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Microbiological assay procedures for vitamin B12

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MICROBIOLOGICAL ASSAY PROCEDURES FOR VITAMIN B_{12}

by

Waldo Charles Friedland

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Biophysical Chemistry

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I. INTRODUCTION

In the years preceding the isolation and crystallization of vitamin B_{12}, a number of unidentified nutritional factors were recognized by their action or effect. Among these unknown and apparently unrelated factors were the antipernicious anemia factor of liver, the animal protein factor (APF) required by chicks and swine, factor X for rats, and the requirement of many bacteria for natural materials such as yeast extract in order to grow in culture media. When crystalline B_{12} became available in 1948, it was shown to be an active principle in all of these diversified systems.

Swine feeding experiments on the Iowa State College campus showed the value of supplementing rations with sources of APF such as meat scraps or fish meal. When B_{12} concentrates, and later crystalline B_{12}, were employed as supplements, they were shown to have much of the activity of crude sources of APF. It is now known that B_{12} is not the only factor in APF. Other unknown factors and antibiotics are involved in the concept termed "APF."

After feeding experiments gave definite proof that B_{12} would increase weight gains and feed efficiency, it

was necessary to undertake a more fundamental study of the role of B_{12} in the nutrition of the pig. The purpose of the work described here was to develop means of determining B_{12} in various materials in connection with swine feeding experiments. A microbiological method of assaying for B_{12} was chosen because of the short assay time, and because some preliminary work on a microbiological assay had been carried out in this laboratory.

II. REVIEW OF THE LITERATURE

In the past ten years, the use of microorganisms to quantitatively and qualitatively analyze for vitamins and amino acids has grown tremendously. Prior to 1940, a few biological assays were in use with rats employed as the test animal. The main advantage of such an assay was the testing of a biological material in a functioning biological system. However, such an assay is expensive, time-consuming, and requires large amounts of materials. After extensive studies on the nutrition of microorganisms, Snell and Strong (1939) developed a microbiological assay for riboflavin using Lactobacillus casei as the test organism. Their work showed reliable results, only minute amounts of riboflavin were necessary, and the assay time was shortened to one to three days. Since this work was published, the microbiological assay has become a major, indeed indispensable, tool in nutritional research. This is well illustrated in the isolation of vitamin B_{12}.

A century ago, Addison (1849) described a type of anemia which has become known as Addisonian pernicious anemia. There was no treatment for the disease until Minot and Murphy (1926) showed that whole liver gave beneficial

results in a matter of a few days. Unfortunately, a pound of liver per day was necessary, and very few individuals could stay on such a diet very long. Fractionation and purification of liver preparations decreased this requirement to only one milligram per day. The extreme potency of such preparations poised an intriguing problem for the chemist. Work in England continued for twenty years on the purification of the active principle. After each step in the purification process, work was halted until a pernicious anemia patient could be located and the material evaluated by clinical testing. Dr. Ungley conducted over eighty clinical tests during this period. Finally, Smith (1948) announced the isolation of one gram of highly active red material from four tons of liver. Although not crystalline, the material appeared to be essentially pure, and was considered to be the liver factor postulated by Minot and Murphy.

Cary, Hartman, Dryden, and Likely (1946) reported that rats on a diet adequate in all known nutrients required an unidentified factor for normal growth. They called it factor X, and found crude casein to be a good source. Hartman and Cary (1946) also reported factor X in various foods and liver extracts. In an attempt to locate a microorganism that required this unknown factor X, Shorb (1947b) found that

Lactobacillus lactis Dorner (ATCC 8000) responded to the liver preparations. She (1947a) had previously shown that this organism required two factors: one, the TJ (tomato juice) factor in clarified tomato juice; the other, the LLD (*Lactobacillus lactis* Dorner) factor in certain liver preparations. Furthermore, the LLD factor was concentrated in refined liver extracts, and the activity closely paralleled the refinement. This suggested that the LLD factor was the active antipernicious anemia principle of liver.

With Shorb's microbiological assay to help follow the various purification procedures, the Merck chemists were able to isolate the active factor of liver in a matter of months. Rickes, Brink, Koniuszy, Wood, and Folkers (1948) announced the isolation of a red crystalline compound just two weeks before the English report. The compound was designated vitamin B_{12}, and it produced positive hematological response in patients with Addisonian pernicious anemia. Analyses of the American and English compounds indicated they were identical, the most interesting feature being 4.5 percent cobalt present, corresponding to one cobalt atom per molecule. Furthermore, West (1948) reported crystalline B_{12} effective in microgram doses against pernicious anemia, which makes this vitamin one of the most potent biological substances known.

With the aid of a rapid microbiological assay for the LLD factor, Folkers and his associates accomplished, in a few months, work that took years for others to complete. Crystalline B_{12} assayed with L. lactis Dorner had a potency of 11,000,000 LLD units per milligram, as reported by Shorb (1948). This high potency made it evident that B_{12} was wholly responsible, or nearly so, for the LLD activity of liver.

The assay, however, was not very satisfactory. L. lactis was not a desirable assay organism in that it dissociated or mutated quite easily. It gave very erratic results, sometimes not responding to B_{12} at all. Besides dissociation, Shorb and Briggs (1948) found high concentrations of folic acid in some crude materials resulted in growth inhibition. They also found high levels of serine, p-aminobenzoic acid, xanthine, manganese, sodium, and iron inhibited growth.

A further complication in the L. lactis assay arose when Shive, Ravel, and Eakin (1948) and also Wright, Skeggs, and Huff (1948) reported that thymidine but not thymine supported growth of the organism. Comparatively large amounts of thymidine (1-3 µg. per 10 ml. of medium) were required, and both groups suggested that B_{12} had a coenzyme function in the conversion of thymine to thymidine.

Other lactic acid organisms were soon found that were more desirable than L. lactis as an assay organism. Skeggs and her associates (1948) reported good correlation with mouse response using Lactobacillus leichmannii (ATCC 4797). This organism responded to thymidine and ascorbic acid, as did L. lactis. The ascorbic acid effect was eliminated by autoclaving the medium. This same organism was found by Capps, Hobbs, and Fox (1949) to be more stable in culture than L. lactis, and, therefore, more suitable for assay work. A very similar organism, Lactobacillus leichmannii 313, was tried by Hoffmann, Stokstad, Franklin, and Jukes (1948) because it was known to require thymidine. Both crystalline B_{12} and liver extracts produced similar growth response, provided folic acid and p-aminobenzoic acid were present in the medium.

A completely different type of organism, the alga Euglena gracilis var. bacillaris, was suggested by Hunter, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes (1949). In a chemically defined medium, Euglena showed a quantitative growth response to B_{12} but not to thymidine. The only other vitamin required in the medium was thiamin. The response of this alga is apparently specific for B_{12}, as shown by work to be mentioned later, and it is more sensitive to B_{12} than is L. leichmannii by a factor of ten.

As a research method, the *Euglena* assay has been used to some extent, particularly by large industrial organizations. It suffers as a routine assay in that the cells are more difficult to handle than a bacterium, the assay tubes must be evenly illuminated during incubation, and strict aseptic techniques are required because of the four day incubation time.

Mutants of *Escherichia coli* have been found by Davis and Mingioli (1950) that are stable enough to be used as assay organisms. In some mutants methionine gave a growth response, but thymidine and homocysteine did not interfere. Experiments with some of the *E. coli* mutants indicated that B_{12} was concerned with the methylation of homocystine to methionine. Recently Johansson (1951) described an *E. coli* assay using a simple medium and with a sensitivity equal to the *L. leichmannii* assay.

Although they will not be discussed here, a number of animal assays have appeared in the literature. Bosshardt, Paul, O'Doherty, Huff, and Barnes (1949) have developed two mouse assays. One involves using mice from mothers that were maintained on an APF-deficient diet to prevent carry-over of APF to the young. The second is based on the ability of APF to counteract the growth inhibition of mice fed thyroid active materials. Rats may also be employed.

Frost, Fricke, and Spruth (1949) found rats on a B_{12}-deficient diet gave equal growth response to either oral or parenteral administration. Tappan, Lewis, Register, and Elvehjem (1950) reported that rat and microbiological assays agreed reasonably well. The chief interference in a rat assay comes from antibiotics, particularly aureomycin, which stimulate rat growth. Miller and Groschke (1950) employed a chick assay to determine APF activity in various materials. The chick assay appears to be an excellent method of measuring APF, but not B_{12}. At least two chick factors in addition to B_{12} have been found in liver by Carlson, Miller, Peeler, Norris, and Heuser (1949) and by Combs, Carlson, Miller, Peeler, Norris, and Heuser (1950). The presence of unknown factors, other than B_{12}, was confirmed by Coats, Harrison, and Kon (1951), and they concluded that the chick and microbiological assays were not measuring the same thing.

The organisms most commonly used in B_{12} assays at present are *L. lactis* Dorner and *L. leichmannii*. Part of the difficulty in maintaining *L. lactis* Dorner cultures has been overcome by carrying the cultures on a tomato juice-yeast extract-skim milk medium as suggested by Greene, Brooke, and McCormack (1949), or dextrose-yeast extract-salts agar supplemented with tomato juice eluate as used

by Koditschek, Henlin, and Woodruff (1949). Unfortunately, neither of these organisms shows a specific requirement for B_{12}, and a considerable amount of work has been done in an effort to get reproducable assays for B_{12}.

As mentioned earlier, thymidine interferes with the *L. lactis* Dorner assay for B_{12}. Shive, Ravel, and Harding (1948) found both thymidine and guanylic acid and, to a lesser extent, purine bases supported the growth of *L. lactis* Dorner. *L. leichmannii* (ATCC 4797), also, could utilize thymidine to replace B_{12}. *L. leichmannii* 313 shows a similar response to thymidine, as already stated. The theory that B_{12} may be a coenzyme in the synthesis of thymidine from thymine for *L. leichmannii* appears quite reasonable, but is not supported by experiments using closely related organisms. Wright (1949) could find no relationship between B_{12} and thymidine for *Streptococcus faecalis* R or *Leuconostoc citrovorum*. Working with over twenty-five strains of lactobacilli, Kitay, McNutt, and Snell (1950), and Kitay and Snell (1950) found at least eighteen required thymidine or other desoxyribosides, while the requirement for B_{12} varied in no uniform manner. It thus appears that any general statement of the role of B_{12} in bacterial nutrition is unwarranted at the present time. An interesting observation on *L. casei* (ATCC 7469)

supports the idea that B_{12} is involved in some fundamental way in the growth and reproduction mechanisms. Both folic acid and B_{12} enhanced the synthesis of desoxyribonucleic acids (DNA), as reported by Rege and Sreenivasan (1950), although B_{12} was not quite as effective as folic acid. Similar results were found by Roberts, Roberts, and Abelson (1949) for L. leichmannii. Measuring radioactive phosphorus uptake by the cell, they found that the DNA fraction was four times as active in the presence of B_{12}.

To further complicate the picture of interfering substances, Winsten and Eigen (1949a) ran paper strip chromatograms of B_{12}, antipernicious anemia, and APF samples using n-butanol as the mobile phase. The strips were laid on nutrient agar seeded with L. leichmannii 313, and at least six zones were distinguishable. The two slowest moving zones were not always separable, and were believed to be different forms of B_{12} as suggested by Smith (1948). Two of the zones were tentatively identified as thymidine and hypoxanthine desoxyriboside by Winsten and Eigen (1949b). The remaining two zones were probably other desoxyribosides, since four zones were found when DNA was hydrolyzed with desoxyribonuclease and mylase P.

The findings of Winsten and Eigen have been confirmed by other workers. Smith and Cuthbertson (1949) tried

different solvents and found substantially the same fractions. Picken and Bauriedel (1950) have repeated and corroborated Winsten and Eigen's work with L. leichmannii 313 and have extended the bioautograph technique to Euglena gracilis. They found four active materials on the paper strips, in addition to B_{12}, by L. leichmannii assay; E. gracilis responded only to the stationary B_{12} zone. Essentially the same results with L. lactis were obtained by Kocher, Karrer, and Muller (1950) using butanol or collidine as the moving solvent.

There is ample evidence to support the conclusions that the substances moving down the paper strips may be various desoxyribosides. Thymidine and the desoxyribosides of hypoxanthine and cytosine were equal in B_{12} activity for eleven lactobacilli tested by Kitay, McNutt and Snell (1949). L. lactis 1175 was found by Kocher and Schindler (1949) to respond to the desoxyribosides of thymine, guanine, cytosine, and hypoxanthine. Of the eighteen strains of lactobacilli investigated by Kitay, McNutt, and Snell (1950) that required thymidine, in most cases the thymidine could be replaced by other desoxyribosides. Recently it was announced that the desoxynucleotides are equally effective. Shive, Sibley, and Rogers (1951) found the desoxynucleotides of cytosine, thymine, guanine, adenine,

and 5-methylcytosine to have the same order of activity as thymidine for *L. leichmannii* and *L. lactis* Dorner. These desoxynucleotides showed no activity for an *E. coli* culture used for B_{12} assays.

Despite the disadvantages associated with the non-specifity of *L. leichmannii* and *L. lactis* for B_{12}, both organisms can be routinely used for assaying most materials. Preferential destruction of B_{12} by alkali is possible, and this coupled with chromatographic analysis of samples readily gives pertinent information about any activity measured by these organisms. It should also be pointed out that B_{12} is 10,000 or more times as effective as desoxyribosides on a weight basis, and that for most materials the dilution used to measure B_{12} is too great to result in interferences by other factors. One method to eliminate the desoxyriboside interference is a cup assay described by Foster, Lally, and Woodruff (1949). The novel feature of the medium is a high sodium chloride concentration (2 gm. per 100 ml.) that apparently eliminates the *L. lactis* Dorner response to these compounds. Another method has been suggested by Skeggs, Nepple, Valentik, Huff, and Wright (1950) for *L. leichmannii* 4797. They recommended incorporation of ribonucleic acid or the nucleotides of guanine, uracil, or cytosine into the medium, along with

thiomalic acid and a special casein hydrolyzate. Finally, *E. coli* or *Euglena* assays may be employed for materials that cause too much trouble with the lactobacilli.

It is very desirable in developing a microbiological assay to know the chemical structure and reactions of the compound in question, and what effect the compound has on the test organism. It has already been stated that we have little information on the role of B_{12} in bacte rial nutrition. The same is true for the chemistry of B_{12}. Because the information is important for a good assay, the chemical information that is available will be briefly reviewed.

