AN INVESTIGATION OF VITAMIN B_{12} SYNTHESIS IN A RUMINANT

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Clara Dodge Refson

1950

This is to certify that the

thesis entitled

AN INVESTIGATION OF VITAMIN B_{12} SYNTHESIS IN A RUMINANT

presented by

CLARA LODGE RELSON

has been accepted towards fulfillment
of the requirements for

M. Sc. degree in Biochemistry

Keith B. McCall.
Major professor

Date November 7, 1950

O-169

AN INVESTIGATION OF VITAMIN B_{12} SYNTHESIS IN A RUMINANT

By

Clara Dodge Refson

A THESIS

Submitted to the School of Graduate Studies of Michigan
State College of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemistry

1950

ACKNOWLEDGMENT

I wish to express my sincere appreciation to Dr. Keith B. McCall and to Dr. Carl A. Hoppert whose constant encouragement and advice have been invaluable.

I am sincerely grateful to Dr. Lester F. Wolterink who donated both material and technical advice for the tracer portion of this project.

Thanks are also due to Dr. Joseph Meites, Dr. Carl F. Huffman, and Mr. Elbert S. Churchill who kindly shared unavailable material.

****
****
******
****
**
*

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The Liver Factor

Vitamin B_{12} as we know it at the present time is yet a fraction of the form whose development is inevitable. Although its life as an independent vitamin has been a short one, the volume of research which has been published in the last three years is indeed impressive.

The study actually began a century ago when pernicious anemia was recognized clinically.[1] The specific importance of diet as an influencing factor was not fully realized until Minot and Murphy[2] announced in 1926 that at least partial alleviation of symptoms could be effected by treatment with whole liver. This discovery was paralled by the work of Koessler et al.[3] and was preceded by that of Whipple et al.[4] and Elders[5] who had also considered pernicious anemia to be a deficiency disease. Subsequent verification was again supplied by Minot and Murphy[6] in 1927 and by Castle and Bowie[7] in 1929.

As must follow, various groups of investigators now took up the task of liver fractionation with hopes of characterizing the unknown factor or factors which must be present. Virtually nothing was excluded in the never ending search as each of the major classifications of compounds was eliminated from each new fraction. By 1945, highly concentrated preparations had been developed, and an extensive review of the painstaking fractionation studies was published by Subbarow, Hastings, and Elkin.[8] The progress thus far attained appears all the more impressive when the only method at that time available for testing these

fractions is considered, - that of direct clinical therapy on pernicious anemia patients.

It was postulated early that the factor sought was not of singular nature when Castle et al.[9,10,11] advanced the classic theory that an extrinsic factor, supplied by dietary constituents, must be combined with an intrinsic factor, supplied by certain glands of the stomach, to be nutritionally effective. Although this theory has been modified somewhat by later research, the initial postulation was a sound one and is still supported at the present time. Present evidence seems to indicate that the intrinsic factor of gastric juice functions primarily as an aid to absorption and hence more complete utilization of the orally administered vitamin.[12,13,14]

The "Animal Protein Factor"

Gradually, investigators in other branches of nutrition took up the task, as the growth effects of an "animal protein factor" were realized. Parkhurst,[15] as early as 1927, discovered that a protein of animal origin was necessary for normal hatchability of eggs. Two years later, a similar factor was reported in codliver meal by McFarlane, Fulmer, and Jukes.[16] Further studies were forthcoming, and in 1935 Van der Hoorn et al.[17] identified a strong growth promoting factor in casein. Nestler et al.[18] postulated a "new factor" and indicated pork liver meal as a good source. Some essential nutrient(s), absent in vegetable protein, was obviously present and associated with animal

protein. During 1940 - 1944, the beneficial effects of sardine meal and fish solubles toward growth and hatchability were determined.[19,20,21,22]

McGinnis[23] and co-workers noted that non-protein-nitrogen levels in the blood of chicks fed a diet deficient in the "animal-protein factor" were increased when soy bean protein or vitamin-free casein was added to the basal diet. Also within this period came the discovery that cow manure was effective for growth under the same conditions in which sardine meal was effective.[24] Rubin, Bird, and co-workers[25,26,27,28,29,30] further distinguished this factor from the known vitamins and succeeded in concentrating it from cow manure by combined drying, precipitative, and extractive methods.

The new factor gradually began to find its way into animal as well as poultry science. As early as 1932, Mapson[31] had recognized that rats maintained on a diet in which yeast and wheat germ were the only sources of B vitamins developed a deficiency which was corrected by feeding liver. Cary and Hartman[32] in 1946, using a purified diet containing alcohol-extracted casein and yeast, demonstrated an unidentified factor required for normal growth of the rat. An anti-pernicious anemia liver extract satisfied this requirement when added to the ration in milligram quantities. Jaffe,[33] Jaffe and Elvehjem,[34] and Sporn, Reugamer, and Elvehjem[35] reported similar observations. Bowland, Esminger, and Cunha[36] also emphasized the association of a growth factor with animal proteins. The rat growth factor was further shown to be present in such foods as milk, beef and pork liver, and egg yolk.[37] The potency of the factor

in egg yolk was observed to be effected by the diet of the hen. The yolk was apparently a storage depot, serving to transmit this nutrient to the chick.

The research of poultry science nutritionists in the same year paralled that of Cary and Hartman when it was demonstrated that the chick growth factor was likewise transmitted by way of the egg to the chick, under some conditions in quantity sufficient to support maximum growth to four weeks of age.[38,39,40,41,42,43]

The Beltsville workers[44] reported the chick growth factor to be present in hen feces. Evidences of bacterial origin were therefore suggested. This was partially verified by McGinnis et al.[45,46] and further in 1948 by workers at the Lederle Laboratories[47] who succeeded in isolating a microorganism which, by aerobic fermentation, formed the animal protein factor. This material, when refined and concentrated, proved therapeutically active against pernicious anemia in man by clinical test.

Although chemical assay was impossible without further characterization of this factor, Shorb,[48] also of Beltsville, was attacking the situation by attempting to find a microorganism for which the rat growth factor was an essential nutrient. Lactobacillus lactis Dorner apparently required two constituents, a "TJ factor" found in tomato juice and casein, and an "LLD factor", active for rat growth, and found in highest concentrations in liver extracts. A linear relationship was observed between the potency of this latter factor and the anti-pernicious anemia

factor in liver concentrates.[49] Further investigations identified the latter factor in other well known animal protein factor source materials.[50] At this point, the various lines of investigation began to converge.

