	39	30	39

[a] 0.15 mμg. vitamin B_{12} = 34.

B_{12} activity, nor did whole blood serum. It is possible that the concentrations of protein were not high enough for detection of inhibition. Precipitate-2R, the only fraction showing inhibition, contained 2.5 times more protein per ml. than any other fraction tested. The inhibitory action towards

vitamin B_{12} was increased slightly by dialysis. This may mean that growth factors, purine and pyrimidine derivatives, were removed. Because of the difficulties encountered in this fractionation and the availability of already purified blood proteins this work was not continued.

Pure blood fractions

Human blood plasma proteins were obtained from Dr. E. J. Cohn. These proteins are described as follows: (I) 60 per cent fibrinogen and 40 per cent sodium citrate; (II) 100 per cent γ-globulin by electrophoresis; (III-3) unknown amounts plasminogen; (IV-7) 90 per cent β_1-metal-combining protein; (V) 95 per cent albumin and 5 per cent α_1-globulin; and acid glycoprotein (93 per cent α_1-globulin). Acid glycoprotein and plasminogen were dissolved in 0.05 M sodium citrate; the others were dissolved in water. The amounts used for testing inhibitory power and the results of this experiment are given in Table 36.

Albumin did not affect the growth of the organism in any amount tested. γ-Globulin increased growth at 12 mg. per tube and had no effect in lower amounts. Plasminogen and fibrinogen at 12 and 4 mg. amounts per tube contained enough impurities to increase the growth. Acid glycoprotein contained more growth promoting substances than any other plasma protein tested.

A definite inhibition of growth occurred with

Table 36.

Effect of Plasma Proteins upon the Growth of L. leichmannii

Protein	Per cent transmission[a] Amount per tube				
	12 mg.	8 mg.	4 mg.	0.4 mg.	0.04 mg.
Albumin	45	42	43	42	40
γ-globulin	29		39	39	39
Plasminogen	20		30	38	38
Fibrinogen	24		32	37	39
β_1-metal combining globulin	84	66	52	37	42
	4 mg.	1 mg.	0.1 mg.	0.01 mg.	
Acid glycoprotein	22	33	38	42	

[a] 0.15 mμg. vitamin B_{12} = 44.

Table 37.

Growth of L. leichmannii after Heating Plasma Proteins with the Basal Medium without Vitamin B_{12}

Protein	Amount per tube mg.	Per cent transmission
Albumin	4	100
γ-globulin	4	100
Plasminogen	4	87
Fibrinogen	4	100
β_1-metal combining globulin	4	86
Acid glycoprotein	1	46

β_1-metal-combining globulin. Amounts of 12, 8, and 4 mg. per tube showed inhibition. This inhibition may not be a specific inhibition of vitamin B_{12} because this protein binds iron and other metal ions. According to Surgenor, Koechlin, and Strong (105) 1.25 µg. of iron(II) ion are bound per mg. of protein at pH 7.0. Iron(III) is bound also. Blood contains 24 mg. of this protein per ml. The medium contains 10 µg. iron per tube. Under optimum conditions this amount of iron could be bound by 8 mg. of protein. However, the pH is not optimum and the medium contains other metal ions also. But the possibility of a linkage to the cobalt of vitamin B_{12} can not be excluded by this experiment.

It has been shown that heating proteins with the assay medium will release vitamin B_{12}. The plasma proteins were heated in the medium without vitamin B_{12} (Table 37). Albumin, globulin, and fibrinogen contained no growth substancees in 4 mg. amounts. β_1-Metal-combining globulin and plasminogen released traces of growth promoting substances. One mg. of acid glycoprotein contained growth factors equivalent to 0.1 mµg. vitamin B_{12}. In the previous experiments where vitamin B_{12} was present, this protein enhanced growth. Schmid (94, 95) has reported the properties of the acid glycoprotein but its function is not known.

Further investigations of the plasma proteins will probably disclose a vitamin B_{12}-protein relationship.

DISCUSSION

Many problems involved in the microbiological assay of vitamin B_{12} are not encountered in the determination of other vitamins with microorganisms. Vitamin B_{12} is not a specific nutrient for L. leichmannii or other organisms used for the assay of this vitamin. Purines, pyrimidines, and their compounds can substitute for vitamin B_{12} as growth factors for lactobacilli. Derivatives of vitamin B_{12}, such as hydroxocobalamin and histidine cobalichrome, have growth promoting properties similar to vitamin B_{12} in animals, whereas different responses are observed in microbiological assay. Pseudovitamin B_{12} and other vitamin B_{12}-like compounds are bacterial growth factors, but are not active for animal growth or in hematopoiesis.