Vitamin B_{12} is an optically active, weakly basic, cobalt coordination compound with a molecular weight of about 1500. Brink, Wolf, Kaczka, Rickes, Koniuszy, and Folkers (1949) reported a chemical analysis corresponding to $C_{61-64}H_{86-92}N_{14}O_{3}PCo$. It was not a peptide and gave color tests similar to five-membered nitrogen rings. Acid degradation has been the principal method of disrupting the molecule to obtain smaller units for analysis. Brink and Folkers (1949) have identified 5,6-dimethylbenzimidazole. Brink, Holly, Shunk, Peel, Cahill, and Folkers (1950) have isolated 1-α-D-ribofuransido-5,6-dimethylbenzimidazole and compared it with the synthetic compound. The conclusion is reached by Buchanan and his associates

(1950) that phosphate is bound to either the number two or three carbon of the sugar. This is a very interesting fragment of B_{12}, if it exists, because of its close similarity to the nucleotides of yeast ribonucleic acid. To have such a structure in a molecule that appears to be involved in nucleic acid synthesis can lead to some intriguing speculation.

Several excellent papers have come from the chemists at the British Drug Houses Ltd. Ellis, Petrow, and Snook (1949a) found phosphate completely liberated by 20 percent hydrochloric acid in six hours. In a later paper they (1949b) reported ammonium chloride and a ninhydrin-reacting moiety also liberated by hydrochloric acid. The ninhydrin-reacting substance moved the same as 2-amino-1-propanol on paper strips in four different solvent systems. This should have been sufficient evidence for the identity of the unknown compound. However, Cooley, Ellis, and Petrow (1950) later reported that a permanganate oxidation of the unknown compound did not give alanine, as would be expected. To date this is the only case where two different compounds appeared to be identical under widely differing chromatographic conditions. The problem was finally resolved by Wolf, Jones, Valiant, and Folkers (1950) who isolated the dibenzoate of D_g-1-

amino-2-propanol from an acid hydrolyzate of B_{12}. Even after acid hydrolysis, the cobalt in B_{12} is organic bound and can be extracted with butanol. No chemical information is available about this fraction of the B_{12} molecule.

Another striking feature of B_{12} is the presence of cyanide. Brink, Kuehl, and Folkers (1950) obtained almost one mole of hydrogen cyanide for each mole of B_{12} by permanganate oxidation or by heating in hydrochloric or oxalic acids. This would indicate a cyanide group coordinated with cobalt in B_{12}, and the C≡N absorption band at 4.69 µ was found. The cyanide must have been tightly bound to cobalt, because injections of B_{12} equivalent to fatal doses of cyanide were not toxic to mice.

All that is known about the B_{12} molecule as a unit is that it is a cobalt coordination compound and has six weakly basic points. A salt of B_{12} containing six moles of perchloric acid per mole of B_{12} has been crystallized from glacial acetic acid by Alicino (1951). Infrared absorption spectrum measurements indicated the cyanide group was still present and not replaced as is the case with B_{12a} and B_{12b}.

To complicate the assay for B_{12} even more, various forms of the vitamin are known. The first one reported was termed B_{12a} by Kaczka, Wolf, and Folkers (1949).

B_{12a} was produced by hydrogenation of B_{12} and appeared to be less active biologically than B_{12}. Vitamin B_{12a} behaves as a weak base, and Kaczka, Wolfe, Kuehl, and Folkers (1950) considered it to have a hydroxyl group instead of a cyanide group coordinated with cobalt. They converted B_{12a} back to B_{12} by treatment with potassium cyanide, and made compounds with a sulfite, chloride, or cyanate group in place of the cyanide. To eliminate some of the nomenclature confusion, they referred to all of the B_{12} molecule except the coordinating group as cobalamin. Thus, B_{12} is cyano-cobalamin and B_{12a} is hydroxo-cobalamin.

Vitamin B_{12b} was first isolated from liver by Pierce, Page, Stokstad, and Jukes (1950). It was later isolated from a Streptomyces aureofaciens fermentation by this same group (1950), and prepared by hydrogenation of B_{12} by Brockman, Pierce, Stokstad, and Jukes (1950). Vitamin B_{12c} was described by Buchanan, Mills, and Todd (1950). By the Merck nomenclature it is nitroso-cobalamin. The fourth one of the series, termed B_{12d} by Smith, Fantes, and Ball (1951), has been isolated from fermentation liquors, as have the other forms of B_{12}. Vitamins B_{12a} and B_{12b} have been shown to be identical by Wijmenga, Veer, and Lens (1950) and also by Kaczka, Denkewalter, Holland, and Folkers (1951). According to Anslow, Ball, Emery,

Fantes, Smith, and Walker (1950), B_{12c} and B_{12d} have the same activity as B_{12}. Thus, under some conditions all the cobalamins have the same microbiological activity, but a considerable variation in activities can be found by using different organisms or different assay conditions. For pure materials the absorption spectra are sufficiently different to be used for analysis, but generally this is not applicable to natural materials.

Considerable progress has been made in improving the media used for B_{12} assays, and this should be included in any discussion of the microbiological assay. The original medium used by Shorb was a modification of several media, reported in the literature, for use with lactobacilli. All the known crystalline B vitamins were used, together with the usual glucose, inorganic salts, purines, and amino acids. In addition, the medium was supplemented with clarified tomato juice to supply the TJ factor for *L. lactis* Dorner.

Pure amino acids are too expensive to use for routine assay medium and they are usually replaced by acid-hydrolyzed casein plus tryptophan and cystine, which are destroyed by acid hydrolysis. Wright, Skeggs, and Huff (1948) used a casein hydrolyzate, clarified tomato juice, and also a tryptic digest of casein for *L. lactis* Dorner. The

tryptic digest of casein, called strepogenin, was supposed to be a polypeptide that improves the utilization of amino acids by some bacteria. At least part of the difficulty experienced in early B_{12} assays could be attributed to strepogenin. This digest, but not tomato juice, was included in the medium described by Skeggs, Huff, Wright, and Bosshardt (1948) for L. leichmannii 4797. Replacing the casein hydroyzate by amino acids in this same medium, Welch and Wilson (1949) found that the casein digest contains DNA or products of its degradation, and also an "oxidized" form of B_{12}. In the presence of such reducing agents as ascorbic acid, thioglycollic acid, and glutathione, the casein digest promoted excellent bacterial growth in the absence of B_{12}. Furthermore, ascorbic acid was without activity when the casein digest was omitted from the medium. This report seemed to clear up some of the difficulties of the B_{12} assay. Surprisingly, the strepogenin difficulty was finally resolved with another organism, L. casei. Peeler, Daniel, Norris, and Heuser (1949) found that strepogenin and glutamine gave almost identical responses to the organism; glutamic acid was less active. Asparagine was then related to strepogenin by Stokes, Koditschek, Rickes, and Wood (1949), and Rickes, Koch, and Wood (1949) speculated that asparagine, serine,

and glutamic acid acted as precursors of strepogenin.

Following this another unknown factor was eliminated. The TJ factor required by L. lactis Dorner gave considerable trouble due to the presence of varying amounts of stimulatory and inhibitory substances in tomato juice eluates. Some TJ preparations were active enough to permit growth in the absence of added B_{12}. This difficulty was overcome by Caswell, Koditschek, and Henlin (1949) by replacing the TJ with fumaric acid and sodium ethyl oxalacetate, in an amino acid medium containing DL-alanine.

Two facts of considerable importance have come out of the studies of Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes (1949). During autoclaving of medium containing glucose, some reducing agent was formed by the glucose and constituents of the medium, which acted as a growth factor for the organism. Little growth was obtained if the glucose was autoclaved separately from the rest of the medium. When a reducing agent such as thioglycollic acid, methyglyoxal, cysteine, or ascorbic acid was added to the medium, growth resulted whether the glucose was autoclaved separately or with the rest of the medium. Furthermore, the added reducing agent protected the B_{12} from destruction during autoclaving.

As a result of this work, all media now contain some reducing agent. In addition to those mentioned above, Thompson, Dietrich, and Elvehjem (1950) suggested ascorbic acid plus fumaric acid, and Skeggs, et al, (1950) preferred thiomalic acid.

The statement appears quite frequently in the literature that a reducing agent is used in the medium to protect the B_{12} during sterilization. In view of the stability of B_{12} to autoclaving and its chemical inertness to ordinary organic qualitative tests for functional groups, this statement seems rather inconsistent. The elucidation of this problem is not complete, but an excellent beginning was made by Broquist, Stokstad, and Jukes (1951). They noted that there is a considerable difference of opinion regarding the comparative activity of the different forms of B_{12} and of samples autoclaved or added aseptically to the medium. Their own experience was that aseptic addition of B_{12} gave less response than autoclaving B_{12} with the medium containing thiomalic acid; whereas, B_{12b}, B_{12c}, ammino-cobalamin, and liver extract gave essentially the same response by either treatment. To explain the B_{12} action, they produced a reaction product from B_{12} and thiomalic acid which moved between B_{12} and B_{12b} on buffered paper strips. This reaction product was more

potent than B_{12} and gave the same response when autoclaved with the medium or added aseptically. This seems to point out at least one role of the reducing agent in the assay medium, but further data are necessary on other reducing agents before any definite conclusions can be reached.

The latest scheme to overcome assay difficulties is the addition of potassium cyanide to the basal medium. Cooperman, Drucker, and Tabenkin (1951) found that potassium cyanide improved *L. lactis* Dorner assays and replaced thioglycollic acid in *L. leichmannii* 313 assays. With potassium cyanide in the *L. leichmannii* medium, B_{12}, B_{12a}, and B_{12b} elicited equivalent growth responses. Their *L. leichmannii* assays gave consistently higher results than *L. lactis* assays, but with potassium cyanide they gave equivalent results. This is in contrast to Henlin and Soars (1951) who found *L. leichmannii* assays generally lower than *L. lactis* Dorner assays, particularly with B_{12a}. Such conflicting reports in the literature about B_{12} assays makes it imperative for one beginning B_{12} work to find out for himself what to expect from his assay conditions.

Several chemical methods for measuring B_{12} are known. In a series of four papers Boxer and Rickards (1950, 1951) have described a colorimetric and fluorometric method for

5,6-dimethylbenzimidazole and a colorimetric method for the cyanide of B_{12}. The cyanide method is quite sensitive and versatile and may replace the microbiological assay as the method of choice.

The development of satisfactory assay conditions was not the only problem associated with B_{12} research. Liberating or extracting B_{12} from its natural environment so that it is suitable for microbiological assaying has presented some difficulties. Materials such as liver extracts and urine may simply be made up to the proper dilution with water. Various animal tissues and vegetable materials require more treatment. Enzymatic digestion with trypsin was used by Dietrich, Nichol, Monson, and Elvehjem (1949) and pancreatin by Couch and Olcese (1950). A combination of papain and takadiastase has been employed for vegetable materials by Pruzansky (1949) and by Peeler, Yacowitz, and Norris (1949). Vijmenja, Veer, and Lens (1950) have suggested that potassium cyanide may be used in place of proteolytic processes for liver. Their work on liver preparations with absorption spectra measurements is very interesting, but as yet has not been followed by a microbiological study. Support for their postulate that B_{12} in liver is bound to a protein or peptide group has recently come from Bird and Hoevet (1951) who have demonstrated

that proteins are capable of binding B_{12} and rendering it microbiologically inactive.

When crystalline B_{12} became available in 1948 and 1949 it was shown to be quite necessary for proper growth and health of many animals. Johnson and Neumann (1949) took two-day-old pigs and maintained them on an APF-deficient diet for three weeks. At the end of the depletion period some of the animals were given daily injections of crystalline B_{12} or reticulogen, a refined liver preparation. The animals receiving the injections gained weight steadily for the duration of the experiment. Of the six pigs not receiving daily injections, three died and the other three lost weight. This work was extended to weanling pigs by Luecke, McMillen, Thorp, and Bonice (1949). The sows and pigs were fed an APF-free diet. At weanling half of the pigs were changed to a diet supplemented with a B_{12} concentrate. The animals on the supplemented diet gained more weight and utilized their food more efficiently than those on the APF-free diet. Essentially the same results have been obtained in swine feeding experiments at this institution. Experiments are continuing with B_{12} and antibiotics in swine nutrition, and this investigation was part of the research program of the Department of Animal Husbandry.

Vitamin B_{12} has been implicated in many biological processes which cannot be covered here. For more general reviews of B_{12} the reader is referred to the publications of Woods (1949) and Strauss (1950).

III. MATERIALS

A. Organisms

The organisms used in this investigation were *Lactobacillus leichmannii* (ATCC 4797) and *Lactobacillus leichmannii* 313 (ATCC 7830).

B. Chemicals

1. Constituents of the basal medium

Amino acids were purchased from Merck and Company, Rahway, New Jersey and Nutritional Biochemicals Corporation, Cleveland, Ohio. The purines, pyrimidines, and vitamins were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. C. P. dextrose was purchased from Pfanstiehl Chemical Company, Waukegan, Illinois. Tomato juice was purchased by the case on the open market.

2. Enzymes

Takadiastase was secured from Parke, Davis and Company, Detroit, Michigan and papain from Nutritional Biochemicals Corporation, Cleveland, Ohio.

3. Special nutrients

Atlas Tween 80 for vitamin B_{12} assays was obtained from Hill Top Laboratories, Incorporated, Cincinnati, Ohio. Difco Bacto Yeast Extract, Difco Bacto Peptonized Milk, and Difco Bacto Agar were purchased from Difco Laboratories, Detroit, Michigan.

4. Vitamin B_{12}

Vitamin B_{12} (Cobione, Merck) was supplied by Dr. D. V. Catron of Iowa State College or purchased from Merck and Company, Rahway, New Jersey. Vitamin B_{12} (Lederle NP 94-73-5) and vitamin B_{12b} (Lederle NP 119-73-6) were obtained through the courtesy of Dr. J. Kastelic of Iowa State College.

C. Apparatus

Turbidity measurements were made with a KWSZ photometer using filter number 7, 650 millimicrons. This instrument is distributed by Wilkens-Anderson Company, Chicago, Illinois. All pH measurements were made with a MacBeth Continuous Indicating pH Meter made by Macbeth Corporation, New York. Oxidation-reduction potentials were measured with a Beckman pH Meter, Model G, distributed by

George T. Walker and Company, Minneapolis, Minnesota. Basal medium and distilled water were dispensed with a Cannon Automatic Dispenser-Titrator purchased from International Instrument Company, Los Angeles, California. Assays were carried out in Kimble lipless 18x150 mm. test tubes.

IV. METHODS

The methods described in this section are those developed from two years of work on B_{12} assays. Part of the experiments to be described were carried out by different methods. Where any major divergence from these methods occurred, it will be pointed out in the experimental section.