The Isolation

In the spring of 1948, after several years of work, Rickes and coworkers[51] announced the isolation of a pure, crystalline material from liver extracts, designated as vitamin B_{12}. First aided by clinical test, then later by microbiological assay[52,53] (using Lactobacillus lactis Dorner), the anti-anemia factor had been isolated. Clinical tests proved it to be effective not only in producing positive hematological responses in pernicious anemia, but in the alleviation of neurological symptoms as well.[54,55,56,57,58,59,60,61,62] It was also effective in the treatment of tropical sprue and nutritional macrocytic anemia. Wetzel[63] reported a statistical analysis of growth failure in school children as associated with vitamin B_{12} deficiency and the responses to oral treatment. As would be expected by the use of Lactobacillus lactis Dorner in fractionation studies, the crystalline material exerted LLD growth factor activity.[64]

At the same time and independently of other investigators, Smith,[65,66] at the Glaxo Laboratories in England announced the isolation of the anti-anemia factor. His fractionation studies were followed by means of clinical testing as well as the characteristic pink coloration.

By means of partition chromatography, he had concentrated one gram of the product from four tons of ox liver.

Reported attempts to determine the relationship between vitamin B_{12} and the animal protein factor were forthcoming. It had already been indicated that vitamin B_{12} and the LLD factor were probably identical. The crystalline material or vitamin B_{12} concentrate were observed to be active in the promotion of both good growth and hatchability in chicks.[67,68,69,70,71,72,73]

Rat growth was stimulated as much as thirteen fold over control animals depending upon the protein concentration in the diet.[74] Tissue distribution studies showed that vitamin B_{12} concentration increased in most organs and tissues when small amounts were added to the diet.[75]

The effectiveness of vitamin B_{12} for pig growth was also reported by several investigators and its efficiency observed to compare favorably with that of the animal protein factor.[76,77,78,79,80] The equivalence of vitamin B_{12} and anti-pernicious anemia liver extract could be demonstrated.[81,82]

Stokstad et al.,[83] however, upon the feeding of more acutely deficient basal rations, found that maximum growth requirements of the chick were not completely satisfied by the addition of vitamin B_{12} to the diet. Some indication of this had been noted by Hill[84] a year earlier. The existence of other factors not replaceable by vitamin B_{12} which may be present in the animal protein factor have been suggested: (1) an additional liver factor[85,86] and (2) a substance in Streptomyces

cultures.[87] Zucker and Zucker,[88] however, did not obtain responses from the above factors in addition to those obtained from vitamin B_{12}. Hartman, Dryden, and Cary[89] have attampted to clarify some of the conflicting evidence previously reported. They concluded that exceedingly high levels of riboflavin may play an important role in bringing about intestinal synthesis of vitamin B_{12} in the rat. Nichol et al.[90] have found considerable variation in chick growth response to the same dosage of anti-pernicious anemia preparation in different experiments. The "multiple nature"[91] of the animal protein factor is still under investigation, being at present inadequately defined.

Zucker and Zucker,[92,93,94] who had also observed the syndrome produced in rats by high protein diets, suggested the name "Zoopherin" for the missing dietary factor. The name was soon abandoned, however, since sources of microbial[95,96] as well as animal origin were involved.

Some relationship appears to exist between the vitamin and growth requirements in hyperthyroid animals.[97,98] Rats in a thyrotoxic condition apparently require one or more factors not needed by normal animals. Purified liver extracts contained this factor, and crystalline vitamin B_{12} satisfactorily replaced the extracts. A similar condition in chicks was counteracted by vitamin B_{12}[99] and conversely, a vitamin B_{12} deficiency could be demonstrated in the chick much more quickly if a thyroxine-active substance was administered.[100]

The isolation of pure, crystalline vitamin B_{12} was followed immediately by further characterization of chemical properties and structure,

as would indeed be expected.[101,102,103,104,105,106] Cobalt and phosphorus were found to be present to the extent of one atom of each per molecule. A similarity in structure to riboflavin was evidenced when a 1,2-diamino - 4,5 - dimethyl benzene moiety was identified.

Evidence of the multiple nature of this vitamin was shown when two other compounds, vitamin B_{12a}[107] and vitamin B_{12b}[108,109] were crystallized. These were later shown to be identical and to differ from vitamin B_{12} only by the absence of a cyano group.[110,111]

The isolation of crystalline B_{12} from liver called forth a search for other more readily available sources. Workers at the Lederle and Merck Laboratories were able to isolate the vitamin from cultures of Streptomyces aureofaciens[112] and from Streptomyces griseus[113] respectively. The further testing of the bacterial production of the animal protein factor and of B_{12} by other workers[114,115] not only resulted in the development of numerous fermentation procedures, but served to further demonstrate the complex nature of the animal protein factor.

Microbiological Assay

Numerous procedures for the assay of vitamin B_{12} and the animal protein factor were developed during 1949. Vitamin B_{12} was found to elicit growth responses by other microorganisms in addition to Lactobacillus lactis Dorner. These include Euglena gracilis,[116] Lactobacillus leichmannii 313, ATCC 7830,[117,118,119] and Lactobacillus leichmannii, ATCC 4797.[120,121] A microbiological assay employing either

Lactobacillus lactis or Lactobacillus leichmannii based upon anti-biotic type procedures[122,123] was later published by Foster et al.[124] A method involving paper chromatography in combination with microbiological procedures was also published.[125,126] Short-term rat assays also were developed.[127,128]

Non-specificity, especially in the case of the earlier broth culture assay procedures, sensitivity to oxygen tension, variation in oxidation-reduction potentials, and culture dissociation introduced troublesome complications.[129,130,131,132,133,134,135] The fact that the growth factor can apparently be stored in the animal body[136,137,138] as well as be transferred through the mother's milk[139] further complicated animal assay procedures and deficiency experiments.

Possible Mechanism of Action

Two years prior to the discovery of vitamin B_{12}, another factor, vitamin M or folic acid was studied and synthesized.[140,141] With its discovery came the justified, although short-lived, hope that the vital factor of liver had at last been isolated. It was still apparent, however, that erythrocyte counts did increase somewhat with folic acid treatment. Also during 1948, the desoxyriboside of thymine was isolated from liver. Shive et al.[142] indicated a functional relationship between this factor and folic acid in that it was highly active in conteracting bacterial growth inhibition by methyl folic acid. Stokes[143] had previously demonstrated that thymine itself, if substituted in large quantities, could

completely replace folic acid in the nutrition of lactic acid bacteria. It was, in fact, capable of replacing folic acid in the treatment of macrocytic anemia if administered in the proper amount.[144] Snell et al.[145] reported thymidine to be an essential growth factor for certain lactic acid organisms.

Vitamin B_{12} was again brought into this series of events when Wright, Skeggs, and Huff[146] interpreted their investigations of Lactobacilli to show replacement of vitamin B_{12} as a growth factor by thymidine. Shive, Raull, and Eaken[147] also reported an interrelationship. Similar information was indicated by Kitay et al.[148] in regard to other desoxyribosides as well as thymidine.