A medium developed by Skeggs and associates (99) and modifications of it were tried as basal media for the microbiological assay of vitamin B_{12} with L. leichmannii. It was found that the sensitivity to vitamin B_{12} was increased when reducing agents were added, but the growth in the controls was also increased. If the enzymatically hydrolyzed casein was left out of the medium, the effect of reducing agents was decreased. Welch and Wilson (115) reported that the effect of reducing agents was to enhance the growth

promoting properties of substances in the enzymatically hydrolyzed casein. Complete elimination of growth in controls with good sensitivity to vitamin B_{12} was not obtained with this medium so the method was replaced by another.

Modifications were made on a basal medium proposed by Peeler, Yacowitz, and Norris (82). This medium contains amino acids in place of casein. Growth effects due to peptides or other substances in the casein hydrolysate are thus eliminated by the chemically defined composition. A study made of the amino acids showed that cystine, norleucine, and hydroxyproline could be omitted because they did not affect the growth of the organism. The cystine requirement can be met by bacterial synthesis from cysteine.

Another difference between the Skeggs and Peeler media is the concentration of the salts. The amounts of iron, manganese, and magnesium salts in the Peeler medium are higher than those in the Skeggs's medium and greatly in excess of the amounts needed by the organism for growth. Several levels of these salts were studied for their growth effect and it was found that wide differences in the amounts did not appreciably affect the growth although an amount five times that used by Skeggs gave the most reproducible results. Sodium citrate, which is used in the Peeler medium is a growth inhibitor for several microorganisms. Since good growth was obtained, it was retained in the medium. It may be that the test organism is adapted to the citrate.

Some of the fractions of the inhibitory factor tested contained citrate and the inclusion of citrate in the medium may cancel out the effect of adding it along with the inhibitory substances.

Additional amounts of vitamins did not show any effects upon the growth. The Peeler medium contained large amounts of B_6 vitamins. When the medium was autoclaved, these compounds promoted growth even in the absence of vitamin B_{12}, the growth being proportional to the amount of heating. For this reason it seemed advisable to decrease the amounts of these vitamins. The amounts of pyridoxine and pyridoxal were cut in half and pyridoxamine was omitted. It was found that the organism could use each of these compounds equally well.

The medium was autoclaved 5 minutes instead of being steamed because the heating was more easily controlled in this way. The conditions for heating the medium are very critical because the activity of vitamin B_{12} depends upon the time and temperature at which it is heated with the medium. Since the assay was used to determine the inhibitory factor, and standards were run in each assay for comparative purposes, absolute values for vitamin B_{12} were never determined.

Materials inhibitory to the growth of L. leichmannii, believed to be proteins, occur in all tissues. Extracts of gastric mucosa were found to be inhibitory to vitamin B_{12}

in bacterial assay. The presence of growth factors in these extracts complicated the assay of the inhibitory factor. Other investigators who used L. leichmannii for the assay of vitamin B_{12} have had similar difficulties with mucosal extracts (15, 96). These growth factors are heat-and alkali-stable so, for the most part, they probably consist of nucleic acid derivatives.

A great deal of effort was expended without success in trying to separate the growth factors from the vitamin B_{12}-inhibitory principle. No precipitating agent nor any of the conditions used have been found to give a concentrate of vitamin B_{12}-inhibitor free of growth substances. Dialysis removed a part of the growth substances, but not enough to prevent interference in the assay. Ammonium sulfate, ethanol, or salt and ethanol were successfully used to precipitate the vitamin B_{12}-inhibitory principle from Ventriculin. Although pure material was not obtained, much microbiologically inactive protein and a portion of the growth substances were removed in the precipitation processes. Dialyzed ammonium sulfate precipitates were used with reproducible results in testing the effect of other substances upon the inhibitor.

The disadvantage of these methods lies in the non-specific action of these precipitating agents in separating various proteins from each other. The Cohn method using ethanol and heavy metal salts appears to be the most useful

method because the handling of large quantities of salt and ethanol are avoided. However, modifications are necessary in order to adapt this procedure to the particular properties of the proteins in the gastric mucosal extracts. Although zinc and ethanol precipitated most of the inhibitory material from the extract, a little remained in the supernatant solution. Subsequent extractions on the precipitate were not satisfactory and additional work in varying the ionic strength and the species of metal ions needs to be performed in order to purify the inhibitory factor. Burkholder (22) also has used Cohn's zinc-ethanol reagent for precipitating the inhibitory factor.

It should be pointed out that Burkholder (22) used a mutant of E. coli to test the potency of vitamin B_{12} inhibitor. This organism is not affected by the growth substances present in the mucosal extracts; hence his assay has doubtful value in following a purification process because it has been shown in the work reported in this thesis that zinc and ethanol will not separate the growth factors from the inhibitory principle.

The acetone and acid precipitation method of Glass and associates (52) applied to Ventriculin yielded a product with little vitamin B_{12}-inhibitory action.

The inhibitory factor was found to be very stable; it will withstand freezing and drying and can be kept for weeks in a refrigerator without loss of activity. In dilute

solutions at neutral and acid pH it will withstand boiling, but its activity is readily destroyed by alkali. Ethanol and heavy metals used in the precipitation process did not affect it.