A. Amino Acids

The following amino acids were dissolved in 235 ml. hot water: 1.0 g. DL-alanine, 1.21 g. L-arginine hydrochloride, 1.0 g. DL-aspartic acid, 1.0 g. glycine, 1.35 g. L-hystidine hydrochloride hydrate, 0.115 g. hydroxy-L-proline, 1.0 g. DL-isoleucine, 0.5 g. L-leucine, 0.64 g. L-lysine hydrochloride hydrate, 1.0 g. DL-methionine, 1.0 g. DL-norleucine, 0.235 DL-phenylalanine, 1.0 g. L-proline, 0.115 g. DL-serine, 0.115 g. DL-threonine, 1.0 g. DL-valine, 0.47 g. L-glutamine, and 0.40 g. L-asparagine. L-Glutamic acid, 2.85 g., and 0.94 g. L-tyrosine were dissolved in hot water by the addition of hydrochloric acid and diluted up to

235 ml. L-cystine, 0.94 g., was dissolved in 60 ml. of water with hydrochloric acid, and 0.115 g. DL-tryptophan was dissolved in 60 ml. of water with potassium hydroxide. All four solutions were preserved under toluene, cystine at room temperature, the other three solutions in the refrigerator.

B. Vitamins

Ten milligrams each of riboflavin and thiamine were dissolved in 250 ml. of hot 0.02 N acetic acid and stored in the refrigerator under toluene. The following vitamins were dissolved in 250 ml. of 25 percent ethanol and refrigerated: 50 µ biotin, 10 mg. calcium pantothenate, 10 mg. nicotinic acid, 5 mg. pyridoxine hydrochloride, 5 mg. pyridoxal hydrochloride, and 5 mg. pyridoxamine dihydrochloride. To this solution was added 2 ml. of a solution containing 0.4 mg. p-aminobenzoic acid and 0.54 mg. folic acid.

C. Inorganic Salts

Salts A contained 28 g. $MgSO_4 \cdot 7H_2O$ and 6 g. $MnSO_4 \cdot 4H_2O$ in 200 ml. of water. Salts B contained 40 g. KH_2PO_4 and 52.5 g. $K_2HPO_4 \cdot 3H_2O$ in 300 ml. of water. Both solutions were kept under toluene at room temperature.

D. Purine and Pyrimidine Bases

One hundred twenty milligrams of uracil was dissolved in a few milliliters of hot concentrated hydrochloric acid; 120 mg. of adenine sulfate and 120 mg. of guanine hydrochloride were added and the volume made up to 200 ml. Xanthine, 120 mg., was dissolved in 200 ml. of water with a few drops of 20 percent potassium hydroxide. Both solutions were preserved under toluene at room temperature.

E. Tween 80

Twenty grams of Tween 80 was made up to 200 ml. with 95 percent ethanol and refrigerated.

F. Tomato Juice Filtrate

The tomato juice filtrate was prepared by centrifuging to remove part of the pulp and suction filtering with filter aid to further clarify it. This filtrate was stirred with Norit A, approximately 15 g. per liter, for 30 minutes, suction filtered with the help of filter aid to remove the Norit, and stored under toluene in the refrigerator. No special effort was made to obtain a colorless or charcoal-free filtrate by repeated filtrations.

G. Preparation of Basal Medium and Assay Tubes

The following ingredients were weighed out for each batch of double strength (d. s.) basal medium: glucose, sodium citrate, sodium acetate, cysteine hydrochloride, and ferrous sulfate. The required amounts of the other solutions were added and the pH adjusted to 5.5 with 20 percent potassium hydroxide. Aliquots of test solution or standard B_{12} solution were pipetted into the assay tubes. Distilled water to make 5 ml. and 5 ml. of d. s. medium were dispensed into each tube with the Cannon

Dispenser, and the tube covered with an aluminum cap. Sterilization was accomplished by autoclaving for five minutes at fifteen pounds steam pressure. The tubes were cooled with tap water and placed in the 37°C. incubator until the inoculum was prepared. All levels of standard B_{12} and test solutions were run in duplicate.

H. Stock Cultures

Stock cultures were maintained as stabs in 1.25 percent nutrient agar consisting of 1 percent glucose, 1 percent yeast extract, 1 percent peptonized milk, 0.2 percent Tween 80, and supplemented with about 0.1 mug. B_{12} per ml. Liquid carrying medium had the same composition except that the agar was omitted. *L. leichmannii* 4797 was transferred every two or three weeks from stab to stab. *L. leichmannii* 313 was transferred weekly. Liquid medium was inoculated from a stab, incubated 12 to 18 hours at 37°C. and then a new agar stab made. The stab was incubated for 18 hours and then refrigerated. Assay inocula for a week's work were prepared from the broth culture.

I. Assay Inoculum

Broth cultures were incubated for 12 to 18 hours at 37°C. The cells were centrifuged, resuspended in sterile 0.9 percent saline, and centrifuged again. After a second washing, the cells were resuspended in saline and diluted about 10 times with saline to give a faint turbidity. Assay tubes were inoculated with one drop of this suspension.

J. Final Determination

After 18 to 24 hours incubation at 37°C., the turbidities of the assay tubes were measured as percent transmission with a photometer. The instrument was set with distilled water at 100 percent transmission. The standard curve was plotted on semi-log paper and the results of assays read from this curve. All results were expressed as millimicrograms of vitamin B_{12}; a more correct statement would be "*L. leichmannii* activity" equivalent to the growth elicited by such an amount of pure B_{12}. Whenever there is evidence that the "*L. leichmannii* activity" is or is not due to B_{12}, it will be mentioned along with the experimental results.

K. Paper Chromatography

Paper chromatography was carried out in a battery jar 15 inches high and 11 inches in diameter, covered with a plastic lid. Several holes in the lid were plugged with rubber stoppers or plastic caps. Two glass dishes, one inside the other, were placed on a ringstand just below the cover of the chamber. A small beaker of water and n-butanol was kept in the bottom of the chamber at all times to keep the atmosphere saturated.

Whatman No. 1 filter paper was cut in strips one inch wide and about 15 inches long. Spots of test solutions were pipetted onto the strips at marked points and air dried. The strips were hung from the glass dishes and allowed to equilibrate for one-half to one hour, then wet n-butanol was added to the inside dish through one of the holes in the jar cover. When the butanol had flowed down the strip for the required length of time, the strips were air dried, cut in sections, and assayed in test tubes. The original spot and one inch below it were cut as the first section, the remainder of the strip was cut in one inch sections. This procedure permitted a semi-quantitative estimation of the activity on the strip and was much more sensitive than the usual agar plate method.

L. Cleaning of Glassware

All assay tubes were cleaned with chromic acid before use. Pipettes used for B_{12} and test solutions were cleaned with chromic acid. All other glassware was cleaned with detergent and occasionally with chromic acid.

V. EXPERIMENTAL

A. Experiments with *Lactobacillus leichmannii* (ATCC 4797)

1. *Basal medium*

The basal medium employed with *L. leichmannii* 4797 was that described by Skeggs, Huff, Wright, and Bosshardt (1948) with the acid-hydrolyzed casein replaced in most experiments by the amino acid mixture of Pruzansky (1949). The sensitivity of the medium was fair, 0 to 1.0 mμg. B_{12} per tube, but growth was quite erratic.

An attempt was made to simplify the medium by using only the trypsin digest of casein as the principal source of nitrogen. This substitution was possible, but not practical. The strepogenin preparation had to be norited a number of times to reduce the blank growth, but had to be checked after each Norit treatment to prevent complete inactivation.

Hoffmann, *et al*, (1949) had reported that thioglycollic acid (TGA) protected B_{12} during autoclaving with the medium. In an effort to increase the sensitivity of the assay, TGA was incorporated into the medium. The first

trial gave no improvement in growth, so a fresh supply of TGA was purchased. At levels of 25 and 50 mg. per 100 ml. of medium it produced more growth than was normally obtained from the highest levels of B_{12} in the standard curve. This was the same effect noted by Welch and Wilson (1949) which led them to speculate on an oxidized form of B_{12} present in casein. These results made it imperative to replace the strepogenin in the medium, since it was apparently the cause of much of the erratic growth of the organism.

At this time a culture of _L. leichmannii_ 313 (ATCC 7830) was obtained from Mr. W. R. Bauriedel, Iowa State College. _L. leichmannii_ 313 gave essentially the same response to a given level of B_{12} in 24 hours as _L. leichmannii_ 4797 gave in 40 hours, so _L. leichmannii_ 313 was used in subsequent studies.

A strepogenin assay has been worked out using _L. casei_ as the test organism. Peeler, Daniel, Norris, and Heuser (1949) and Stokes, Koditschek, Rickes, and Wood (1949) found glutamine and asparagine could replace strepogenin for this organism. Experiments with glutamine and asparagine in place of strepogenin for _L. leichmannii_ were not very encouraging. Consequently, this medium was discarded.

2. <u>Assay of Sow's Milk</u>

A description of the nutritional aspects and conclusions of this problem has been reported by Vohs (1950). The primary concern of this investigation was to determine the amount of B_{12} in sow's milk, since it was believed that there might be a relationship between the B_{12} content of the sow's milk and the mortality of the newborn pigs. *L. leichmannii* 4797 and the medium of Skeggs, *et al*, (1948) were employed. The acid-hydrolyzed casein in the medium was replaced by the amino acid mixture of Pruzansky (1949). Assay tubes were plugged with cotton, sterilized for 10 minutes at 10 pounds pressure, and incubated 40 to 48 hours at 37°C.

A preliminary experiment in which the casein of the milk was removed by acetic acid precipitation at pH 4.5 proved to be undesirable. The casein was incompletely precipitated and inhibited the growth of the organism. Rennin was tried, but this gave rapid curdling of the milk and it seemed reasonable to assume that B_{12} was trapped in the curds.

Since papain and takadiastase had been used successfully by Pruzansky (1949) for grains, this combination was tried on milk. It was hoped that the small amount of rennin in takadiastase would cause slow coagulation of the casein

and that the papain would digest the proteins of milk enough to release the B_{12} if it were bound in any manner. A 12 hour digestion of 25 ml. samples of milk at 37°C. was carried out with 10 mg. of rennin and with 10 mg. each of papain and takadiastase. The combination of enzymes did result in slow precipitation of the casein and increased the B_{12} activity of the sample.

The method of preparing milk samples for assay as finally used was as follows: 25 ml. of milk and 25 ml. of water were mixed with 50 mg. each of papain and takadiastase in a 125 ml. flask, layered with benzene, and incubated 12 hours at 37°C. At the end of this time the flasks were steamed 10 minutes to remove benzene, centrifuged to pack the curd, and filtered through fluted filter paper. For the assay conditions described, a 1:10 dilution of the filtrate was made (total, 1:20 dilution of the milk) and assayed at 1, 2, 3, and 5 ml. levels, in duplicate. Blanks on the enzymes were carried through the same procedure, except omitting the milk sample, and showed no activity. Results of these assays are shown in Table 1.

The disturbing feature of the milk assay was that the higher levels of milk samples gave higher values of B_{12} than lower levels. This could be accounted for in two ways: either the activity was not due to B_{12} or the milk

Table 1

Vitamin B_{12} Content of Sow's Milk[a]

Days of lactation	Lot			
	1	2	3	4
2	2.95	1.05	3.05	6.25
14	2.75	0.95	3.15	7.25
28	2.85	1.90	2.50	8.75
56	2.60	4.60	6.20	4.80

[a]Composite samples of 4-6 sows, expressed as µg. of B_{12} per liter.

supplied additional nutrients for the assay organism. In an attempt to answer the first possibility, 50 ml. of a milk preparation were treated with 1.0 g. of Norit A for 15 minutes at pH 3. This treatment removed all of the activity, as would be expected if B_{12} were present. Alkaline hydrolysis of a milk sample with approximately 0.2 N sodium hydroxide lowered the activity somewhat. However, in a later control experiment, a 5 percent lactose solution subjected to a similar hydrolysis produced a definite stimulus for the organism. Consequently, this procedure did not yield any information. A paper chromatogram of a milk sample carried out by Mr. W. R. Bauriedel did show a zone corresponding to B_{12}. In the light of more recent knowledge, this cannot be considered conclusive proof that B_{12} is present in the milk.

Several later experiments, conducted with the improved assay to be described, indicated that the activity of the milk was due to B_{12} and to other nutrients in insufficient amounts in the basal medium. The milk assay was not pursued further because the results of the swine lactation experiments did not warrant further investigation along this line. There was no significant difference in livability of nursing pigs when the sows were fed graded levels of B_{12}.

B. Experiments with *Lactobacillus leichmannii* 313 (ATCC 7830)

1. Basal medium

The original medium and methods employed were those of Peeler, Yacowitz, and Norris (1949). Working with the same medium and organism, Bauriedel (1950) found a significantly greater amount of growth was obtainable with a hydrolyzate of DNA than with B_{12}. This indicated that the basal medium was deficient, probably in nitrogen. Since B_{12} has been implicated in the conversion of thymine to thymidine, the first attempt to overcome the deficiency in the basal medium was the addition of thymine. In concentrations of 1.0 and 1.5 mg. per ml. of d. s. basal

medium, thymine was ineffective. Consequently, increased amounts of the bases already present--adenine, guanine, uracil, and xanthine--were tried. The requirement for additional bases was quite apparent, as shown in Table 2. Further experiments with varying amounts of bases in the medium indicated that five times (5X) the original concentration was optimum, and this change in the basal medium was used for a number of assays.

Table 2

Effect of Purine and Pyrimidine Bases on Growth of *L. leichmannii*

mμg. B_{12} per tube	Per cent transmission			
	0X	1X	5X	10X
0.00	88.7	86.5	86.6	86.6
0.01	88.7	86.3	86.0	85.7
0.05	86.7	71.2	68.4	69.6
0.10	82.7	52.3	47.6	47.5
0.50	64.4	17.6	11.0	11.8

This work was repeated several times in the course of about a year and the same results noted each time. In one experiment a low level of the bases was employed with various tubes supplemented with additional amounts of one of the four bases. The lack of good growth could not be attributed to any one individual base. However, additional

xanthine seemed to give slightly poorer growth. The amount of xanthine was then lowered to be equal to the other bases. The response to various concentrations of the bases is shown in Figure 1. In a number of experiments the best growth was obtained with 3X bases. Generally, 5X gave equal or slightly less growth, so 3X or 3 mg. of each per 100 ml. of d. s. medium was used as the optimal concentration.

Since this medium, as reported in the literature, was found to be deficient in one respect, it was open to suspicion on other grounds. No other major changes were found necessary, but several possibilities were investigated. In order to have more freedom for making up solutions for the basal medium, the amount of tomato juice was lowered from 60 to 50 ml. per 100 ml. d. s. medium without any effect on the growth of the organism.