The growth characteristics of certain other organisms (Streptococcus faecalis R,[149] Leuconostoc citrovorum,[150] and Lactobacillus bifidus[151]) has not indicated a specific thymidine - B_{12} relationship. Hoff-Jorgensen[152] reported irregularities in regard to three other strains of lactic acid bacteria. Similarly, thymine will not replace thymidine for Lactobacillus leichmannii 313, Lactobacillus leichmannii 327, or Lactobacillus citrovorum.[153] The possibility still exists, however, of a series of reactions in the animal body in which the above mentioned components are involved. In consideration of the apparent interrelationship of folic acid, thymine, thymidine (or nucleosides in general), and vitamin B_{12}, Skeggs et al.[154] have proffered a possible mechanism by which these substances may function: "The biochemical defect in pernicious anemia may well be inability to synthesize certain nucleosides,

particularly thymidine, from parent purines and pyrimidines. The curative effects observed in this disease with folic acid may arise from increased thymine snythesis, which, by mass action effects, yields more thymidine. The effectiveness of large amounts of thymine in pernicious anemia similarly may be explained." The possibility is further suggested that vitamin B_{12} may enter the above scheme as a co-enzyme in the synthesis of thymidine from thymine.[155,156]

Shive et al.[157] presented further evidence of the interrelationship of purines and vitamin B_{12}, but did not preclude the possibility of a reverse situation in which purines or derivatives are involved in the biosynthesis of the vitamin.

Also in accord with the foregoing theory, Roberts et al.[158] employing radioactive tracer techniques have demonstrated an increase in phosphorous uptake in the desoxyribonucleic acid fraction of Lactobacillus leichmannii cultures when supplemented with varying amounts of vitamin B_{12} although bacterial growth remained constant.

Minute amounts of anti-pernicious anemia concentrate produce excellent growth of rats given folic acid and succinylsulfathiazole. Consideration of the quantity of concentrate needed indicates that vitamin B_{12} is the factor which is responsible for this increased growth as well as the fact that vitamin B_{12} may be necessary for the proper functioning of folic acid in the animal body. Jones et al.[159] have interpreted their data in part as follows: "The mechanism by which succinylsulfathiazole increased the need for the growth factor is unknown at the present time.

The most logical explanation is that intestinal bacteria form small amounts of this factor, and that the bacteria are inhibited by the sulfa drug from doing so".

A lipotropic effect in rats with dietary-induced liver injury was reported as another of the properties of liver extract.[160] Endeavoring to reduce the number of possible factors to a minimum, repeat experiments using vitamin B_{12} concentrate continued to demonstrate a significant lipotropic effect comparable to earlier results.[161] Vitamin B_{12} is known to exert a sparing effect on the requirement for choline and methionine in the prevention of hemorrhagic kidney. The addition of vitamin B_{12} to subprotective levels of choline produced significant weight increases although this was not the case when adequate choline levels were maintained.[162] Other investigations of a choline, vitamin B_{12} relationship served to substantiate this fact.[163,164] Similarly, an increased requirement of methyl group donors (choline and betaine) for chicks made partially deficient in the animal protein factor was observed.[165] Cunha et al.[166] reported that pigs on a corn, peanut meal ration showed growth response to either animal protein factor or methionine supplements, the latter eliciting the lesser response.

Stokstad and Jukes[167] found vitamin B_{12} to be involved in the methylation of homocystine to methionine. Homocystine promoted growth only if vitamin B_{12} was supplied.

Vitamin B_{12} was found to exert a protective effect on hepatic injury produced by carbon tetrachloride.[168] The development of characteristic

histologic changes such as fatty metamorphosis and depletion of ribose nucleic acid were prevented. Shaefer et al.[169] have expressed the belief that vitamin B_{12} exerts its maximum effect in the presence of folacin. This was indicated a year earlier by Nichol et al.[170] in regard to hemoglobin regeneration.

Since it was known earlier that desoxyribonucleic acid synthesis was important in the multiplication of a type of bacteriophage (T4r), Roberts and Sands[171] investigated the possibility that vitamin B_{12} might likewise be involved. The vitamin was found to be one of the rate limiting factors of virus synthesis in the presence of resting cells.

The research already accomplished, although rather broad in scope, leaves many questions unanswered as does it call forth many new ones for future investigation. There can be little doubt that the initial problem has been solved, that of finding the active anti-pernicious anemia factor present in liver.

The recent biosynthesis of radioactive vitamin B_{12}[172] containing isotopic cobalt, Co^{60}, has made available another technique for new and different types of research.

HISTORY

The importance of cobalt as a trace element in the nutrition of ruminants was late in receiving recognition. There is no definite evidence that cobalt is a dietary essential in the non-ruminant although its action in producing polycythemia would indicate that some mechanism for the utilization of this element does exist.[173] Attempts to produce cobalt deficiency in laboratory animals (non-ruminants) have proven unsuccessful,[174,175,176] and according to Marston,[177] cobalt deficiency in horses does not occur.

The syndrome characteristic of insufficient cobalt in the diet of ruminants, however, has been reported in many parts of the world under such titles as Denmark disease, coastal disease, enzootic marasmus, bush sickness, salt sickness, Nakuritis, and pining disease. A rather complete summary of the disease characteristics as well as areas of occurrence has been published by the National Research Council.[178]

The characteristic digestive system of the ruminant at once suggests the possibility that cobalt is involved in bacterial metabolism. McCance and Widdowson[179] in 1944 suggested that cobalt may function in this connection. An investigation of possible changes in the rumen flora of cobalt deficient sheep in 1949 showed that a marked alteration in the types and numbers of bacteria did occur.[180] Although the retention of orally administered cobalt was shown to be negligible, it was not nutritionally effective when injected.[181,182,183,184]

The announcement that the vitamin B_{12} molecule contained an atom of cobalt at once revealed another possibility. The vitamin is readily

metabolized by many microorganisms. It would therefore seem quite possible that the influence of cobalt is exerted through microbial synthesis of vitamin B_{12} which is, in turn, utilized further to support growth of rumen microflora.

It was in support of this contention that the research reported in this paper was begun in the fall of 1949. If it could be definitely established that vitamin B_{12} was present in the ruminant stomach, further steps would be taken to demonstrate synthesis of the vitamin, provided that the metabolic pool was large enough at any one time to insure working quantities. In the event that results proved positive, it was hoped that this might be the starting point for future work, perhaps the isolation of the organisms involved.

In November of 1949, Burroughs et al.[185] and Hale et al.[186] reported their investigations of this same problem derived from two entirely different approaches to the situation. Burroughs attempted to duplicate in so far as possible the conditions existant in the rumen. The cellulose digestion of poor quality roughages was studied. Two of the most potent supplements effecting maximum cellulose digestion were: (1) a complex mineral mixture and (2) an autoclaved water extract of cow manure. Cow manure, as mentioned before, contained a factor which promoted growth and hatchability of chicks. Hale conducted direct chick growth assays of the rumen contents from sheep fed cobalt-deficient and cobalt-supplemented diets. A significant growth difference was observed. Growth retardation could be completely overcome by adding vitamin B_{12} to the

ration of the chicks fed the rumen contents from the cobalt deficient sheep. Additions of inorganic cobalt were ineffective.