No change in the absorption spectrum of vitamin B_{12} was observed in the presence of gastric mucosal extracts. The extracts used in this laboratory probably did not contain enough inhibitory principle to react with all of the vitamin B_{12} if a stoichiometric reaction is assumed to occur. Wolf and associates (119) incubated vitamin B_{12} with gastric juice for a long period of time with the assumption that some enzymatic change would take place in the vitamin B_{12} molecule and observed no change in the absorption spectrum from pH 2.0 to 7.0. Since these workers used only 25 ml. of gastric juice a change in spectrum could not have been detected if the reaction is stoichiometric.

Ascending chromatography on paper of the vitamin B_{12} inhibitor was used to remove inactive substances. The inhibitory principle would appear to be protein because it did not move on paper in concentrated salt solutions and was colored by ninhydrin. It is probable that the material colored by ninhydrin, which moved up on the filter paper contained the growth substances, but the latter were not detected because of the small amount of material applied to the spot.

The filter paper pile appears to be a useful method for

purifying larger amounts of material than can be obtained on single sheets of filter paper. The inhibitory principle is absorbed on filter paper and can be washed out with water. In these processes the material was diluted and it was not determined whether or not all of the growth materials were removed. On cellulose columns equilibrium was not established and presumably everything ran through the column so that the product was more dilute than the sample and still contained growth factors.

Ion exchange has not received enough study for definite conclusions to be drawn as to its possibilities in concentrating the inhibitory factor. However, the small number of experiments performed did not separate the growth and inhibitory factors. Robinson and associates (88) have attempted to separate alkali-stable factors from liver extracts using five different resins at pH values from 3.0 to 8.0. The factors ran rapidly through the column without reacting with the resin.

Experiments in this laboratory showed that Ventriculin extracts could be treated with trypsin and still retain vitamin B_{12}-inhibitory activity. The assay of the inhibition was complicated by growth factors released by proteolytic action. It should be recalled that enzymatically hydrolyzed casein, containing growth stimulating peptides, was eventually omitted from basal media because high blanks were obtained. Burkholder (22) found that papain, trypsin,

and pepsin did not destroy the vitamin B_{12}-binding activity of gastric extracts. Bethell and associates (14) reported that trypsin did not destroy the hematopoietic activity of intrinsic factor incubated with vitamin B_{12}. On the other hand Gregory, Ford, and Kon (53) reported inactivation of vitamin B_{12}-binding factor in sow's milk by papain. However, the milk factor is probably different from the gastric factor.

Heating experiments in this laboratory showed that the vitamin B_{12}-inhibitory factor is not inactivated by heat in dilute solutions when heated separately. Heating in a boiling water bath for 10 minutes and autoclaving for 5 minutes at pH 5.5 to 7.0 resulted in no significant loss in activity whereas boiling at alkaline pH destroyed the vitamin B_{12}-inhibitory principle. Several investigators have found that heating does not prevent the reaction of vitamin B_{12} with gastric factors and others report that vitamin B_{12} cannot be completely recovered for microbiological assay by heating the complex. Castle and associates (26) claimed that the intrinsic factor was destroyed when gastric juice was heated. However, subsequent work on concentrates of intrinsic factor have shown that mixtures of vitamin B_{12} and gastric extracts were still hematopoietically active after heating. Klein and Wilkinson (72) found that alcohol precipitates of the press juice of hog stomach were still active after heating to 80° C. Hall, Bethell, and others

(14, 56) have shown that intrinsic factor and vitamin B_{12} are still hematopoietically active after being heated to 100° C. Shaw (96) and Spray (104) did not recover by heating all of the vitamin B_{12} for bacterial assay from gastric extracts. An evaluation of the effects of heating is difficult because many investigators have not specified the conditions under which heating took place.

From the differences observed by heating the vitamin B_{12}-inhibitory principle in the basal medium and heating it alone, it appeared that heat was not the destructive agent but that other substances were responsible for the inactivation when the mixture was heated. The constituents of the basal medium were investigated in order to determine the nature of the agents causing the destruction of the inhibitory action. Among the substances tested only histidine in the presence of iron(II) or manganese(II) caused inactivation of the vitamin B_{12}-inhibitor upon heating. The similarity of these compounds to components of the vitamin B_{12} structure led to the testing of benzimidazoles and cobalt(II) which are found in the vitamin B_{12} molecule. Cobalt and benzimidazole were found to be even more effective than iron and histidine. These reactions occurred at acid pH at 100° C., but not at 37° C.