The magnesium sulfate concentration of this medium was considerably higher than used in most media described for _L. leichmannii_ or _L. lactis_. This concentration, 280 mg. $MgSO_4 \cdot 7H_2O$ per 100 ml. d. s. medium, was quite satisfactory, as shown in Table 3. It is apparent that the magnesium concentration can be varied quite widely with little effect on the organism.

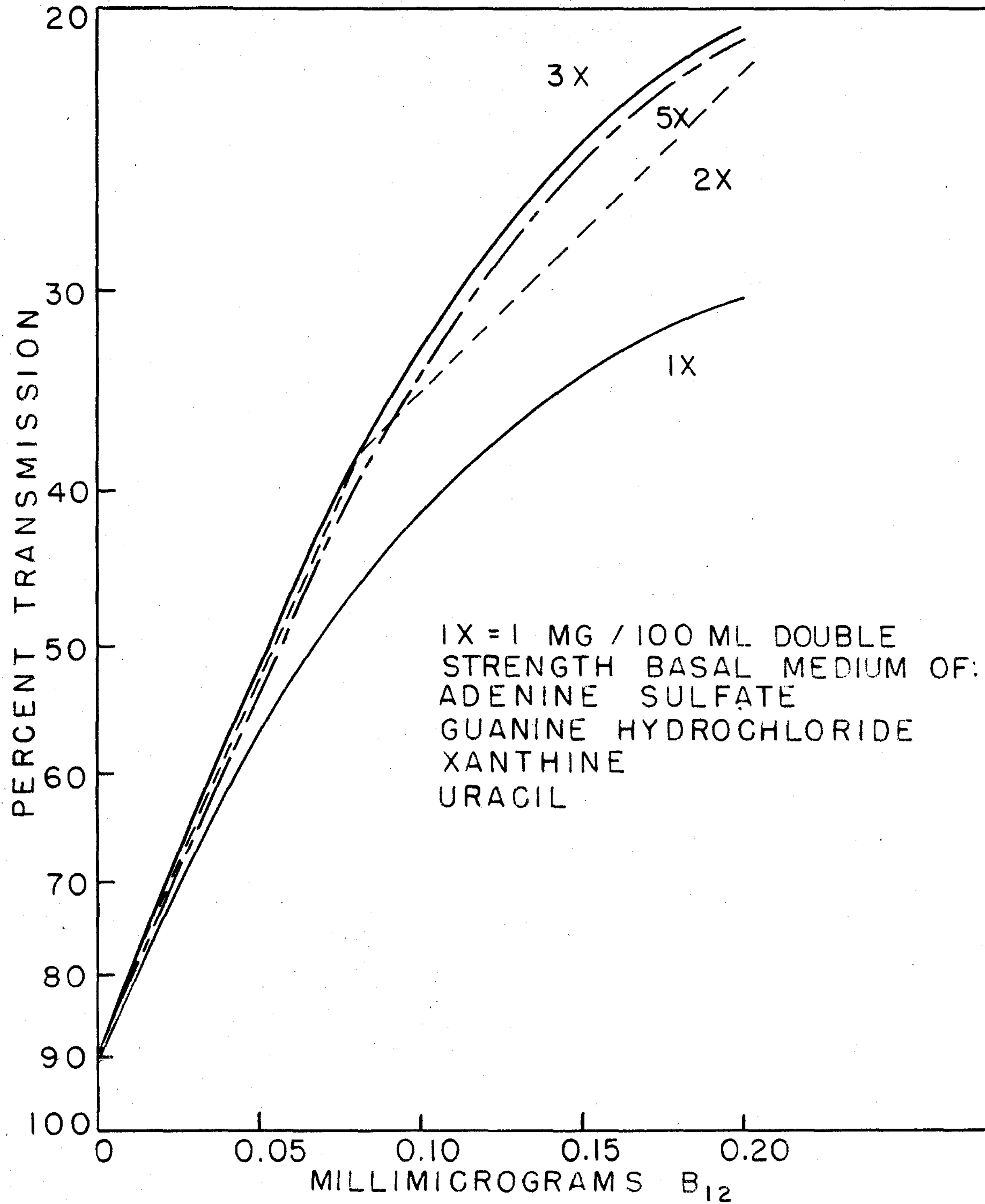


Figure 1. Requirement for Purine and Pyrimidine Bases.

Table 3

Effect of Magnesium Sulfate on the Growth of *L. leichmannii*

mμg. B_{12} per tube	Per cent transmission			
	mg. $MgSO_4 \cdot 7H_2O$ per 100 ml. d. s. medium			
	0	40	100	280
0.00	91.3	91.9	92.5	91.5
0.04	64.0	66.5	66.0	61.3
0.08	45.6	45.6	41.2	39.2
0.12	35.4	34.6	30.6	30.0
0.20	24.9	22.1	20.9	21.3

The concentration of p-aminobenzoic acid was varied from 8 to 500 μg. per 100 ml. d. s. medium without effect, either with or without tomato juice in the medium. This is understandable if the role of p-aminobenzoic acid is in the synthesis of folic acid, since folic acid is included in the medium.

Variable results were obtained by changing the amount of DL-tryptophan in the medium. The over-all conclusion was that the original concentration was adequate.

Tomato juice contains a number of known as well as unknown nutrients for *L. leichmannii*. The added response produced by tomato juice in the medium is illustrated in Figure 2. Good growth was obtained without tomato juice,

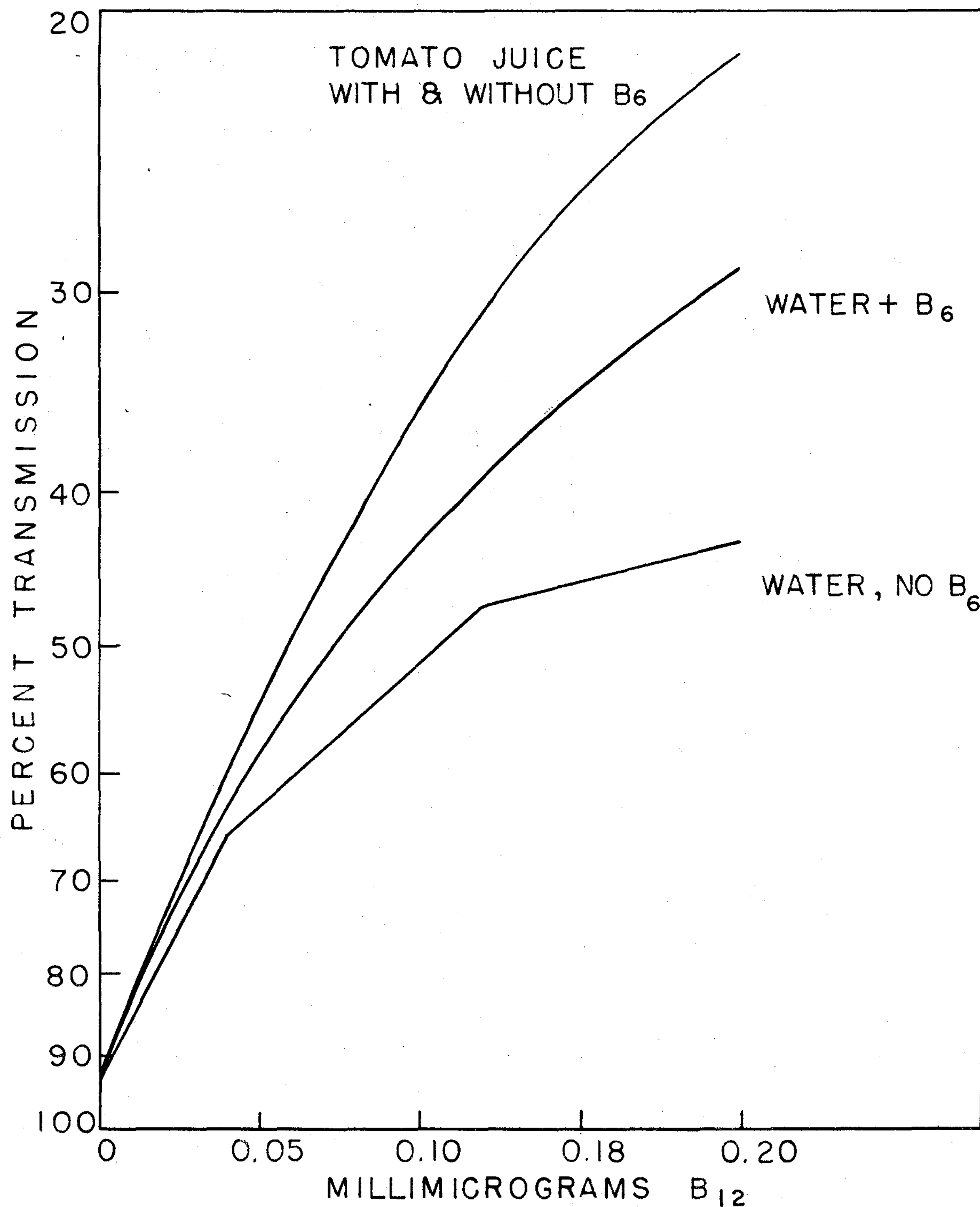


Figure 2. Requirement for Tomato Juice and Vitamins B_6.

but it seemed desirable to have the TJ factor present. Caswell, *et al*, (1949) found that fumaric acid plus ethyloxalacetate would replace the TJ factor for *L. lactis* Dorner. An experiment was tried replacing the tomato juice with these two compounds, but they were without stimulus for *L. leichmannii*. This result has been confirmed by Peeler and Norris (1951). They also point out that the requirement for this TJ factor(s) is eliminated by a heavy inoculum or by long incubation periods. For this investigation it was desirable to use a short incubation time and a light inoculum, so tomato juice was used in all media employed for assay purposes. However, tomato juice complicated the study of the nutritional requirements of the organism and was frequently omitted in such experiments.

The medium described by Peeler, Yacowitz, and Norris (1949) included all three of the B_6 vitamins. Snell and Rannefeld (1945) and Stokes and Gunness (1945) have reported pyridoxamine and pyridoxal to be much more active than pyridoxine for a number of lactobacilli. Furthermore, Snell (1945a) and Winsten and Eigen (1948) have found pyridoxal and pyridoxamine in equilibrium in the presence of amino acids. This equilibrium is apparently quite fundamental in bacterial transamination reactions.

In view of these findings, a study of the vitamin B_6 requirements of *L. leichmannii* was undertaken.

In a preliminary experiment with tomato juice in the medium, each individual vitamin was compared with a combination of all three. Pyridoxine gave slightly poorer growth, but any one of the vitamins was almost as good as all three combined. The results of the next experiment are shown in Figure 2. The organism would grow in a chemically defined medium without a B_6 vitamin present, but growth was not normal. The addition of B_6 vitamins increased the growth of the organism, and the growth curve with B_{12} had a normal shape. The growth of the organism was the same with or without added vitamins B_6 with tomato juice in the medium. It seemed apparent that there was an ample supply of the B_6 group in tomato juice, in addition to the TJ factor which promotes more rapid growth. *L. leichmannii* did not have an absolute requirement for a B_6 vitamin, however. In a medium without tomato juice, the data in Table 4 show that the organism was able to synthesize its B_6 requirements.

Alanine has been implicated in the synthesis of pyridoxine for some lactobacilli by Snell (1945b) and by Stokes and Gunness (1945). An 18 hour assay, without tomato juice in the medium, showed that alanine was not

Table 4

Effect of Time on the Requirement for Vitamins B_6 by *L. leichmannii*

mμg. B_{12} per tube	Per cent transmission			
	Without B_6		With B_6	
	18 hr.	36 hr.	18 hr.	36 hr.
0.00	95.9	92.0	95.6	92.3
0.04	72.0	46.2	65.3	52.8
0.08	61.7	31.4	49.8	35.7
0.12	55.2	24.6	39.2	26.8
0.20	49.3	18.6	29.8	17.5

essential, but beneficial. In a 48 or 72 hour assay as frequently employed for measuring the acid produced by the organism, alanine could probably replace the B_6 requirement of *L. leichmannii*. This was of no concern for a short turbidimetric assay.

It was of some interest to find out if any one of the B_6 vitamins was preferred by the organism. As mentioned earlier, pyridoxamine and pyridoxal are frequently more active than pyridoxine, suggesting that pyridoxine must be converted into a more active form before utilization by the organism. Several experiments gave essentially identical growth with each individual vitamin or all three combined. In these experiments the medium was steamed for 5 minutes for sterilization. Snell (1945a) ran his

pyridoxal-pyridoxamine reactions by autoclaving for 30 minutes at 15 pounds pressure. This is considerably more drastic than the conditions employed for sterilization of the medium. However, a slow non-enzymatic reaction was found by Winsten and Eigen (1948) even at refrigerator temperatures. Consequently, sterile filtration of the medium was tried in an effort to reduce any reactions that might be taking place in the medium, thus changing the form of the added vitamin. Vitamin B_{12} and necessary water were autoclaved in the tubes. The basal medium was made up with the B_6 vitamin present. Half of the tubes were steamed 5 minutes after the basal medium was added. The other half of the basal medium was sterile filtered and aseptically added to the sterile tubes. Growth of the organism was identical when the medium and B_6 vitamin were steamed or added aseptically. With the experimental conditions employed for this assay, it was apparent that the organism could use any of the forms of B_6 with equal facility.

A strange effect was observed when the medium was sterile filtered. Blank tubes, containing no B_{12}, consistently showed a slight growth, whereas corresponding tubes that had been steamed showed no visible growth. No explanation for this growth was found.

The preceding experiments had shown that the organism was not specific in its requirement for a particular form of B_6, and that the tomato juice was a good source of this group. Some paper chromatography was undertaken to find out which one(s) of the B_6 group was present in tomato juice, with the idea of supplementing the medium with the missing form(s). Pyridoxine moved at about the rate reported by Winsten and Eigen (1948). Pyridoxal and pyridoxamine smeared almost the length of the strip. Because the standard vitamins did not behave satisfactory the method was abandoned.

Since the B_6 content of tomato juice was unknown, but apparently adequate, the amounts of vitamin B_6 were lowered in the vitamin solution. A concentration of 100 µg. of each per 100 ml. d. s. medium was selected. This represented a sufficient amount to support good growth without any other source of the vitamins, and covered the possibility of varying amounts in different batches of tomato juice.

The medium, as reported by Peeler, Yacowitz, and Norris, contained cystine, 40 mg., and a rather high level of cysteine, 200 mg. per 100 ml. d. s. medium. For economy and convenience it seemed quite possible that the cystine could be dropped. In several experiments using water in place of the tomato juice, cystine appeared unnecessary.