In May of 1950, Henlin and Ruger[187] were successful in culturing rumen isolants which synthesized LLD-active substances and demonstrated an increased synthesis with the addition of cobalt salts.

EXPERIMENTAL

The study of cobalt metabolism in the ruminant at the time of initiation of this project was quite incomplete. Further, opinions concerning the involvement of vitamin B_{12} in this phase of animal nutrition were many and varied. Several approaches to the problem had been published, some of them more or less indirect.

This project was planned as a direct investigation of the situation which would at least serve to elucidate and solve some of the minor issues involved. Direct isolation of the vitamin from ruman contents was beyond the scope of this project. Attention was turned to the preparation of suitable extracts whose potency could be determined by microbiological assay. The successful demonstration of a significant concentration of the factor active for Lactobacillus leichmannii would at least serve to establish the presence of an LLD active substance. By the use of radioactive tracer techniques involving Co^{60}, the incorporation of cobalt into the molecule of the active substance could perhaps be demonstrated.

The Microbiological Assay

It was apparent that the choice of a suitable assay procedure was one of the most important steps to be taken. The cup assay of Foster, Lally, and Woodruff[188] was finally selected since it appeared to be the most workable method.

The rate of growth of either of the test organisms (Lactobacillus lactis or Lactobacillus leichmannii) has been reported to be affected

by oxidation-reduction potential, by degree of aeration, and by the accumulation of peroxides in liquid media. The cup assay procedure permits better control of these factors and hence a greater degree of accuracy.

Although animal assays have been developed, the diet has been known to affect markedly the weight gains produced by varying concentrations of the vitamin. Until such a time as the importance of intestinal synthesis has been determined and the multiple nature of the animal protein factor has been more clearly defined, the microbiological assay would appear to involve less question than other procedures. To this end, the situation was further complicated by the fact that the exact nature of impurities present in the extracts to be tested was unknown. The number and types of substances present in the rumen as a result of bacterial fermentation were without doubt many and varied.

The assay organism used throughout the entire project was Lactobacillus leichmannii A.T.C.C. #4797. The media of Foster et al.[189] was used for the first few trial assay procedures, in so far as it could be duplicated. It was desirable to prepare fresh batches of media for each usage in 150 to 200 milliliter quantities but the proportions of the ingredients were such that this could be accomplished only by weighing aliquots from a mixture of the dry ingredients. Other methods were laborious and uniformity could be ascertained only by preparing the dry mixture in rather large quantities. For simplification, the choice of media was changed to the prepared vitamin B_{12} Agar Experimental of Difco

and Company, a medium essentially the same as that of Capps, Hobbs, and Fox.[190]

The stock cultures were prepared by stab inoculation of Bacto-Micro Assay Culture Agar. Each new culture was incubated at 35°C for 48 hours and then stored in the refrigerator. Cultures stored in this manner were satisfactory if used within a period of thirty days. Slightly better results were obtained, however, by preparing fresh cultures immediately before each assay.

Although good stab growth was observed during the first several trial procedures, the assays themselves were very unsuccessful, apparently due to culture dissociation. The incorporation of .01 - .02% of Tween 80 into the Micro-Assay Culture Agar was found to correct the difficulty after the first transfer.

The inoculum for each assay was prepared by subculturing from the stock culture into a tube containing ten milliliters of Bacto-Micro Inoculum Broth. This broth was supplemented with Tween 80 in the same concentration as employed in the preparation of the Micro-Assay Culture Agar. This was observed to produce an exceptionally large growth increase over that of the dissociated culture. The tubes were then incubated for 24 hours at 35°C.

Following incubation, the broth was centrifuged at about 2500 r.p.m. for ten minutes. The cells were then resuspended in ten milliliters of sterile physiological saline and centrifuged as before. This was repeated a second time. The procedure varied slightly at this point in

that saline was added until a final volume of ten milliliters was reached. After the second washing, the volume was again made up to a ten milliliter level with saline. One milliliter of this suspension was used to inoculate each one hundred milliliters of melted vitamin B_{12} Agar, Experimental held at 45 - 50°C. Aseptic conditions were maintained throughout.

One to one and one-half percent of sodium chloride was added to the assay medium before plating. This had a tendency to diminish indistinct growth zones. The use of abnormal salt concentrations was also reported by Foster et al.[191] to eliminate the diffuse growth response of the test organism to desoxy ribonucleic acid or its corresponding nucleosides. A sufficient volume of a sterile sodium chloride solution was added to the autoclaved assay medium to produce the desired salt concentration. This was added immediately before the inoculation and at a temperature of 45-50°C. Occasionally a flocculent precipitate was observed to form after the media was autoclaved. This condition was improved to some degree by adding the sodium chloride as a sterile solution after autoclaving. Any flocculent material which formed after these precautions had been taken was easily resuspended by swirling the flask containing the mixture and this material apparently did not interfere with assay results.

Twenty-five milliliters of the inoculated medium was pipetted into each of four sterile petri dishes. Precautions were taken to be sure that the surface on which the plates were resting was perfectly level.

Since final results are dependent upon an even diffusion downward and outward from the assay cylinders, uniformity in depth of the agar was important. The agar was allowed to harden for ten minutes.

Four to six stainless steel assay cylinders were then distributed evenly on the surface of the agar by dropping from a uniform height equidistant from the outer edge of the petri dish. The weight of the cylinder was sufficient to produce a tight seal between the lower edge of the cylinder and the agar surface. Heating of the cylinder before placing in position as advocated for antibiotic assay procedures was not found to be necessary, and a more uniform settling was obtained without it.

The number and choice of standards was more or less arbitrary, depending upon the concentration of the unknown solution and the accuracy desired. Slightly better results were obtained when both the standards and the test sample were included on the same plate. This was not absolutely necessary, however, if care was taken to keep the procedure as uniform as possible. Standards usually ranged from 0.0 to 2.0 gamma of vitamin B_{12} per milliliter. Dilution of the test solution, when necessary, and use of the lower standards (.0. to 0.5 gamma per milliliter) gave greater accuracy. Standards were run with each new assay since growth sometimes varied from day to day.

The cylinders were then filled with the test solutions from sterile dropping pipettes. Air bubbles sometimes formed in the bottom of the assay cups if care was not taken to prevent it. Bubbles inhibited proper diffusion of the liquid through the agar, and the volume of liquid which

could be placed in the cylinders was also diminished. Splattering from the dropper occurred occasionally. The resultant growth areas appearing after incubation of the plates were, however, easily distinguished from the true growth zones which were to be measured.