A hypothesis for the action of these compounds is as follows. The inhibitory substance unites with vitamin B_{12} at two points, with the cobalt and the 5,6-dimethyl-

benzimidazole, thus making the vitamin unavailable to the test organism and resulting in inhibition of growth. In the presence of both cobalt and benzimidazole the two reactive sites of the inhibitor are tied up, it becomes inactive toward vitamin B_{12}, and no inhibition of B_{12} activity results. However, neither cobalt nor benzimidazole alone can inactivate the inhibitor and thus prevent its inhibition of vitamin B_{12}. Additional work needs to be done with other compounds such as the ribosides and ribotides of imidazoles, in order to establish a physiological relationship.

Blood is known to contain small amounts of vitamin B_{12}, which are not available to bacteria until the blood is heated or subjected to enzyme action. Fractionation of blood did not aid in locating the binding substances in the experiment performed. Several pure protein fractions from blood were tested for vitamin B_{12}-inhibitory activity but were found to be less active than the mucosal extracts. β_1-Metal-combining protein showed a definite inhibition of growth of L. leichmannii. This protein may cause only an indirect inhibition of growth by removing metal ions from the medium. The acid glycoprotein increased the growth; perhaps it contained nucleotides or vitamin B_{12} attached to it. When heated, it gave excellent growth even without vitamin B_{12} in the medium. Schmid (95) reported that this protein is not denatured by boiling. There are several similarities between this protein and those of the vitamin

B_{12}-binding substance. Although stability to heat is not a common property of proteins, there is yet no evidence that the vitamin B_{12}-binding factors are not proteins.

The identity of intrinsic factor with the vitamin B_{12}-binding factor in gastric mucosal extracts cannot be established from the results obtained in this laboratory. Their purification and testing on pernicious anemia patients would be necessary in order to determine a relationship. Welch (112) denied that any relationship exists because his intrinsic factor concentrates did not have any microbiological activity. Bethell and associates (14) believe that the intrinsic factor activity and the microbiologically determined vitamin B_{12}-inhibitory activity are parallel. If such a relationship were established, the purification of the intrinsic factor and its relationship to vitamin B_{12} could be more easily studied.

SUMMARY AND CONCLUSIONS

1. A microbiological method for the assay of vitamin B_{12} using Lactobacillus leichmannii (modified from a method proposed by Peeler) has been adapted for use in vitamin B_{12} inhibition experiments.

2. Inhibitory principle concentrates, which prevent the use of vitamin B_{12} by bacteria, have been prepared from hog stomach mucosa (Ventriculin).

3. Ammonium sulfate, ethanol, and ethanol with salts with controlled ionic strength were effective precipitating agents for the vitamin B_{12}-inhibitory principle, whereas acetone-isoelectric precipitation produced largely inactive material.

4. Growth promoting substances which are non-dialyzable, alkali and heat stable, were precipitated with the vitamin B_{12}-inhibitory factor under all the conditions used.

5. The growth substances could be removed from the inhibitory principle by ascending chromatography on paper, but not by cellulose columns or ion exchange resin IR-120.

6. Tryptic digestion of gastric mucosal extracts did not destroy the vitamin B_{12}-inhibitory principle.

7. The absorption spectrum of vitamin B_{12} was not changed after incubation with gastric mucosal extracts.

8. The vitamin B_{12}-inhibitory principle was inactivated when autoclaved in the basal medium but not when autoclaved alone.

9. The vitamin B_{12}-inhibitory principle was stable to boiling in neutral or acid solutions.

10. The inhibitory principle was inactivated by boiling in the presence of histidine or benzimidazole with either iron(II), cobalt(II), or manganese(II) at acid pH.

11. Fractionation of bovine blood plasma did not aid in locating the vitamin B_{12}-inhibitory substances in plasma.

12. Albumin, γ-globulin, fibrinogen, and plasminogen were not effective as vitamin B_{12} inhibitors, but β_1-metal-combining globulin was inhibitory to the growth of Lactobacillus leichmannii and unheated or heated acid glycoprotein contained growth substances.

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to Dr. Leland A. Underkofler for his advice and encouragement in the experimental work and for suggestions in preparing this manuscript.

The author is grateful also for the advice and assistance of the late Dr. Robert R. Sealock.

Appreciation also is extended to the Atomic Energy Commission for the financial aid which made this investigation possible.

LITERATURE CITED

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

2. Baudet, P. and Giddey, Cl. Le dosage colorimétrique (reaction du biuret) de l'azote protéique. Helv. Chim. Acta 31:1879-1884. 1948.

3. Bauriedel, W. R. Microbiological assay of vitamin B_{12} in blood. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library. 1952.

4. Beaven, G. H., Holiday, E. R., Johnson, E. A., Ellis, B. and Petrow, V. The chemistry of anti-pernicious anemia factors. Part VI. J. Pharm. Pharmacol. 2:944-955. 1950.