In contrast, when tomato juice was present, both cystine and cysteine were necessary for maximum growth. Further experiments, in which the levels of cystine and cysteine were varied, indicated additional cystine improved growth at lower levels of B_{12}. The results of one such experiment are given in Table 5. Based on these experiments, the cystine content of the medium was increased to 120 mg. per 100 ml. d. s. medium for a number of assays.

Table 5

Effect of Cystine and Cysteine on the Growth of *L. leichmannii*

	Per cent transmission					
	0.00	0.04	0.08	0.12	0.20	mμg. B_{12} per tube
1X cystine[a] 1X cysteine[b]	87.8	58.6	36.0	26.1	18.5	
1X cystine 3X cysteine	87.6	53.0	32.3	24.4	17.4	
1X cystine 5X cysteine	87.6	51.7	31.8	23.5	17.6	
3X cystine 1X cysteine	87.9	52.9	34.7	24.3	17.0	

[a]40 mg. cystine per 100 ml. d. s. medium.

[b]200 mg. cysteine hydrochloride per 100 ml. d. s. medium.

It was customary to check any changes made in the medium on several occasions, so that the results could be confirmed with a completely new set of solutions for the basal medium. When cystine and cysteine were reinvestigated several months later, no requirement for additional cystine was noted. In fact, cystine did not appear to be necessary, as was originally expected. To cover a possible requirement for cystine, the original level of 40 mg. per 100 ml. d. s. medium suggested by Peeler, Yacowitz, and Norris was used in all subsequent work.

Ascorbic acid and TGA are extensively used as reducing agents in B_{12} media. During the course of this investigation both were tried on a number of occasions, and never were satisfactory substitutes for cysteine. Cysteine apparently acted both as a necessary nutrient and as a favorable oxidation-reduction potential agent. With low levels of cysteine, sufficient for nutrient purposes, the addition of ascorbic acid resulted in slightly less growth rather than more growth as expected from an additional amount of reducing agent. Measurements of the oxidation-reduction potential of assay tubes showed that with cysteine the potential leveled off at about -75 millivolts (against sat. calomel), compared to about -120 millivolts for ascorbic acid or TGA. In the light of the work by

Broquist, Stokstad, and Jukes (1951) showing that thiomalic acid converts B_{12} into a microbiologically more active substance, the role of reducing agents in B_{12} media needs some fundamental study.

The composition of the basal medium finally developed from this work is given in Table 6. This medium was quite sensitive, gave very reproducable results, and supported luxuriant growth of the organism in a relatively short incubation time. A typical standard curve is shown in Figure 3. Generally an 18 hour assay was employed; 16 hours would be satisfactory. For an 18 hour assay the curve usually had a sharper break at about 0.10 mμg. than shown in this figure. By running a 22 or 24 hour assay, the curve rounded out much nicer. If the levels of a test solution ran beyond 0.10 or 0.12 mμg. of B_{12}, a 22-24 hour assay gave more consistent results.

A sample of B_{12b}, compared with B_{12} in several assays, consistently showed only about 35 percent of the activity of B_{12}. Treatment of the B_{12b} with a large molar excess of potassium cyanide at neutrality did not result in any increased activity. It is possible that this B_{12b} was approximately equivalent to B_{12} when the sample was originally prepared, but slowly became inactivated. When

Table 6

Basal Medium for *L. leichmannii*, 100 ml. double strength

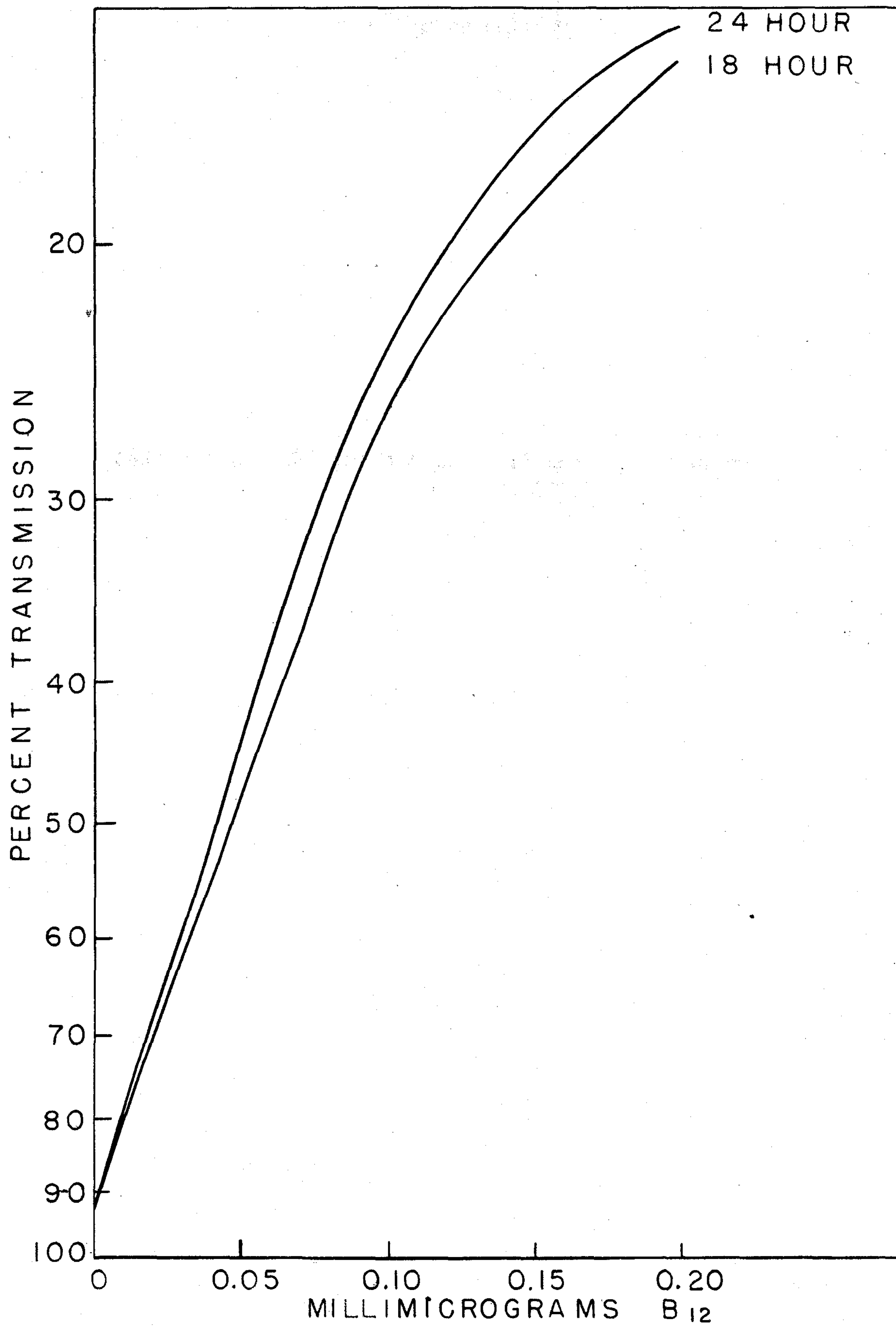
Component	Amount
Glucose	4.0 g.
Sodium citrate	1.0 "
Sodium acetate (anhydrous)	720.0 mg.
Cysteine	200.0 "
$FeSO_4 \cdot 7H_2O$	80.0 "
DL-alanine	42.5 "
L-arginine	42.5 "
DL-aspartic acid	42.5 "
L-cystine	121.3 "
Glycine	42.5 "
L-histidine	42.5 "
L-hydroxyproline	5.0 "
DL-isoleucine	42.5 "
DL-leucine	42.5 "
L-lysine	21.3 "
DL-methionine	42.5 "
DL-norleucine	42.5 "
DL-phenylalanine	10.0 "
L-proline	42.5 "
DL-serine	5.0 "
DL-threonine	5.0 "
DL-tryptophan	5.0 "
L-tyrosine	40.0 "
DL-valine	42.5 "
L-aspargine	15.0 "
L-glutamine	20.0 "
$MgSO_4 \cdot 7H_2O$	280.0 "
$MnSO_4 \cdot 4H_2O$	60.0 "
KH_2PO_4	400.0 "
K_2HPO_4	400.0 "
Adenine sulfate	3.0 "
Guanine hydrochloride	3.0 "
Uracil	3.0 "
Xanthine	3.0 "

Table 6 (Cont'd)

Tween 80	0.2 gm.
Tomato juice filtrate	50.0 ml.
Pyridoxal	100.0 µg.
Pryidoxamine	100.0 "
Pyridoxine	100.0 "
Nicotinic acid	200.0 "
Riboflavin	200.0 "
Thiamine	200.0 "
Calcium pantothenate	200.0 "
Biotin	1.0 "
Folic acid	11.0 "
p-Aminobenzoic acid	8.0 "

employed as a standard for later paper chromatography work, it slowly lost its potency.

Most of the samples assayed during this investigation were from animals that were fed large amounts of penicillin, streptomycin, aureomycin, and terramycin. In the B_{12} assay range for the test organism, a combination of about 1 µg. per tube of each of the antibiotics could be tolerated. Penicillin is the most effective of these antibiotics against _L. leichmannii_, and also the least stable. Autoclaving the medium at pH 5.5 with a large amount of cysteine should have destroyed even fairly high levels of penicillin. None of the difficulties encountered in this investigation could be attributed solely to antibiotics, although several samples were known to contain appreciable amounts. More


24 HOUR
18 HOUR
PERCENT TRANSMISSION
20
30
40
50
60
70
80
90
100
0
0.05
0.10
0.15
0.20
MILLIMICROGRAMS B 12

complete data on the antibiotic content of the samples would have been very helpful.

2. <u>Assays of materials from swine feeding experiments</u>

The purpose of the swine feeding experiments was to establish the nutritional requirement for B_{12} of the weanling pig. During the experiments the pigs were kept in individual wood crates with wire floors. The basal ration consisted of ground yellow corn, soybean meal, vitamins, and minerals. Additions to this ration and an outline of the experiments are shown in Table 7. In experiment 491 the pigs were carried from weanling to 75 pounds. The pigs in experiment 512 were carried from weanling to 100 pounds.

The following analyses were made on the animals in these experiments: weight gain, feed consumption, hemoglobin, red blood cell count, while blood cell count, blood nitrogen, and the B_{12} content of liver, urine, and feces. The B_{12} content of the intestinal tract was determined for some of the animals in experiment 512. It was the purpose of the investigation reported in this thesis to develop assay procedures for the B_{12} assays. A more detailed report on the nutritional aspects and results of these experiments may be found in the thesis by Richardson (1951) and on experiment 491 in the paper by Richardson, Catron, Underkofler, Maddock, and Friedland (1951).

Table 7

Outline of Swine Feeding Experiments

Lot	Number of pigs	µg. B_{12} per lb. feed	mg. Antibiotic mixture[a]
		Experiment 491	
1	3	0	0
2	3	5	0
3	3	10	0
4	3	20	0
5	3	0	40
6	3	5	40
7	3	10	40
8	3	20	40
		Experiment 512	
1	6	0	80
2	6	2	80
3	6	4	80
4	5	6	80

[a]Antibiotic mixture made up of equal weights of aureomycin hydrochloride, procaine penicillin G, streptomycin sulfate, and terramycin hydrochloride.

In brief, these experiments showed the weanling pig requires about 4 µg. of B_{12} per pound of ration when antibiotics are also fed. The requirement for B_{12} seemed to be higher in the absence of antibiotics. This indicated that the antibiotics controlled certain intestinal microorganisms which compete with the pig for B_{12}. In addition, the antibiotics inhibit B_{12}-producing microorganisms in the intestine, since the antibiotics without B_{12} produced more

extreme symptoms of deficiency than the basal ration alone.

a. Feed samples. Feed samples were assayed by the method of Peeler, Yacowitz, and Norris (1949) as follows: a 2 g. sample was autoclaved for 30 minutes at 20 pounds pressure in 100 ml. of acetate buffer (pH 4.5). The mixture was cooled and 40 mg. each of papain and takadiastase plus 2 mg. of cysteine hydrochloride added and well mixed. The enzymatic digestion was incubated for 40 hours at 37°C. under benzene. At the end of this time, the pH was adjusted to 5.5 and the benzene steamed off for 5 minutes. After cooling, the volume was made up to 200 ml. and the solution filtered. The preliminary autoclaving for 30 minutes at 20 pounds pressure was eventually eliminated because it seemed to have no effect on the results.

The assay was not satisfactory, as shown by the results given in Table 8. Each value is the calculated B_{12} content for a particular level of the sample. These samples were a commercial B_{12}-fortified chicken feed produced by growing a culture of Bacillus subtilis on regular feed. A small drift in values frequently occurs when the levels of a sample extend beyond the straight line portion of the standard curve. But a 100 percent difference such as illustrated in Table 8 made it doubtful that B_{12} was being measured. Paper chromatograms were then run on a concentrate

Table 8

Vitamin B_{12} Activity of Feed Samples

Sample	mμg. B_{12} per gram
1	24, 48
3	16, 30
5	15, 30
8	10, 20
9	15, 26
10	37, 32

of sample 10, Table 8. One very slow and one faster moving zone were distinguishable, in addition to the activity at the original spot. The non-moving activity was presumably B_{12}; the fastest moving spot had an R_f value of about 0.5, which may have been thymidine (R_f = 0.54). A quantitative estimation of the strip indicated that B_{12} accounted for less than half of the activity. It was thus evident that there was little B_{12} activity in these feed samples, and that an accurate assay would be difficult, if not impossible, to attain.

As a check on this method for assaying feed samples, the following experiment was conducted. A solution of B_{12} was made up and aliquots containing 20 mμg. of B_{12} were treated as follows: A, stored in the refrigerator; B, carried through the assay procedure, including autoclaving

at 20 pounds pressure for 30 minutes, but the enzymes were omitted; C, same as B, only with enzymes. The assay values for a sample from each of these solutions were as follows: A, 0.052; B, 0.050; C, 0.024 mμg. B_{12}. Autoclaving and incubation had little effect upon B_{12}, but the enzyme digestion destroyed about half of the B_{12}. This cast considerable doubt on the usefulness of this enzyme combination for B_{12} work. However, in the presence of another substrate, the enzymes may not appreciably destroy B_{12}.

Assays of a number of the rations employed in swine feeding experiments were no more successful than with the feeds described above. These rations were composed mainly of corn and soybean meal. Little, if any, B_{12} has been found in these materials, but they apparently contain enough interfering substances for L. leichmannii to make an assay extremely difficult.