After the first cylinder was once filled, the others were filled as rapidly as possible. Results were dependent upon the growth time of the organism as well as the diffusion of the test solution. The more quickly the assay cylinders were filled, the less the difference in growing time of the organism for each test solution.

To facilitate transfer from the working surface to the incubator, and to avoid any possible jarring, the plates were set on small racks prior to the positioning of the cylinders upon the agar. All the plates could thus be moved in a single operation.

Glass petri dish covers were replaced by sterile porous covers to prevent subsequent condensation and dripping into the test solutions.

After incubation at 35°C. for 24 to 48 hours, the diameters of the growth zones were measured as these values indicate the amount of vitamin B_{12} present when compared with standard solutions. Measurements were best made over an ordinary plate counter or ruled scale such as is used for standard anti-biotic assay procedures. Good growth zones were white, distinct areas with smooth, regular edges. (Figure 1) This is not the case if culture dissociation is present.

Tests for false positive reactions were made using both desoxy ribonucleic acid, ribo nucleic acid, and a mixture of the two plus vitamin B_{12}.

Nucleic acid concentrations as high as 4000 gamma per milliliter were used. The results are recorded in Figure 2. Despite the fact that measureable growth due to desoxy ribonucleic acid did occur, it was of an entirely different quality than that obtained from any concentration of vitamin B_{12} and could easily be distinguished from it. Growth was very slight, faint, and not easily measured. There was no significant additive effect as a result of combining the three compounds.

Sample Preparation

A rumen-fistula goat was selected as the experimental animal for this project. Four weeks were allowed to elapse after the rumenotomy before the first sample was withdrawn. From consideration of the physiology of the rumen, it was apparent that a definite time for removal of each sample must be set if results were to be consistent. It has been estimated[192] that both the bacterial and protozoan populations in the rumen declined to their lowest numbers during a ten to twelve hour period after feeding. Also at this point, the volume of oxygen present in the rumen would have reached a maximum value due to the cessation of bacterial gas production. Whether or not the presence of aerobic organisms was of any importance was of course not known. However, the fact that the rate of disappearance of vitamin B_{12} due to bacterial metabolism would have reached a minimum value during this period was important. It was also possible that an excess of the vitamin was present at this time. Samples were therefore drawn directly from the rumen, eleven to twelve hours after feeding.

The animal was fed twice daily. The diet consisted of alfalfa hay, water ad libitum, and a ground mash containing the following constituents:

oats	-	400 pounds
corn	-	400 "
linseed oil meal	-	200 "
salt	-	2%

An attempt was made to limit each feeding to a quantity such that it would be completely consumed by the animal within an hour's time. This, however, was not always possible.

Preparation of Extract

The fluid portion of each rumen sample was used in the preparation of extracts. In spite of this fact, samples were found to be very difficult to handle. Attempts to filter the sample were unsuccessful. Centrifugation resulted in the formation of several layers of material, and a clear solution could not be obtained. After numerous attempts, a successful extraction procedure was finally developed. Ruben, et al.[193] reported that the growth factor in cow manure could be precipitated by virtue of the association of the factor with protein, by adjusting the pH of a water extract to 3.0. Following this same suggestion, the rumen sample was acidified to pH 3.0 with 1N hydrochloric acid. Earlier use of .1N hydrochloric acid led to excessive dilution of the samples. Centrifugation at this point yielded two distinct layers, a brownish-gray

precipitate and a cloudy supernatant. The supernatant was discarded, and the precipitate extracted seven to eight times with n-butanol. Warming to about 50°C hastened the separation of the butanol layer. The extraction was completed as quickly as possible, and the pH of the butanol extract adjusted again to 7.0 with .1N sodium hydroxide. The extract was then evaporated to dryness by vacuum distillation below 50°C. The dry residue was extracted with redistilled water, and this solution autoclaved at 121°C for 15 minutes followed by an assay for the vitamin B_{12} content.

Attempts to obtain an accurate concentration value would have been of little significance since the vitamin B_{12} concentration in the rumen, without doubt, varied from day to day. A vitamin B_{12} triturate* (95% minimum purity) was used in the preparation of standards. All standards were prepared by serial dilution so that consistent results would be obtained. Assay results determined the vitamin B_{12} concentration to be approximately three gamma per milliliter of extract (Figures 3 and 4). Since one liter of rumen sample was used, and the total volume of extract was 55 milliliter, the concentration of vitamin B_{12} in the liquid portion of the raw rumen material was calculated to be .17 gamma per milliliter.

The Use of Radioactive Cobalt

Attempts to demonstrate bacterial synthesis of vitamin B_{12} by the use of tracer cobalt indicated early that some modification of the

*Merck & Co., Rahway, New Jersey.

extraction procedure was necessary. Cobalt^{60} (as a water solution of cobalt sulfate) was added to a fresh rumen sample after removal from the rumen, and the material extracted in the usual manner. A much larger quantity of radio-cobalt was added to these samples than had been proposed for the actual tracer study. It was found that a large part of the inorganic radio-cobalt was dissolved in the butanol extract. This was assumed to be due to the partial miscibility of butanol and water. It was not necessary to make an accurate count of the extract to demonstrate the presence of inorganic cobalt. Merely holding the flask containing the extract up to the G.M. tube of an ordinary laboratory monitor was sufficient.

Radio-cobalt was added to a second rumen sample, and the pH again adjusted to 3.0. This material was then added to 500 milliliter centrifuge bottles, centrifuged, and the supernatant discarded. The remaining precipitate was shell frozen and lyophilized. This operation was completed as rapidly as possible. By the end of the lyophilization period, it was apparent that smaller samples must be used since only partial drying had been accomplished. Nevertheless, the samples were extracted, and a large reduction in count was noted as compared to the previous sample.

A third sample was prepared and treated in the same manner as the second sample, with only three exceptions. A 500 milliliter sample, before pH adjustment, was placed in a fixed position near the G.M. tube of the laboratory monitor. Radio-cobalt was added until the scale

registered 1000 counts per minute. This sample was to be treated as a blank for future work if necessary. Exactly 100 milliliter of sample was added to each of five centrifuge bottles. The supernatant was discarded after centrifugation as before, and the remaining precipitate lyophilized to complete dryness. This was then extracted with dry butanol. Dry butanol was prepared according to the following steps:

1. Fractionally distilled, and the fraction collected which boiled above 114°C.
2. Allowed to stand over potassium carbonate for forty-eight hours, with occasional shaking.
3. Redistilled from drying agent immediately before using. Fraction collected which boiled above 116.7°C.

The extract was evaporated to dryness, extracted with 120 milliliter of water, and autoclaved, as previously described. This sample was then refrigerated until the actual tracer sample could be prepared.