5. Beerstecher, E. Jr. A biologically active product of vitamin B_{12}. Fed Proc. 10:161. 1951.

6. Beerstecher, E. Jr. and Altgelt, S. Apoerythein in saliva. J. Biol. Chem. 189:31-34. 1951.

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

8. Beerstecher, E. Jr. and Edmonds, E. J. Evidence for the existence of a salivary apoerythein-inactivating factor. J. Biol. Chem. 195:185-189. 1952.

9. Beerstecher, E. Jr. and Edmonds, E. J. Evidence for existence of an apoerythein precursor in gastric mucosa. Fed. Proc. 11:185. 1952.

10. Beiler, J. M., Moss, J. N. and Martin, G. J. Formation of a competitive antagonist of vitamin B_{12} by oxidation. Science 114:122-123. 1951.

11. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R. and Epstein, M. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. X. Activity of vitamin B_{12} as food (extrinsic) factor. New Eng. J. Med. 239:911-913. 1948.

12. Berk, L., Denny-Brown, D., Finland, M. and Castle, W. B. Effectiveness of vitamin B_{12} in combined system disease. Rapid regression of neurologic manifestations and absence of allergic reactions in a patient sensitive to injectable liver extracts. New Eng. J. Med. 239:328-330. 1948.

13. Bessel, C. J., Harrison, E. and Lees, K. A. Assay of vitamin B_{12} with a mutant of Escherichia coli. Chemistry and Industry p. 561. 1950.

14. Bethell, F. H., Swenseid, M. E., Miller, S. and Citron-Rivera, A. A. Cobalamin (vitamin B_{12}) and the intrinsic factor of Castle. Ann. Internal Med. 35:518-528. 1951.

15. Bird, O. D. and Hoevet, B. The vitamin B_{12}-binding power of proteins. J. Biol. Chem. 190:181-189. 1951.

16. Brink, N. G., Kuehl, F. A. and Folkers, K. Vitamin B_{12}: the identification of vitamin B_{12} as a cyanocobalt complex. Science 112:354. 1950.

17. 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.

18. Broquist, H. P., Stokstad, E. L. R. and Jukes, T. H. Further observations on the microbiological assay for vitamin B_{12} using Lactobacillus leichmannii 18639. Proc. Soc. Exp. Biol. Med. 76:806-811. 1951.

19. Burk, D., Hearon, J., Caroline, L. and Schade, A. L. Reversible complexes of cobalt, histidine and oxygen gas. J. Biol. Chem. 165:723-724. 1946.

20. Burk, D., Hearon, J., Levy, H. and Schade, A. L. Reversible oxygenation of cobaltodihistidine to oxy-biscobaltodihistidine and comparisons with other metal-amino acids, oxyhemoglobin and oxyhemocyanin. Fed. Proc. 6:242. 1947.

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

22. Burkholder, P. R. Microbiological studies on materials which potentiate oral vitamin B_{12} therapy in Addisonian anemia. Arch. Biochem. Biophys. 39:322-332. 1952.

23. Castle, W. B. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. I. The effect of administration to patients with pernicious anemia of the contents of the normal stomach recovered after the ingestion of beef muscle. Am. J. Med. Sc. 178:748-764. 1929.

24. Castle, W. B. and Ham, T. H. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. V. Further evidence for the essential participation of extrinsic factor in hematopoietic responses to mixtures of beef muscle and gastric juice and to hog stomach mucosa. J. Am. Med. Assn. 107:1456-1463. 1936.

25. Castle, W. B., Heath, C. W. and Strauss, M. B. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. IV. A biologic assay of the gastric secretion of patients with pernicious anemia having free hydrochloric acid and that of patients without anemia or with hypochromic anemia having no free hydrochloric acid, and of the role of intestinal impermeability to hematopoietic substances in pernicious anemia. Am. J. Med. Sc. 182:741:764. 1931.

26. Castle, W. B., Heath, C. W., Strauss, M. B. and Heinle, R. W. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. VI. The site of interaction of food (extrinsic) and gastric (intrinsic) factors failure of in vitro incubation to produce a thermostable hematopoietic principle. Am. J. Med. Sc. 194:618-625. 1937.

27. Castle, W. B. and Townsend, W. C. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. II. The effect of the administration to patients with pernicious anemia of beef muscle after incubation with gastric juice. Am. J. Med. Sc. 178:764-777. 1929.

28. Castle, W. B., Townsend, W. C. and Heath, C. W. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. III. The nature of the reaction between normal human gastric juice and

beef muscle leading to clinical improvement and increased blood formation similar to the effect of liver feeding. Am. J. Med. Sc. 180:305-336. 1930.

29. Chow, B. F. Sequelae for the administration of vitamin B_{12} to humans. J. Nutr. 43:323-343. 1951.

30. Chow, B. F., Rosenblum, C., Silber, R. H., Woodbury, D. J., Yamamoto, R. and Lang, C. A. Oral administration of vitamin B_{12} containing cobalt^{60} to rats. Proc. Soc. Exp. Biol. Med. 76:393-395. 1951.