The problem of representative samples also arose in connection with these samples. The rations were mixed several hundred pounds at a time, and a few grams taken out for assay purposes. The poor sampling became evident when an assay was run on rations supplemented with crystalline B_{12}. In this case an acetate buffer extraction of the samples was employed, and there was practically no recovery of the crystalline B_{12}.

b. Urine samples. In order to establish a nutritional requirement for B_{12} for the pig, it was necessary to know both the amount of B_{12} ingested and the amount excreted. For this reason, B_{12} assays were run on urine and feces samples collected during a 24 hour period. Crystalline B_{12} was added to the feed, and the B_{12} intake for any period could be calculated from the records of feed consumed by the animal.

Urine samples were adjusted to a pH of 5.5 and diluted to the necessary volume. In a few cases, the pH was not adjusted and no differences in the assay results were noted.

There was a definite drift in the values for different levels of the urine samples from feeding experiment 491. This was partly due to a poor choice of levels for assaying the samples. The results of these assays are given in Table 9.

All of the animals in experiment 512 were on a ration containing penicillin, aureomycin, streptomycin, and terramycin. Unfortunately, antibiotic assays were not carried out, although they were included in the original plans for this feeding experiment. The urine of these animals must have contained appreciable quantities of the antibiotics, but at the dilution levels employed they did not affect the assay. The results of one assay are shown in Table 10.

Table 9

Vitamin B_{12} Content of Swine Urine

μg. B_{12} per lb. ration	mμg. B_{12} per ml.	mμg. B_{12} per ml.[a]
No antibiotics		
0	1.25	1.50
5	1.60	1.80
10	2.75	2.40
20	2.80	2.30
Antibiotics		
0	0.60	0.83
5	1.15	1.50
10	1.80	1.50
20	3.45	2.00

[a]Assays on same samples by Chas. Pfizer and Co., Inc., Brooklyn, New York.

Table 10

Vitamin B_{12} Content of Swine Urine

Level of assay tube (ml.)	mμg. B_{12} per ml.			
	1	2	3	4
1	0.75	1.75	0.90	0.85
3	0.70	1.70	0.90	0.90
5	0.75	1.65	0.95	0.85

The three levels selected in this assay covered the most usable portion of the standard curve, and do not show any drift in values that might indicate non-B_{12} activity or antibiotic interference.

The final assay results for experiment 512 are presented in Table 11. The urine samples were composite samples of the five or six animals in each lot. In the assay in which the pH of the samples was not adjusted, the pH values of the dilutions used for the assay were all about 7.5. From these results it appears that adjustment of the pH of urine samples was unnecessary. Successful assays may be run merely by diluting the urine to whatever volume is necessary to have aliquots of the proper activity.

Table 11

Vitamin B_{12} Content of Swine Urine

Lot	mμg. B_{12} per ml.[a]	mμg. B_{12} per ml.[b]
1	0.55	0.55
2	1.90	1.90
3	1.05	1.05
4	1.05	1.10

[a]pH of samples not adjusted.

[b]pH of samples adjusted to 5.5.

c. Liver samples. Liver samples from experiment 512 were prepared for assay by a method suggested by Dr. J. Kastelic (1951). Five grams of liver and water to make a total volume of 250 ml. were mixed 3 minutes in a blender. To a 50 ml. aliquot, 40 mg. of potassium cyanide was added and the pH adjusted to 7. The solution was autoclaved for 5 minutes at 15 pounds pressure, cooled, and the pH lowered to 5.5. The solution was shaken several hours in the dark, and then diluted to 200 ml. An aliquot of this solution was diluted to the required volume and filtered. The potassium cyanide was added to protect the B_{12} during autoclaving and to bring about the conversion of other forms of B_{12}, if present, into B_{12}. The preliminary autoclaving with potassium cyanide appeared to be necessary to get the B_{12} activity out of the tissue. Digestion with pancreatin gave no improvement in the liberation of B_{12}, and pancreatin preparations ordinarily contained enough B_{12} to make an enzyme correction necessary on low potency samples.

Some typical assay values are given in Table 12. In general, the values for different levels of a sample were reasonably consistent. A drift in values occasionally occurred, such as with sample 952, Table 12. A repeat assay on this sample gave the following values: 167, 173, and 167 mμg. per g.

Table 12

Vitamin B_{12} Content of Swine Liver

Level of assay sample (ml.)	mµg. B_{12} per gram				
	735	711	736	774	952
1	125	100	120	75	167
3	120	100	127	80	160
5	120	105	127	80	153

Liver normally contains appreciable amounts of nucleic acids or their components, which may interfere with a *L. leichmannii* assay. Hoffmann, *et al*, (1949) found about 3 percent of the activity of liver extracts was alkali stable, presumably desoxyribosides. The characteristic effect of desoxyribosides is a drift down in values with higher levels of samples. No consistent drifting of values was observed, and, therefore, no correction was attempted.

d. *Feces samples*. For all assays concerned with experiment 491, the assay tubes were sterilized by steaming for 5 minutes, as recommended by Peeler, Yacowitz, and Norris (1949). When assays of fecal samples were undertaken, the problem of contamination arose. Preliminary experiments were performed by simple acetate buffer extraction of the sample for 24 hours under toluene. In order

to prevent bacterial growth during the extraction, the samples were first mixed with the buffer solution and then autoclaved at 15 pounds pressure for 10 minutes.

It was necessary to prepare feces samples in dilutions of about 1 g. per 50,000 ml. Even in this extreme dilution, there was a noticeable amount of suspended material. Two samples were prepared, half of each filtered, and the filtered and non-filtered solutions assayed. There were a 15 and a 25 percent loss of activity as the result of filtering. On the basis of experiments on both feces and other materials, filtration of samples was generally avoided.

In an effort to improve the release of B_{12} from the fecal material, an enzyme digestion was compared with buffer extraction. A 2 g. sample was digested with 40 mg. each of papain and takadiastase. The enzyme digestion gave a value of 280 mμg. of B_{12} per g., compared to 340 mμg. per g. for buffer extraction. As a result of this experiment enzyme digestions were abandoned.

To avoid contamination, the samples were autoclaved, as stated at the beginning of this section. Since cysteine was used in the medium to protect B_{12}, the effect of cysteine on the samples was investigated. The effect was quite apparent, as shown in Table 13. The amount of cysteine hydrochloride given in the table was mixed with a

Table 13

Effect of Cysteine on Autoclaving Feces Samples

mg. Cysteine hydrochloride	mμg. B_{12} per gram
0	330
50	360
200	1080
500	1240

2 g. sample in 100 ml. of acetate buffer before autoclaving. Other experiments with graded levels of cysteine indicated that 250 mg. of cysteine hydrochloride per gram of feces was sufficient for maximum protection of the B_{12}.

All of the samples that were assayed during the course of this investigation were quick frozen and maintained in a frozen state until prepared for assay. In experiment 491 there was some delay in collecting fecal and urine samples, with the resulting possibility of bacterial growth and synthesis of B_{12}. The amount of B_{12} found in feces up to this point was surprisingly high compared to the one published report about swine. Using a chick growth test, Groschke, Thorburn, Luecke, Thorpe, and McMillen (1950) found incubated pig manure highly potent, whereas fresh manure contained no B_{12}. Therefore, a strictly fresh

sample was assayed along with a frozen sample. The fresh sample had the same order of activity as the frozen sample, indicating that no bacterial synthesis had occurred in the samples.

Both before and after the experiments on the addition of cysteine to the samples, the effect of pH on autoclaving and incubating the samples was studied. With cysteine present, the pH could be varied at least from 4.5 to 6.5. Without cysteine, the pH range was limited from 5.0 to 6.0 for maximum activity.

Mechanical mixing of the samples in a Whizmix blender was compared with the 24 hour extraction in an effort to streamline the assay procedure. The same results were obtained by either method, so the shorter mechanical mixing was adopted.

The following procedure was employed for the feces assay of experiment 491 samples. The samples were composites of the three pigs in each lot. To lessen sampling errors, a 5 g. sample was used. The sample was mixed with 250 ml. of acetate buffer, pH 4.5, for 10 minutes, then 1250 mg. of cysteine hydrochloride was added. The pH was adjusted to 5.0 and the mixture autoclaved for 10 minutes at 15 pounds pressure. After cooling, the pH was readjusted to 5.5 and the mixture diluted to 500 ml. For experiment 491

samples, a 2 ml. aliquot was diluted to 1 liter and assayed at 1, 3, and 5 ml. levels.

Throughout the development of this assay procedure, samples were frequently checked by paper strip chromatography. Aliquots representing as much as five times the activity that could be assayed in a tube did not show the presence of any moving activity on the paper strip. As a further check on the nature of the activity, a sample of Lot 4 was mixed with an equal volume of 0.4 N potassium hydroxide and steamed for 30 minutes. Less than 3 percent of the original activity remained after this treatment. The nature of the assay, the alkali sensitivity of the activity, and the lack of movement on a paper strip all characterized the activity of these samples as B_{12}.

The results of the final assays for experiment 491 are shown in Table 14. The assays by the Pfizer research group were carried out by a different procedure and with a different basal medium. The agreement between the two sets of data is excellent, since differences of 50 or 100 percent have not been uncommon in B_{12} assays run on the same sample by different groups.

When the feces samples of experiment 512 were available, the assay procedure was further simplified. Several experiments showed that the medium could be autoclaved at

Table 14

Vitamin B_{12} Content of Swine Feces

µg. B_{12} per lb. of ration	mµg. B_{12} per gram	mµg. B_{12} per gram[a]
No antibiotics		
0	850	860
5	950	990
10	1000	1100
20	1350	1260
Antibiotics		
0	800	800
5	1150	1240
10	700	520
20	2500	2070

[a]Assays on same samples by Chas. Pfizer and Co., Inc., Brooklyn, New York.

15 pounds pressure for 5 or 10 minutes with equal or slightly better growth than steaming for 5 minutes. A comparison was then made with a feces sample between the method employed previously and a new procedure. The new procedure consisted of mixing the sample in a Whizmix in acetate buffer, pH 5.5, and diluting the mixture to the required volume. Samples and standard B_{12} tubes were autoclaved for 10 minutes at 15 pounds pressure. The calculated results, in terms of B_{12} per gram, were equal or slightly better for the new procedure.

Several paper chromatograms of the feces samples showed only the non-moving zone corresponding to B_{12}. In addition, samples were treated with potassium cyanide to convert any of the less active cobalamins into B_{12}. One gram samples were treated with 10 mg. and 0.1 mg. of potassium cyanide at pH 5.5 and shaken for 22 hours. This did not result in any improvement in the assay.

For the assays of experiment 512 samples, either 2 or 5 g. samples were weighed into the Whizmix. The samples were mixed for 5 minutes with about 200 ml. of acetate buffer, pH 5.5. After mixing, the samples were diluted to the required volume and aliquots pipetted into the assay tubes. The results of one such assay appear in Table 15. Except for Lot 1, this assay was quite satisfactory. It must be remembered that a difference of 50 mμg. of B_{12} in Table 15 represents a difference of only 0.001 mμg. at the assay level. This is about as accurate a value as could conveniently be read from the standard curve, and the values were generally rounded off to two or three significant figures to avoid the appearance of unattainable accuracy. The reproducibility of most assays was within 10 percent, although differences as great as 100 percent were encountered.

Table 15

Vitamin B_{12} Content of Swine Feces

Level of assay sample (ml.)	mμg. B_{12} per gram			
	I	II	III	IV
1	1150	850	850	900
2	1050	850	850	950
3	1050	850	850	1000
4	950	800	850	950
5	950	800	800	950

e. Intestinal contents. The results of the urine and feces assays of experiment 491 showed that the pig excreted as much as ten times more B_{12} than was in the feed consumed. If the B_{12} assays were reliable, it appeared that bacterial synthesis of B_{12} by the intestinal flora of the pig occurred in excess of the pig's requirement, but that this B_{12} was unavailable to the animal. The excellent agreement between the assays run in this institution and by Chas. Pfizer and Company was very encouraging, in view of the opinion in the literature that fresh pig manure had no B_{12} activity. Therefore, one phase of experiment 512 was to determine where the synthesis of B_{12} took place. The original plan called for assays of the contents of the stomach, small intestine, cecum, large intestine, and feces of the animals

that were sacrificed at the end of the feeding experiment. Because feed assays had been unsuccessful, the stomach contents were not investigated, since they differed little from the raw feed. The fecal assays were reported in the preceding section.

Preliminary experiments were run on a large intestine sample to determine the necessary dilution and nature of the assay. A drift in values was observed that suggested either non-B_{12} activity or antibiotic interference. Paper strip chromatography showed some diffusion of the original spot, but no moving zones of activity characteristic of desoxyribosides. A recovery experiment was then set up to find out if the sample contained inhibitory substances for the assay organism. No inhibition was noted, as shown in Table 16.

Although potassium cyanide treatment did not affect the feces assays, it was tried on the intestinal samples. This experiment is shown in Table 17. Each sample was run at the 1, 3, and 5 ml. levels. The treatments were as follows: a 5 g. sample was mixed with 250 ml. of acetate buffer for 3 minutes; A, 50 ml. (equivalent to 1 g.) was diluted to the necessary volume; B, 50 ml. was autoclaved for 5 minutes at 15 pounds pressure, cooled, diluted to 100 ml., and shaken for 2 hours. An aliquot of the solution

Table 16

Recovery of B_{12} in Large Intestine Sample

mμg. B_{12} in sample	mμg. B_{12} added	mμg. B_{12} calculated	mμg. B_{12} found
0.038	0.02	0.058	0.056
0.075	0.02	0.095	0.091
0.098	0.02	0.118	0.117

Table 17

Effect of Potassium Cyanide and Autoclaving on Intestinal Samples

	mμg. B_{12} per gram		
Sample	A	B	C
Small intestine	24	12	23
	20	11	24
	19	11	23
Cecum	340	10	380
	340	16	380
	320	16	360
Large intestine	500	0	700
	530	33	670
	500	66	630

was diluted to the volume required for the assay; C, same as B, except 25 mg. of potassium cyanide added before autoclaving. The small intestine gave a drift in values which had been observed previously; the cecum and large intestine gave satisfactory assays. Autoclaving the samples, treatment B, destroyed most of the activity, as was expected. It is noteworthy that the percentage of stable activity in the small intestine is much greater than in the other two sections. The reason for this will be presented later. A significant increase in activity resulted from the addition of potassium cyanide, treatment C, suggesting that less active cobalamins were converted into B_{12}. The possibility of various forms of B_{12} being present was investigated at a later date.