Although any attempt to calculate the quantity of cobalt which must be administered as a tracer dose would be rather futile, some sort of estimation was necessary since several factors were involved. There was little danger of introducing artifacts due to over exposure of the animal, since only a matter of days would elapse between the time of administration of the tracer dose and the actual withdrawal of the test samples. According to the data of Comar et al.,[194] only 51.2% of a total cobalt dose remained twenty-four hours after rumen administration. Forty-eight

hours after administration, 20.7% remained, and none was absorbed through the rumen walls. The gamma energies of Co^{60} were sufficiently high as to completely penetrate the animal, leaving only the beta energies to be considered. Since no attempt was made to eliminate cobalt from the diet, this fact alone introduced a sizeable dilution factor, and it was quite obvious that a large dose must be given. Once placed in the rumen, the dose would be further diluted by the mass of material which was present. In addition, the vitamin B_{12} concentration in the rumen would, at best, be small.

A trial dose, calculated to give a maximum exposure of .1 milliroentgen per hour at a distance of one inch, or approximately 7.1×10^{-9} curies of cobalt was placed directly into the rumen. The material was inserted by means of a hypodermic needle through the rubber stopper which closed the fistula opening. The needle was washed through into the rumen several times with distilled water after the cobalt injection.

Twenty-four hours after adding the radio-cobalt, a sample of rumen contents was withdrawn and checked for radioactivity. The count on this raw sample was far too weak to produce an extract with a significant count.

The second dose was calculated to give a maximum exposure of approximately 1.6 milliroentgens per hour at one inch, or approximately 1.14×10^{-7} curies. This was about sixteen times as great as the first dose given, or about sixteen times the maximum exposure level at a distance of one inch. After a twenty-four hour period, another rumen sample was taken

and measured (as was the blank) from a fixed position on the laboratory monitor. Fortunately, a 500 milliliter sample counted 1400 counts per minute on the monitor scale. This was very close to the count previously obtained for the sample which was to be treated as a blank. This sample was extracted in the same manner as was the blank, using the same quantities of material for each step. The final extract volume in each case was 120 milliliters.

When assayed for vitamin B_{12} content, the extract was found to contain approximately .03 gamma per milliliter of extract, or approximately .07 gamma per milliliter of liquid rumen sample. (Figures 5 and 6). Apparently, a sizeable quantity of vitamin B_{12} had been lost through the added lyophilization step. It was found that the water extract of lyophilized samples had a tendency to gel after autoclaving. To prevent this, and to obtain a clear liquid sample which would diffuse readily, a larger quantity of water had to be used in the water extract than was previously intended. Even after this precaution, the extract had to be filtered, and the gelatinous material washed thoroughly with water. This step undoubtedly accounted for the loss of an appreciable amount of the vitamin content. It was evident that any further work beyond that reported in this paper would necessitate the use of improved extraction procedures and the further purification of extracts.

The two solutions (blank and sample) were plated on aluminum disks, by repeatedly applying one-half milliliter of solution at a time and drying until a total of twelve milliliters of material had been dried on each. Each disk was then counted, and the data listed as shown in

Figure 7. This data gives definite indication that some radioactive cobalt had been bound either in the form of vitamin B_{12}, or some other organic compound which was extracted by the same treatment.

Chromatography

Further identification of the Lactobacillus leichmannii growth factor present in rumen digest was attempted. A type of filter paper chromatography similar to that of Winsten and Eigen[195] was employed. A filter paper cylinder was used instead of filter paper strips, and Vitamin B_{12} Agar was used to replace the media of Winsten and Eigen.

The solutions (pH 5.0) were spotted at the base of a filter paper cylinder (Watman #1) and allowed to dry. The cylinder was then placed upright in a museum jar and wet n-butanol was slowly added. The top of the jar was then sealed tightly, and the chromatogram developed for approximately sixteen hours. The cylinder was then removed, allowed to dry in air for one hour at 30 - 35°C, and cut into strips. These strips were then laid on agar plates seeded with Lactobacillus leichmannii #4797. The paper strips were allowed to soak for six minutes on the moist agar surface in order to allow some of the test solutions to transfer from the strip to the agar. After the strip was removed, its imprint could be plainly seen on the agar. The plates were then incubated for twenty-four hours at 35°C. The location of the vitamin B_{12} on the filter paper strip was identified by the corresponding zone of growth on the agar surface. Standard solutions of one gamma per milliliter of vitamin B_{12} developed perfectly, showing R_f values of .015 (based upon the distance

traveled by the solvent). Growth resulting from the extracts tested was not found. Repeated tests of rumen extracts were completely void of growth, even when quantities as large as .04 milliliter were spotted on the chromatogram by repeatedly adding and drying .005 milliliter portions of the solution. This type of result was completely unexpected since good positive results had been obtained by microbiological assay on the same media. Apparently the vitamin was not released from the filter paper onto the agar when present in this crude extract.

Four per cent solutions of ribonucleic acid, desoxyribonucleic acid, and a mixture of the two plus vitamin B_{12} were chromatogrammed in this same manner and negative results again observed wherever vitamin B_{12} was absent.

DISCUSSION

The use of an agar cup assay method as a means of testing for the presence of vitamin B_{12} in rumen digest has revealed the presence of the vitamin in significant concentration. This is in accordance with the theory that inorganic cobalt may function in rumen metabolism as a part of the vitamin B_{12} molecule. Attempts to trace the dietary cobalt by administering radioactive Co^{60} indicated that the element was involved in a bacterial synthesis.

An extraction procedure was developed by means of which vitamin B_{12} could be extracted from rumen samples and at the same time remove only a very insignificant quantity of inorganic cobalt. It was evidenced, however, that further purification of these extracts was to be desired. This fact was demonstrated further when chromatography was attempted.

The extraction of wet rumen samples with ordinary n-butanol resulted in a clear, liquid solution which could be easily tested by microbiological assay. Attempts to chromatogram these extracts were unsuccessful, although standard solutions of vitamin B_{12} gave clear, positive results.

The extraction of lyophilized samples with dry n-butanol apparently removed more of the organic material present than did the wet extraction. Gelatinous material was observed to form in these solutions upon standing, but could be removed by filtration. Although this gelatinous material was extracted thoroughly by washing with water, assays proved the vitamin B_{12} concentration to be much lower under these conditions.

CONCLUSION

1. A microbiological assay for the determination of vitamin B_{12} was tested and modified.

2. A procedure for the extraction of vitamin B_{12} from rumen samples was developed.

3. A tracer study was performed in an attempt to demonstrate bacterial synthesis of vitamin B_{12}.

4. A chromatographic study of rumen extracts proved unsuccessful and demonstrated the need for further improvement of extraction procedures.