31. Coates, M. E., Ford, J. E., Harrison, G. F., Kon, S. K., Porter, J. W. G., Cuthbertson, W. F. J. and Pegler, H. F. Vitamin B_{12} activity for chicks and different micro-organisms of gut contents and faeces. Biochem. J. 49:lxvii. 1951.

32. Coates, M. E., Ford, J. E., Harrison, G. F., Kon, S. K. and Porter, J. W. G. Some properties of vitamin B_{12}-like factors from calf faeces. 2. Biological activities and interrelationships. Biochem. J. 51:vi. 1952.

33. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. and Uroma, E. A system for the separation of the components of human blood: quantitative procedures for the separation of the protein components of human plasma. J. Am. Chem. Soc. 72:465:474. 1950.

34. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C. and Lang, C. A. Observations on the absorption, utilization and excretion of vitamin B_{12}. J. Lab. Clin. Med. 38:84-94. 1951.

35. Cooley, G., Ellis, B., Petrow, V., Beaven, G. H., Holiday, E. R. and Johnson, E. A. The chemistry of some anti-pernicious anaemia factors. VII. Some transformation of vitamin B_{12b}. J. Pharm. Pharmacol. 3:271-285. 1951.

36. 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. 3:607-608. 1951.

37. Cooperman, J. M., Drucker, R. and Tabenkin, B. Microbiological assays for vitamin B_{12}: a cyanide enhancement effect. J. Biol. Chem. 191:135-141. 1951.

38. Cooperman, J. M., Tabenkin, B. and Drucker, R. Growth response and vitamin B_{12} tissue levels in vitamin B_{12}-deficient rats and chicks fed riboflavin, 5,6-dimethylbenzimidazole and related compounds. J. Nutr. 46:467:478. 1952

39. Couch, J. R., Olcese, O., Witten, P. W. and Colby, R. W. Vitamin B_{12} content of blood of various species. Am. J. Physiol. 163:77-80. 1950.

40. Dacie, J. V. and Witts, L. J. Personal communication and quotation. Foreign newsletter. Blood 6:580-582. 1951.

41. Davis, B. D. Utilization of pseudovitamin B_{12} by mutants of Escherichia coli. J. Bact. 64:432-433. 1952.

42. Davis, B. D. and Mingioli, E. S. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bact. 60:17-28. 1950.

43. Davis, R. L. and Chow, B. F. Some applications of the rapid uptake of vitamin B_{12} by resting Lactobacillus leichmannii organisms. Science 115:351-352. 1952.

44. 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.

45. 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.

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

47. Emerson, G. and Folkers, K. Watersoluble vitamins. Ann. Rev. Biochem. 20:559-598. 1951.

48. Ford, J. E., Kon, S. K. and Porter, J. W. G. The multiple nature and potency for different micro-organisms of the vitamin B_{12} activity of calf rumen contents and faeces. Biochem. J. 50:ix. 1951.

49. Franklin, A. E. and Quastel, J. H. Paper chromatography of proteins and enzymes. Science 110:447-451. 1949.

50. Friedland, W. C. Microbiological assay procedures for vitamin B_{12}. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library. 1951.

51. Girdwood, R. H. Vitamin B_{12} and folic acid in the megaloblastic anemias. Edinburgh Med. J. 58:309-335. 1951.

52. Glass, G. B. J., Boyd, L. J., Rubinstein, M. A. and Svigals, C. S. Relationship of glandular mucoprotein from human gastric juice to Castle's intrinsic anti-anaemic factor. Science 115:101-108. 1952.

53. 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.

54. Gyorgy, P. Vitamin methods II. N. Y., Academic Press Inc. 1951.

55. Hall, B. E. Studies on the nature of the intrinsic factor of Castle. Brit. Med. J. ii:585. 1950.

56. Hall, B. E., Bethell, F. H., Morgan, E. H., Campbell, D. C., Swenseid, M. E., Miller, S. and Citron-Rivera, A. A. Observations on the presence of intrinsic factor in extracts of hog stomach and duodenum. Proc. Staff Meetings Mayo Clinic 25: 105-113. 1950.

57. Hall, B. E., Morgan, E. H. and Campbell, D. C. Oral administration of vitamin B_{12} in pernicious anemia. I. Presence of intrinsic factor in Berkefeld-filtered pooled human gastric juice: preliminary report. Proc. Staff Meetings Mayo Clinic 24: 99-107. 1949.

58. Hausman, K. and Mulli, K. The haemopoietic effect of vitamin B_{12} prepared from fish. Lancet 262: 185-188. 1952.

59. Helmer, O. M., Fouts, P. J. and Zerfas, L. G. Relationship of intrinsic factor to hematopoietic material in concentrated human gastric juice. Am. J. Med. Sc. 188:184-193. 1934.

60. Hirs, C. H. W., Stein, W. H. and Moore, S. Chromatography of proteins. Ribonuclease. J. Am. Chem. Soc. 73:1893. 1951.