To be consistent with the feces assays, the same assay procedure was adopted for the intestinal samples. The only change in the procedure, as reported for feces, was that some of the small intestine samples were filtered to remove suspended material. A summary of these assays is presented in Table 18. It is apparent that synthesis of B_{12} occurs in the cecum and large intestine. This data emphasizes one error in sample technique that had been suspected from urine and feces assays. There is a considerable variation between the animals in any one lot.

Table 18

Vitamin B_{12} Content of Digestive Tract of the Pig

		mμg. B_{12} per gram		
Lot	Pig	Small intestine[a]	Cecum	Large intestine
1	710	19	420	730
1	774	9	460	1050
1	953	8	340	900
2	712	48	360	1600
2	737	6	110	500
2	950	17	240	400
3	711	21	280	470
3	735	42	260	370
3	951	19	320	325
4	713	52	360	1050
4	736	40	240	530
4	952	10	280	770

[a]Activity not due solely to B_{12}.

Each animal is an individual and must be treated as such. Consequently, mixing composite samples of all the pigs in one lot does not give a clear picture of that lot. An animal such as 712 or 713 exerts too much influence in a composite sample to be considered just an average pig.

Alkaline hydrolysis of large intestine and cecum samples resulted in 95-99 percent destruction of the activity. This hydrolysis was carried out by adding 2 or 5 ml. of a sample to 50 ml. of 0.2 N potassium hydroxide, steaming for 30

minutes, cooling, and adjusting the pH to 5.5. The solution was then diluted to the desired volume. The actual pH of the potassium hydroxide solution during hydrolysis was about 12.5.

Assays of small intestine samples resulted in too much drifting of values to have been due only to B_{12}. Alkaline hydrolysis of a number of samples was carried out by adding 5 or 10 ml. of a sample to 20 ml. of potassium hydroxide of such a strength as to make the resulting solution about 0.2 N. The pH of these solutions varied from 12.0 to 12.5. The solutions were steamed 30 minutes, cooled, the pH adjusted to 5.5, and diluted up to 100 ml. Pure B_{12} was destroyed by these conditions. There was almost complete destruction of B_{12} added to samples; actual amounts of B_{12} were difficult to estimate in the presence of such a large proportion of desoxyribosides. Results of some hydrolysis experiments are shown in Table 19. Two levels of each sample were assayed. The amount of B_{12} varied from sample to sample, but generally represented only a small part of the total activity. Because a correction factor for these samples would have been so large, no attempt was made to calculate the actual amount of B_{12} in these samples. It was sufficient to know that there was very little B_{12} present in the small intestine compared to the remainder

Table 19

Effect of Alkaline Hydrolysis on Small Intestine Contents

Pig	mμg. B_{12} activity	mμg. B_{12} activity after KOH	mμg. B_{12} destroyed	Percent alkali sensitive activity
735	40.0	38.0	2.0	5
	34.0	30.0	4.0	12
712	48.0	42.0	6.0	12
	38.0	34.0	4.0	11
736	48.0	46.0	2.0	4
	36.0	34.0	2.0	6
951	23.0	16.0	7.0	30
	19.5	13.0	6.5	33
711	22.0	18.5	3.5	16
	20.0	16.0	4.0	20
710	16.5	8.0	8.5	51
	14.0	8.0	6.0	43
713	53.0	46.0	7.0	13
	42.0	41.0	1.0	2
950	22.5	19.5	3.0	13
	17.5	17.0	0.5	2

of the digestive tract. There is also a direct correlation between the drift of assay values and the B_{12} content of the sample: the greater the percentage of desoxyribosides, the greater the drift in assay values.

Further confirmation of the interferences in the small intestine was given by paper chromatograms. Paper

strips of 712, 713, 735, and 736 showed at least two moving zones in addition to a stationary B_{12} zone. The moving zones had R_f values of about 0.70 and 0.35. The fastest moving zone could have been thymidine, the slower one the desoxyriboside of cytosine or hypoxanthine. These R_f values were difficult to compare with those recorded in the literature, because this chromatography was carried out at 36-37°C. instead of at room temperature as is the usual custom.

Four forms of B_{12} have been isolated as the products of fungal synthesis. Since the data in Table 17 suggested that less active forms of B_{12} might be present, an attempt was made to further identify the activity of the intestinal samples. The method employed was that of Woodruff and Foster (1950) for separating B_{12} and B_{12a} on KH_2PO_4-buffered (pH 4.6) paper strips. Descending chromatography was carried out at 36-37°C. for 36 to 40 hours. Reference samples of B_{12} and B_{12b} could be separated by 5 or 6 inches in this length of time, B_{12} moving the fastest.

Two cecum samples and three large intestine samples showed very little movement. One large intestine sample had some activity corresponding to B_{12b}, but most of the activity did not move. There apparently was no B_{12} in the samples; however, these samples were in acetate buffer, and

it was felt that the buffering action of the samples might have changed the diffusion characteristics of the B_{12} activity.

Fresh samples of a large intestine and cecum were then made up in water. Part of each solution was autoclaved at pH 7 with potassium cyanide (25 mg. per g.) for 5 minutes at 15 pounds pressure. These solutions were cooled and the pH adjusted to 5.5. Paper strips of the samples mixed with water showed only the stationary or very slow moving zone, less movement than B_{12b}. The potassium cyanide treatment changed the character of the sample in that it produced moving activity, but no conclusions could be reached.

A new approach was tried in the next experiment. A 5 g. large intestine sample was made up to 250 ml. with water and mixed for 3 minutes in a Whizmix. The pH was adjusted to about 5.5 with 5 N sulfuric acid and treated as follows: A, a 50 ml. aliquot (equivalent to 1 g.) was diluted to 100 ml.; B, 20 mg. of potassium cyanide was added to another 50 ml. aliquot, the pH adjusted to 7.1, and the mixture placed in the dark. After 5 hours the pH was readjusted to 5.5 with 5 N sulfuric acid and the solution diluted to 100 ml. This treatment should have converted any of the cobalamins into B_{12}. Assays of the two aliquots gave values of 1250 and 1350 mμg. of B_{12} per gram, respectively,

indicating that potassium cyanide had very little effect on the assay. A paper chromatogram of A yielded only the expected very slow moving spot. However, the potassium cyanide treatment, B, gave moving activity, but poorly distinguishable zones.

Portions of the two aliquots were also extracted with n-butanol. Twenty-five milliliters was made three-fourths saturated with ammonium sulfate, and extracted twice with 10 ml. of butanol. The ammonium sulfate caused a flocculation of proteinaceous matter, most of which was carried along with the butanol layer. Assays on the residues of the extractions showed about 80 percent of the activity had been removed. The butanol extracts were combined and spotted on paper strips. The extract of A again had only the stationary spot, whereas, the extract of B had two moving spots. The slowest moving spot corresponded to B_{12b}, the fastest spot moved a little behind B_{12}. More than half of the activity on the strip was in the B_{12} spot.

This data suggested that the activity of the sample was bound in some manner, probably to proteins. This combination did not move on a paper chromatogram, and apparently was not broken by butanol extraction. Potassium cyanide could break the combination and release the activity, possibly converting part of the activity into B_{12}. The

hypothesis of a B_{12}-protein combination is supported by the filtration data given in Table 20. The more efficient the filter, the more activity it removed.

Table 20

Effect of Filtration on Large Intestine Sample

	mμg. B_{12} per gram		
Pig	No filtering	Filtered through Whatman No. 1	Filtered through Sietz filter
712	1400	900	200

Although potassium cyanide could liberate the activity in the sample, it also could convert various cobalamins into B_{12}, thus complicating the chromatography results. In an attempt to release the activity without this complication, a 1 g. sample was digested with 25 mg. each of papain and takadiastase plus 1 mg. of cysteine hydrochloride for 29 hours at pH 4.5. This enzyme digestion resulted in about a 35 percent loss of activity. Paper chromatograms were prepared both from the final solution and a butanol extract of the final solution. Two spots were visible, corresponding to B_{12} and B_{12b}. Approximately 75 percent of the activity was due to B_{12b}.

This work was repeated on a cecum sample with essentially the same results. In this case, the enzymatic digestion did not release any activity that moved down the strip; the potassium cyanide treatment liberated mainly a B_{12b} zone.

VI. DISCUSSION

The organism employed during most of this investigation, *Lactobacillus leichmannii* 313, was very satisfactory in its general characteristics. It grew rapidly and consistently, and it was quite sensitive to B_{12}. However, it lacked a specificity for B_{12} which made it unsuitable for materials containing more than a few percent of interfering substances.

The nutritional requirements of *L. leichmannii* 313 are complex in the sense that this organism requires many preformed amino acids and vitamins. These requirements are known and the organism may be grown in a chemically defined medium. Such a medium has a marked advantage over earlier media which contained unknown and variable ingredients, such as tomato juice and a trypsin digest of casein. Although the medium developed during this investigation contained a tomato juice filtrate of unknown composition, this did not appear to be a disadvantage. The medium without the tomato juice filtrate supported good growth; the tomato juice supplied a factor(s) promoting more rapid growth, which was desirable from the standpoint of saving time. The growth obtained in this medium for any level of B_{12} compared very favorably with any medium described in the literature.

The microbiological assay for B_{12} is still troublesome and uncertain. One of the principle uncertainties is the problem of what change occurs in the B_{12} molecule in the medium to make the vitamin more active for the organism. Aseptic addition of sterile B_{12} to the assay tubes never results in as much growth as when the B_{12} is autoclaved with the medium, provided a reducing agent is present in the medium. A further complication is found in the variable response to other cobalamins. The data available in the literature indicates that these compounds have about the same activity either when added aseptically or autoclaved with the medium. Furthermore, they are not as active as B_{12} on a weight basis. In the experiments reported here, B_{12b} was only about 35 percent as active as B_{12} when autoclaved with the medium. This suggests that the difference in activity must be due to the group coordinated to cobalt, but the reducing agents exert their influence on some other part of the molecule. If the reducing agents acted only through the coordinating group, perhaps replacing it, then all cobalamins would have the same activity when autoclaved with a medium containing a reducing agent.

Absorption spectra of the various cobalamins show some differences that may be due to changes in the molecule other than the coordinating group. These differences might account

for the different microbiological activities. The solution of this problem must wait for a tremendous amount of chemical analysis.

Preparation of samples for assay is a major problem, but the voluminous literature on B_{12} offers very little practical advice. Mixing, extraction, heating, and enzymatic digestion have been employed. With the possible exception of liver tissue, the materials that were assayed in this study were adequately handled by thorough mixing in either water or buffer solutions. A complete comparison of the different materials was not made, but extractions with acetate buffer for 24 hours or more did not appear to be any better than simple mixing of the samples in a mechanical mixer. If a conjugate or combined form of B_{12} exists, steaming or autoclaving the samples with the basal medium released it for utilization by the test organism. The enzyme combination of papain and takadiastase was tried on several materials and consistently resulted in less activity.

Tissues such as liver present a more formidable problem. The release of B_{12} in this case depends upon the rupture of the cell walls which is not easily accomplished by mixing or blending. Digestion with trypsin or pancreatin have been widely used to release B_{12} from the cell contents of

liver, kidney, heart, and muscle tissue. A serious objection to pancreatin is the necessity for an enzyme correction with low potency samples. Trypsin may be recrystallized until the enzyme blank is negligible. The experiments with papain and takadiastase reported in this investigation indicated that B_{12} was destroyed by the enzymes. If this is true, other enzymes may be suspected also.

The method for the preparation of liver samples employed in this investigation offers one possibility to avoid the use of enzymes. The samples were autoclaved with potassium cyanide at pH 7 and then shaken in the dark for several hours at pH 5.5. Potassium cyanide appeared to be an unique reagent in that it liberated B_{12} from any protein combination, acted as a reducing agent to protect B_{12} during autoclaving, and it might have converted other cobalamins into B_{12}, which is the standard employed in all assays.

One of the most significant results of this study was the discovery that the pig excretes in feces much more B_{12} than is consumed in the feed. A small amount of ingested B_{12} is recovered in the urine. Assays of the digestive tract showed that the intestinal synthesis of B_{12} occurred in the cecum and large intestine. If this B_{12} were available to the pig, there would be no need to supplement the feed with B_{12}. Apparently, B_{12} is adsorbed largely in the

small intestine and perhaps to a limited extent in the cecum. There may be some synthesis of B_{12} in the small intestine, but this was not investigated.

If the studies of the intestinal tract are extended, there are an unlimited number of interesting problems. The work reported in this investigation should be done on a more quantitative basis. The amount of material in the intestinal sections should be determined and more B_{12} assays made throughout each section. It would also be desirable to know the antibiotic content of the intestinal contents, and the effect of the antibiotics on the microflora. Some of these microorganisms must be potent producers of B_{12}, and it would be interesting to isolate cultures and compare them with the organisms presently employed for the production of B_{12}.

The condition of the B_{12}, combined or free, is helpful information when developing an assay procedure. The evidence for the intestinal contents suggested that the activity was present as B_{12} and B_{12a} or B_{12b} combined to proteinaceous material. The possibility exists that the B_{12} is still associated in some manner with the cell that synthesized it. Extension of the techniques employed in this investigation to chromatography with different solvents and release of the activity by other proteolytic enzymes could establish the hypothesis that the intestinal activity is due to several forms of B_{12} bound to proteins.

VII. SUMMARY AND CONCLUSIONS

1. The vitamin B_{12} content of some materials concerned with swine nutrition was determined with *Lactobacillus leichmannii* 313 as the test organism. *Lactobacillus leichmannii* (ATCC 4797) could be used, but required a much longer incubation period.

2. Based upon a medium reported in the literature, an improved basal medium for *L. leichmannii* 313 was developed. The medium included an amino acid mixture, a tomato juice filtrate, and a high level of adenine, guanine, uracil, and xanthine. Cysteine was the preferred reducing agent for this medium; ascorbic acid and thioglycollic acid were effective, but never as satisfactory as cysteine.

3. *L. leichmannii* 313 was shown to utilize pyridoxine, pyridoxal, and pyridoxamine equally well for rapid growth. The organism did not have a prerequisite for a B_6 vitamin, since it could synthesize its B_6 requirement if given sufficient time.

4. Satisfactory B_{12} assays of feed samples were not accomplished because the feeds contained too large a proportion of interfering desoxyribosides for the test organism.

5. Sow's milk, assayed after digestion with papain and takadiastase, contained from 1 to 8 µg. of B_{12} per liter.

6. Urine was assayed for B_{12} after diluting to the necessary volume. The B_{12} content of urine varied from about 0.5 to 3 mµg. per ml. Qualitatively, the B_{12} content of urine increased with the B_{12} content of the feed.