Figure 1

Figure 2

Solution Tested	Growth Diameters*(cm.)	Description
1. Desoxyribonucleic acid (4,000 r/cc)	2.4	Growth very faint
2. Ribonucleic acid (4,000 r/cc)	negative	
3. Vitamin B_{12} (.1r/cc)	1.9**	Clear, distinct growth
4. Desoxyribonucleic acid (4,000 r/cc) + Ribonucleic acid (4,000 r/cc) + Vitamin B_{12} (.1r/cc)	2.5	More distinct growth than 1

* Average of two values
** Average of six values

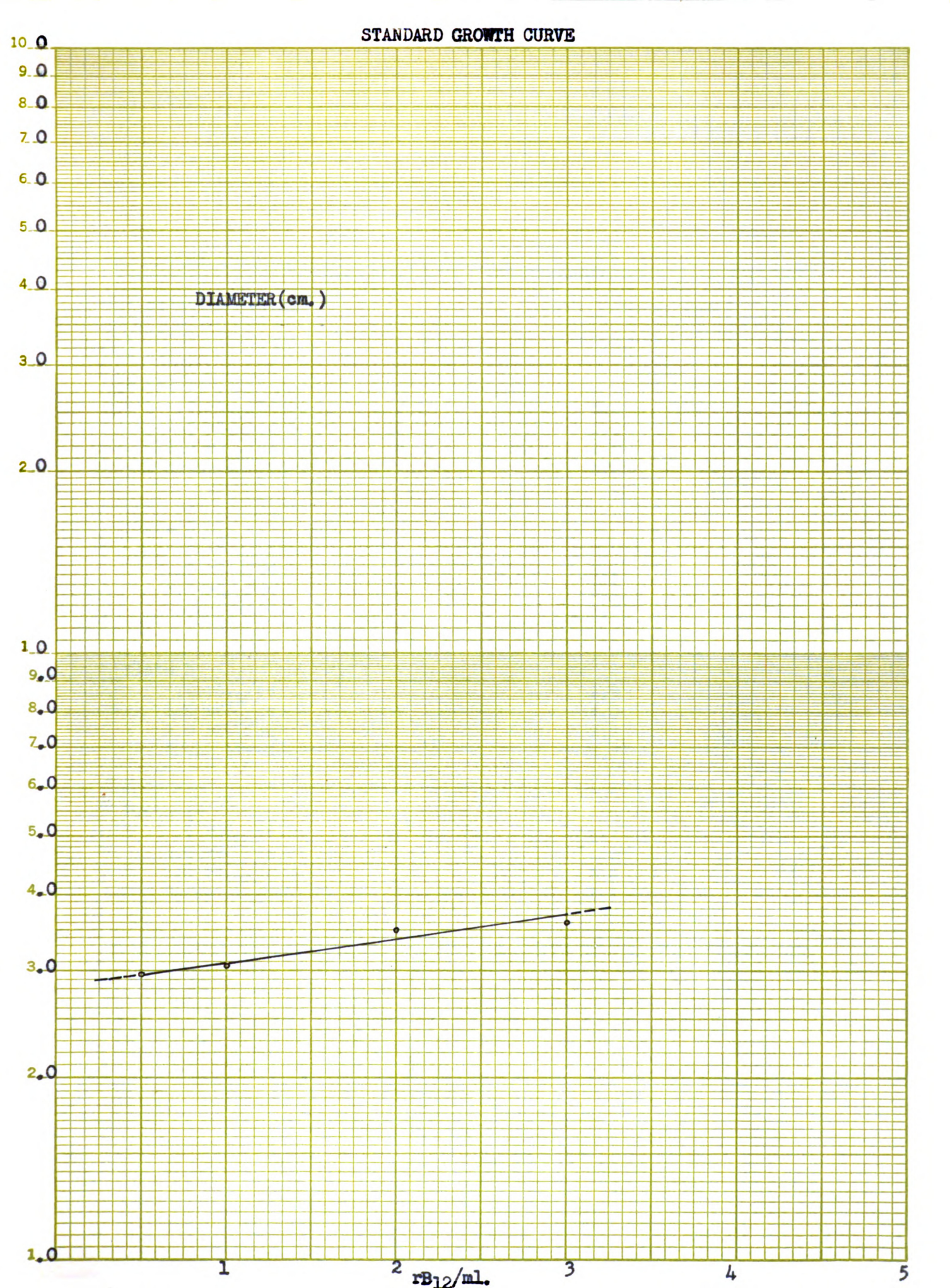
STANDARD GROWTH CURVE
DIAMETER(cm.)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1
2
3
4
5
rB12/ml.

Figure 4

Solution Tested	Growth Diameter (cm.)
Plate 1	
.5 rB_{12}/cc	3.0
1.0 rB_{12}/cc	3.0
3.0 rB_{12}/cc	3.6
Rumen extract	3.6
Plate 2	
.5 rB_{12}/cc	3.0
1.0 rB_{12}/cc	3.1
3.0 rB_{12}/cc	3.6
Rumen extract	3.7
Plate 3	
.5 rB_{12}/cc	2.9
1.0 rB_{12}/cc	3.0
2.0 rB_{12}/cc	3.5
Rumen extract	3.7
Plate 4	
.5 rB_{12}/cc	2.9
1.0 rB_{12}/cc	3.0
2.0 rB_{12}/cc	3.5
Rumen extract	3.8