61. Hoff-Jørgensen, E. The effect of "intrinsic factor" on the absorption of vitamin B_{12} by wild type Escherichia coli. Arch. Biochem. Biophys. 36: 235-236. 1952.

62. Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L. and Jukes, T. H. Response of Lactobacillus leichmannii 313 to the anti pernicious anemia factor. J. Biol. Chem. 176:1465-1466. 1948.

63. Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbusch, A. C. and Jukes, T. H. The microbiological assay of vitamin B_{12} with Lactobacillus leichmannii. J. Biol. Chem. 181:635-644. 1949.

64. Horrigan, D., Jarrold, T. and Vilter, R. W. Direct action of vitamin B_{12} upon human bone marrow. J. Clin. Inv. 30:31-36. 1951.

65. Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L. and Jukes, T. H. Assay of anti-pernicious anemia factor with Euglena. Proc. Soc. Exp. Biol. Med. 70:118-120. 1949.

66. Jukes, T. H. and Stokstad, E. L. R. The role of vitamin B_{12} in metabolic processes. Vitamins and Hormones 9:1-26. 1951.

67. 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. 73:335-337. 1951.

68. Kaczka, E. A., Wolf, D. E. and Folkers, K. Vitamin B_{12}. V. Identification of crystalline vitamin B_{12a}. J. Am. Chem. Soc. 71:1514-1515. 1949.

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. Kitay, E., McNutt, W. S. and Snell, E. E. The nonspecificity of thymidine as a growth factor for lactic acid bacteria. J. Biol. Chem. 177:993-994. 1949.

71. Kitay, E., McNutt, W. S. and Snell, E. E. Desoxyribosides and vitamin B_{12} as growth factors for lactic acid bacteria. J. Bact. 59:727-738. 1950.

72. Klein, L. and Wilkinson, J. F. Investigations on the nature of haemopoietin, the anti-anaemic substance in hog's stomach. II. The production of a thermostable haemopoietically active substance similar to or identical with the anti-anaemic principle of liver by action of thermolabile haemopoietin in beef. Biochem. J. 28:1684-1692. 1934.

73. Lajtha, L. G. An inhibitory factor in pernicious anaemia serum. Clin. Science 9:287-297. 1950.

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

75. Lewis, U. J., Tappan, D. V. and Elvehjem, C. A. Properties and distribution of vitamin B_{12f}. J. Biol. Chem. 199:517-530. 1952.

76. Lichtman, H., Ginsberg, V. and Watson, J. Therapeutic effect of aureomycin in pernicious anemia. Proc. Soc. Exp. Biol. Med. 74:884-888. 1950.

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

78. Minot, G. R. and Murphy, W. P. Treatment of pernicious anemia by a special diet. J. Am. Med. Assn. 87:470-476. 1926.

79. Mitchell, H. K., Gordon, M. and Haskins, F. A. Separation of enzymes on the filter chromatopile. J. Biol. Chem. 180:1071-1076. 1949.

80. Oginsky, E. L. Uptake of vitamin B_{12} by Escherichia coli. Arch. Biochem. Biophys. 36:71-79. 1952.

81. Peeler, H. T., Norris, L. C. A growth stimulating factor for Lactobacillus leichmannii. J. Biol. Chem. 188:75-82. 1951.

82. Peeler, H. T., Yacowitz, H. and Norris, L. C. A microbiological assay for vitamin B_{12} using L. leichmannii 17845. Proc. Soc. Exp. Biol. Med. 72: 515-521. 1949.

83. Pennington, R. J. A heat-labile vitamin B_{12} complex in faeces. Biochem. J. 48:xviii. 1951.

84. 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.

85. Pierce, J. V., Page, A. C. Jr., Stokstad, E. L. R. and Jukes, T. H. Studies on some characteristics of vitamin B_{12b}. J. Am. Chem. Soc. 72:2615-2616. 1950.

86. Prusoff, W. H., Chicago, Illinois. Information on concentration of intrinsic factor. Private communication. 1950.

87. Prusoff, W. H., Meacham, G. C., Heinle, R. W. and Welch, A. D. Concentration of the intrinsic factor of powdered stomach. Abstracts 118th. Meeting Am. Chem. Soc. Chicago, Illinois, Sept. 8, 1950. p. 27 A.

88. Robinson, F. A., Williams, B. W. and Brown, L. H. The microbiological assay of vitamin B_{12} in crude liver extracts. J. Pharm. Pharmacol. 4:27-36. 1952.

89. Rosenthal, H. L., Sarett, H. P. The determination of vitamin B_{12} activity in human serum. J. Biol. Chem. 199:433-442. 1952.

90. Ross, G. I. M. Vitamin B_{12} assay in body fluids. Nature 166:270-271. 1950.

91. Scheid, H. E. and Schweigert, B. S. Some factors affecting the potencies of vitamin B_{12} and leuconostoc citrovorum factor of certain natural products. J. Biol. Chem. 185:1-8. 1950.