7. Liver tissue was prepared for analysis by thorough mixing, followed by autoclaving and shaking with potassium cyanide. The B_{12} content of liver increased from 30 to 160 mµg. per gram as the B_{12} intake of the animal increased. There was maximum storage of B_{12} in the liver at a level of about 5 µg. of B_{12} per pound of ration.

8. Fecal samples were adequately prepared for assay by thorough mixing in water or buffer. The B_{12} activity of feces was confirmed by alkaline hydrolysis and paper chromatography.

9. The contents of the small intestine, cecum, and large intestine were assayed by the same procedure as employed for feces. The activity of the small intestine was due principally to desoxyribosides; the activity of the cecum and large intestine was due to B_{12}.

10. Intestinal synthesis of B_{12} in the pig occurred in the cecum and large intestine.

11. The B_{12} activity of the intestinal contents was confirmed by alkaline hydrolysis and paper chromatography. Evidence was accumulated that the activity in the intestine was mainly B_{12b}, bound in some manner to protein material.

VIII. LITERATURE CITED

Addison, T. 1849. Anemia, disease of the supra-renal capsules. London Med. Gazette 43:517. (Original not available, cited in Woods, p. 11.)

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

Anslow, W. K., Ball, S., Emery, W. B., Fantes, K. H., Smith, E. L. and Walker, A. D. 1950. The nomenclature of the vitamins B_{12}. Chem. and Ind. p. 574.

Bauriedel, W. R. 1950. Ames, Iowa. Information on vitamin B_{12} assay. (Private communication.)

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

Bosshardt, D. K., Paul, W. J., O'Doherty, K., Huff, J. W. and Barnes, R. H. 1949. Mouse growth assay for the APF. J. Nutrition 37:21-25.

Boxer, G. E. and Rickards, J. C. 1950. Chemical determination of vitamin B_{12}. I. Determination of 5,6-dimethylbenzimidazole by colorimetric and fluorimetric methods. Arch. Biochem. 29:75-84.

Boxer, G. E. and Rickards, J. C. 1951. Chemical determination of vitamin B_{12}. II. The quantitative isolation and colorimetric determination of milligram quantities of cyanide. III. Methods for the quantitative and specific release of the cyano group of vitamin B_{12}. IV. Assay of vitamin B_{12} in multivitamin preparations and biological materials. Arch. Biochem. 30:372-381, 382-391, 392-401.

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

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

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

Brink, N. G., Wolf, D. E., Kaczka, E., Rickes, E. L., Koniuszy, T. R. and Folkers, K. 1949. Vitamin B_{12}. IV. Further characterization of vitamin B_{12}. J. Am. Chem. Soc. 71:1854-1856.

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

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

Buchanan, J. G., Johnson, A. W., Mills, J. A. and Todd, A. R. 1950. The isolation of a phosphorous-containing degradation product from vitamins B_{12} and B_{12c}. Chem. and Ind. p. 426.

Capps, B. F., Hobbs, N. L. and Fox, S. H. 1949. A method for the microbiological assay of vitamin B_{12}. J. Biol. Chem. 178:517-518.

Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C. and Heuser, G. F. 1949. The complex nature of the animal protein factor. Poultry Sci. 28:750-752.

Cary, C. A., Hartman, A. M., Dryden, L. P. and Likely, G. D. 1946. An unidentified factor essential for rat growth. Fed. Proc. 5:128.

Caswell, M. C., Koditschek, L. K. and Henlin, D. 1949. The microbiological estimation of LLD activity with vitamin B_{12} as a standard. J. Biol. Chem. 180:125-131.

Coats, M. E., Harrison, G. F. and Kon, S. K. 1951. The chick assay of vitamin B_{12} and the animal protein factor. Analyst 76:146-149.

Combs, G. F., Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C. and Heuser, G. F. 1950. Studies of unidentified chick growth factors. J. Biol. Chem. 182:727-737.

Cooley, G., Ellis, B. and Petrow, V. 1950. Further observations on the "ninhydrin-reacting" hydrolytic fragment of vitamin B_{12}. J. Pharm. Pharmacol. 2:128.

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

Couch, J. R. and Olcese, O. 1950. The vitamin B_{12} content of chick tissues as influenced by the diet. J. Nutrition 42:337-346.

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

Dietrich, L. S., Nichol, C. A., Monson, W. J. and Elvehjem, C. A. 1949. Observations on the interrelationships of vitamin B_{12}, folic acid, and vitamin C in the chick. J. Biol. Chem. 181:915-940.

Ellis, B., Petrow, V. and Snook, G. F. 1949a. Chemistry of antiperniciuos anemia factor. I. Liberation of phosphorous as phosphate from B_{12} by acid hydrolysis. J. Pharm. Pharmacol. 1:287-291.

Ellis, B., Petrow, V. and Snook, G. F. 1949b. The chemistry of antipernicious anemia factors. Part II. The "ninhydrin-reacting" hydrolytic fragment of vitamin B_{12}. J. Pharm. Pharmacol. 1:950-956.

Foster, J. C., Lally, J. A. and Woodruff, H. B. 1949. Cup assay with vitamin B_{12} as a standard. Science 110: 507-509.

Frost, D. V., Fricke, H. H. and Spruth, H. C. 1949. Rat-growth assay for vitamin B_{12}. Proc. Soc. Exp. Biol. Med. 72:102-106.

Greene, R. D., Brooke, A. J. and McCormack, R. B. 1949. Conditions which affect the assay of vitamin B_{12} with _Lactobacillus lactis_. J. Biol. Chem. 178:999-1000.

Groschke, A. C., Thorburn, L. A., Luecke, R. W., Thorpe, F. Jr. and McMillem, W. N. 1950. The occurrence of vitamin B_{12} activity in pig manure as measured by chick growth response. Mich. Agr. Exp. Sta. Quart. Bull. 32:503-506.

Hartman, A. M. and Cary, C. A. 1946. Occurrence in foods of an unidentified factor essential for rat growth. Fed. Proc. 5:137.

Henlin, D. and Soars, M. H. 1951. Comparative microbiological studies with vitamins B_{12} and B_{12a}. J. Biol. Chem. 188:603-610.

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

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

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

Johansson, K. R. 1951. A turbidimetric assay for vitamin B_{12} employing a mutant of Escherichia coli. Bacteriological Proc., Abstracts, p. 146.

Johnson, B. C. and Neumann, A. L. 1949. Crystalline vitamin B_{12} compared to antipernicious anemia liver extract for pig growth. J. Biol. Chem. 178:1001-1002.

Kaczka, E. A., Denkewalter, R. G., Holland, A. and Folkers, K. 1951. Vitamin B_{12}. XIII. Additional data on vitamin B_{12a}. J. Am. Chem. Soc. 73:335-337.

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

Kaczka, E. A., Wolf, D. E., Kuehl, F. A. Jr. and Folkers, K. 1950. Vitamin B_{12}: reactions of cyano-cobalamin and related compounds. Science 112:354.

Kastelic, J. 1951. Ames, Iowa. Information on vitamin B_{12} assay. (Private communication.)

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

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

Kitay, E. and Snell, E. E. 1950. Some additional requirements of certain lactic acid bacteria. J. Bact. 60:49-56.

Kocher, V., Karrer, R. and Muller, H. R. 1950. Paper chromatography of vitamin B_{12} and the desoxyribonucleosides with phenol, butanol and collidine. Inter. Z. Vitaminforsch. 21:403-409.

Kocher, V. and Schindler, O. 1949. Desoxyribonucleoside as a growth factor for L. lactis in B_{12}-free nutrient solution. Intern. Z. Vitamin Forsch 20:441-443.

Koditschek, L. K., Henlin, D. and Woodruff, H. B. 1949. Investigations on the nutrition of Lactobacillus lactis Dorner. J. Biol. Chem. 179:1093-1102.

Lueche, R. W., McMillen, W. N., Thorp, F. Jr. and Boniece, J. R. 1949. The effect of vitamin B_{12} concentrate on the growth of weanling pigs fed corn-soybean diets. Science 110:139-140.

Miller, D. C. and Groschke, A. C. 1950. The occurrence of animal protein factor in horse manure as measured by chick growth response. Mich. Agr. Exp. Sta. Quart. Bull. 32:279-286.

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

Peeler, H. T., Daniel, L. J., Norris, L. C. and Heuser, G. F. 1949. Unidentified factors required by Lactobacillus casei. J. Biol. Chem. 177:905-916.

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

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

Picken, J. C. Jr. and Bauriedel, W. R. 1950. Comparative bioautography of vitamin B_{12} and related growth factors using *Euglena gracilis* and *Lactobacillus leichmannii*. Proc. Soc. Exp. Biol. Med. 75:511-515.

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

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

Pruzansky, J. J. 1949. Microbial growth factors in commercial products of bacterial and fungal origin. Unpublished Ph.D. Thesis. Ames, Iowa. Iowa State College Library.

Rege, D. V. and Sreenivasan, A. 1950. Folic acid, vitamin B_{12}, and nucleic acid synthesis in *Lactobacillus casei*. Nature 166:1117.

Richardson, D. 1951. Vitamin B_{12} requirement of the weanling pig. Unpublished Ph.D. Thesis. Ames, Iowa. Iowa State College Library.

Richardson, D., Catron, D. V., Underkofler, L. A., Maddock, H. M. and Friedland, W. C. 1951. Vitamin B_{12} requirement of the male weanling pig. J. Nutrition 44:371-381.

Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R. and Folkers, K. 1948. Crystalline vitamin B_{12}. Science 107:396-397.

Rickes, E. L., Koch, P. J. and Wood, T. R. 1949. Additional observations on the rate of growth of *Lactobacillus casei*. J. Biol. Chem. 178:103-111.

Roberts, I. Z., Roberts, R. B. and Abelson, P. H. 1949. Effect of vitamin B_{12} on the phosphorous metabolism of _Lactobacillus leichmannii_. J. Biol. Chem. 58:709-710.

Shive, W., Ravel, J. M. and Eakin, R. E. 1948. Interrelationships of thymidine and B_{12}. J. Am. Chem. Soc. 70:2614-2615.

Shive, W., Ravel, J. M. and Harding, W. M. 1948. An interrelationship of purines and vitamin B_{12}. J. Biol. Chem. 176:991-992.

Shive, W., Sibley, M. E. and Rogers, L. L. 1951. Replacement of vitamin B_{12} by desoxynucleotides in promoting growth of certain lactobacilli. J. Am. Chem. Soc. 73:867-869.

Shorb, M. S. 1947a. Unidentified essential growth factors for _Lactobacillus lactis_ found in refined liver extracts and certain natural products. J. Bact. 53:669.

Shorb, M. S. 1947b. Unidentified growth factors for _Lactobacillus lactis_ in refined liver extracts. J. Biol. Chem. 169:455-456.

Shorb, M. S. 1948. Activity of vitamin B_{12} for the growth of _Lactobacillus lactis_. Science 107:397-398.

Shorb, M. S. and Briggs, G. M. 1948. The effect of dissociation in _Lactobacillus lactis_ cultures on the requirement for vitamin B_{12}. J. Biol. Chem. 176: 1463-1464.

Skeggs, H. R., Huff, J. W., Wright, L. D. and Bosshardt, D. K. 1948. The use of _Lactobacillus leichmannii_ (ATCC 4797) in the microbiological assay of APF. J. Biol. Chem. 176:1459-1460.

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

Smith, E. L. 1948. Purification of antipernicious anemia factors from liver. Nature 161:638-639.

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

Smith, E. L., Fantes, K. H. and Ball, S. 1951. Vitamin B_{12}. Additional factors. Biochem. J. 48:1 (abstracts).

Snell, E. E. 1945a. Vitamin B_6 group. V. The reversible interconversion of pyridoxal and pyridoxamine by transamination. J. Am. Chem. Soc. 67:194-197.

Snell, E. E. 1945b. The vitamin B_6 group. VII. Replacement of vitamin B_6 for some microorganisms by d(-)-alanine and an unidentified factor from casein. J. Biol. Chem. 158:497-503.

Snell, E. E. and Rannefeld, A. N. 1945. The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. J. Biol. Chem. 157:475-489.

Snell, E. E. and Strong, F. M. 1939. A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed. 11:346-350.

Stokes, J. L. and Gunness, M. 1945. Pyridoxamine and the synthesis of amino acids by lactobacilli. Science 101:43-44.

Stokes, J. L., Koditschek, L. K., Rickes, E. L. and Wood, T. R. 1949. Factors affecting the rate of growth of Lactobacillus casei. J. Biol. Chem. 178:93-101.

Strauss, M. B. 1950. Vitamin B_{12} and pernicious anemia. New Engl. J. Med. 243:187-194, 222-229.

Tappan, D. V., Lewis, U. J., Register, U. D. and Elvehjem, C. A. 1950. Rat growth assay for vitamin B_{12} activity. Arch. Biochem. 29:408-412.

Thompson, H. T., Dietrich, L. S. and Elvehjem, C. A. 1950. The use of Lactobacillus leichmannii in the estimation of vitamin B_{12} activity. J. Biol. Chem. 184:175-180.

Vohs, R. L. 1950. Vitamin B_{12} in swine nutrition. Unpublished M.S. Thesis. Ames, Iowa. Iowa State College Library.

Welch, A. D. and Wilson, M. F. 1949. 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.

West, R. 1948. Activity of vitamin B_{12} in Addisonian pernicious anemia. Science 107:398.

Wijmenga, H. G., Veer, W. L. C. and Lens, J. 1950. Vitamin B_{12}. II. The influence of HCN on some factors of the vitamin B_{12} group. Biochem. et Biophys. Acta 6:229-236.

Winsten, W. A. and Eigen, E. 1948. Paper partition chromatographic analysis and microbial growth factors: the vitamin B_6 group. Proc. Soc. Exp. Biol. Med. 67: 513-517.

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

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

Wolf, D. E., Jones, W. H., Valiant, J. and Folkers, K. 1950. Vitamin B_{12}. XII. Degradation of vitamin B_{12} to D_g-1-amino-2-propanol. J. Biol. Chem. 72:2820.

Woodruff, H. B. and Foster, J. C. 1950. Analysis for vitamin B_{12} and vitamin B_{12a} by paper strip chromatography. J. Biol. Chem. 183:569-576.

Woods, R. 1949. The story of vitamin B_{12}. Borden's Rev. Nutritional Research 10: No. 1, 1-12.

Wright, L. D., Skeggs, H. R. and Huff, J. W. 1948. The ability of thymidine to replace B_{12} as a growth factor certain lactobacilli. J. Biol. Chem. 175:475-476.

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