92. Schilling, R. F., Fruton, J. S., Hofstee, B. H. F., Welch, A. D., Harris, J. W., Gardner, F. H. and Castle, W. B. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. XII. Failure of thymus aminopolypeptidase to act as intrinsic factor. J. Lab. Clin. Med. 36:942-949. 1950.

93. Schindler, O. Eine kristallisierte Säure aus Vitamin B12, die noch biologische Aktivität besitzt. Versuche mit Vitamin B_{12}. I. Mitteilung. Helv. Chim. Acta 34:101-108. 1951.

94. Schmid, K. Preparation and properties of an acid glycoprotein prepared from human plasma. J. Am. Chem. Soc. 72:2816. 1950.

95. Schmid, K. Preparation and properties of serum and plasma proteins. XXIX. Separation from human plasma of polysaccharides, peptides and proteins of low molecular weight. Crystallization of an acid glycoprotein. J. Am. Chem. Soc. 75:60-68. 1953.

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

97. Shive, W., Sibley, M. E. and Rogers, L. L. Replacement of vitamin B_{12} by desoxynucleotides in promoting growth of certain lactobacilli. J. Am. Chem. Soc. 73:867-868. 1951.

98. 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.

99. Skeggs, H. R., Huff, J. W., Wright, L. D. and Bosshardt, D. K. The use of Lactobacillus leichmannii in the microbiological assay of the animal protein factor. J. Biol. Chem. 176:1459-1460. 1948.

100. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W. and Wright, L. D. Observations on the use of Lactobacillus leichmannii 4797 in the microbiological assay of vitamin B_{12}. J. Biol. Chem. 184:211-221. 1950.

101. Smith, E. L., Ball, S. and Ireland, D. M. B_{12} vitamins (cobalamins). 2. Neutral, basic and acidic cobalamins. Biochem. J. 52:395-400. 1952.

102. Smith, E. L. and Cuthbertson, W. F. J. Paper chromatography of the vitamin B_{12} group of factors. Biochem. J. 45:xii. 1949.

103. Snell, E. E., Kitay, E. and McNutt, W. S. Thymine desoxyriboside as an essential growth factor for lactic acid bacteria. J. Biol. Chem. 175:473-474. 1948.

104. Spray, G. H. The effect of heat on the microbiological and anti-anaemic properties of human gastric juice mixed with vitamin B_{12}. Biochem. J. 50:587-590. 1952.

105. Surgenor, D. M., Koechlin, B. A. and Strong, L. E. The metal-combining globulin of human plasma. J. Clin. Inv. 28:73. 1949.

106. Tallan, H. H. and Stein, W. H. Studies on lysozyme. J. Am. Chem. Soc. 73:2976-2977. 1951.

107. 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.

108. Thompson, R. B. Addisonian pernicious anemia. Confirmatory evidence of a factor inhibiting erythropoiesis. Clin. Science 9:281-285. 1951.

109. Ungley, C. C. Absorption of vitamin B_{12} in pernicious anemia; the administration into buccal cavity, into washed segments of intestine, or after partial sterilization of bowel. Brit. Med. J. ii:905-919. 1950.

110. 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 6:225-228. 1950.

111. Von Bonsdorff, B. Pernicious anemia caused by Diphyllobothrium latum, in the light of recent investigations. Blood 3:91-102. 1948.

112. Welch, A. D. and Heinle, R. W. Hematopoietic agents in macrocytic anemias. Pharm. Rev. 3:345-441. 1951.

113. Welch, A. D. and Nichol, C. A. Water-soluble vitamins concerned with one and two-carbon intermediates. Ann. Rev. Biochem. 21:633-686. 1952.

114. 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. 11:308-309. 1952.

115. Welch, A. D. and Wilson, M. F. Mechanism of the growth-promoting effect of ascorbic acid on Lactobacillus leichmannii and the reduction of oxidation products of vitamin B_{12}. Arch. Biochem. 22:486-489. 1949.

116. 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 6:229-236. 1950.

117. Winsten, W. A. and Eigen, E. Vitamin B_{12} and related factors in the nutrition of Lactobacillus leichmannii-313. J. Biol. Chem. 177:989-990. 1949.

118. Winsten, W. A. and Eigen, E. Paper chromatography of vitamin B_{12} and related bacterial growth factors. J. Biol. Chem. 181:109-120. 1949.

119. Wolf, D. E., Wood, T. R., Valiant, J. and Folkers, K. Vitamin B_{12} and the intrinsic factor. Proc. Soc. Exp. Biol. Med. 73:15-17. 1950.

120. Wright, L. D. Antimetabolites of nucleic acid metabolism. Vitamins and Hormones 9:131-159. 1951.

121. 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. 45:507-519. 1951.