STANDARD GROWTH CURVE

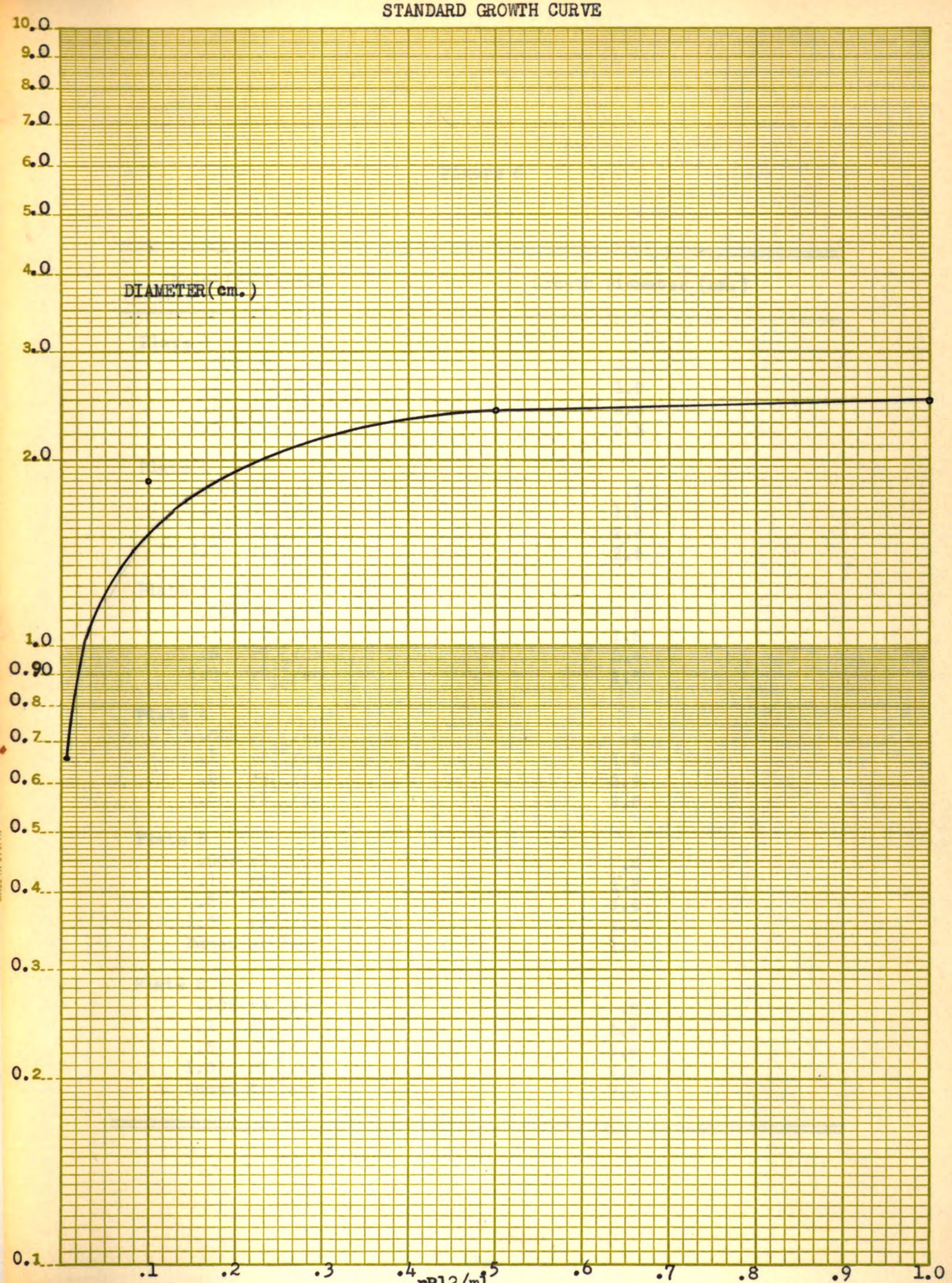

Figure 6

Solution Tested	Growth Diameter (cm.)
Plate 1	
.005 rB_{12}/cc	---
.1 rB_{12}/cc	1.9
.5 rB_{12}/cc	2.4
1.0 rB_{12}/cc	2.5
Plate 2	
.005 rB_{12}/cc	.7
.1 rB_{12}/cc	1.8
.5 rB_{12}/cc	2.4
1.0 rB_{12}/cc	2.5
Plate 3	
.005 rB_{12}/cc	.6
.1 rB_{12}/cc	1.8
.5 rB_{12}/cc	2.4
1.0 rB_{12}/cc	2.5
Plate 4	
.005 rB_{12}/cc	.6
.1 rB_{12}/cc	1.9
.5 rB_{12}/cc	2.4
1.0 rB_{12}/cc	2.5
Plate 5	
.005 rB_{12}/cc	.6
.1 rB_{12}/cc	1.8
.5 rB_{12}/cc	2.4
1.0 rB_{12}/cc	2.5
Rumen extract	1.1
Plate 6	
.005 rB_{12}/cc	.8
.1 rB_{12}/cc	1.8
.5 rB_{12}/cc	2.4
1.0 rB_{12}/cc	2.5
Rumen Extract	1.1

Figure 7

Sample	Volume Plated	Counts/minute above background
1	12.0 cc.	31.7
2	12.0 cc.	31.8
3 (Blank)	12.0 cc.	19.8
4 (Blank)	12.0 cc.	13.4
5 (Blank)	12.0 cc.	20.5

Sample Average Nos. 1 and 2	31.8
Blank Average Nos. 3, 4 and 5	17.9
Average Count Difference	13.9

Specific activity (based upon vitamin B_{12} concentration as indicated by assay values) 6.43 counts/sec/gamma.

BIBLIOGRAPHY

1. Addison, T., "Anemia, disease of the supa-renal capsules", London Med. Gaz. 43:517 (1849).

2. Minot, G. R. and W. P. Murphy, "Treatment of Pernicious Anemia by a special diet", J. Am. Med. Assoc. 87:470 (1926).

3. Koessler, K. K., S. Bauer, and R. Laughlin, "Relation of anemia, primary and secondary, to vitamin A deficiency", J. Am. Med. Assoc. 87:476 (1926).

4. Whipple, G. H. and F. S. Robscheit-Robbins, "Blood regeneration in severe anemia. I. Standard basal ration bread and experimental methods, Am. J. Physiol. 72:395 (1925).

5. Elders, C., "Tropical Sprue and pernicious anemia, etiology and treatment", Lancet I:75 (1925).

6. Minot, G. R. & W. P. Murphy, "A diet rich in liver in the treatment of pernicious anemia", J. Am. Med. Assoc. 89:759 (1927).

7. Castle, W. B. & M. A. Bowie, "Domestic liver extract for use in pernicious anemia; Method of preparation", J. Am. Med. Assoc. 92:1830, (1929).

8. Subbarow, Y., A. B. Hastings, and M. Elkin, "Chemistry of anti-pernicious anemia substances of liver", Vitamins and Hormones 3:257 (1945).

9. Castle, W. B., "Observations on etiologic relationship of achylia gastrica to pernicious anemia; effect of administration to patients with pernicious anemia of contents of normal human stomach recovered after investigation of these muscle", Am. J. Med. Sci. 178:748 (1929).

10. Castle, W. B. & W. C. Townsend, "Observations on etiologic relationship of achylia gastrica to pernicious anemia. II. Effect of administration to patients with pernicious anemia of beef muscle after incubation with normal human gastric juice", Am. J. Med. Sci. 178:764 (1929).

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

12. Campbell, D. C., B. E. Hall, and E. H. Morgan, "Oral administration of vitamin B_{12} in pernicious anemia. II. Studies on the nature and source of intrinsic factor." Proc. Cen. Soc. Clin. Research 22:18 (1949). Abstracted Annotated Bibliog. of Vit. B_{12}, Merck & Co., 17 (1950).

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

14. Gardner, F. H., J. W. Harris, & W. B. Castle, "Erythropoietic activity of extrinsic factor on parenteral administration in pernicious anemia", Am. J. Med. 7:421 (1949).

15. Parkhurst, R. T., Univ. Idaho Agr. Exp. Sta. Bull. 149, (1927). "Borden's Rev. of Nutrition Research" X, No. 1:5 (1949).

16. McFarlane, W. D., H. L. Fulmer, & T. H. Jukes, "Studies in embryonic mortality in the chick. I. The effect of diet on the nitrogen, amino nitrogen, tyrosine, tryptophane, cystine, and iron content of the proteins and on the total copper of the hen's egg", Biochem. J. 24:1611 (1930).

17. Van der Hoorn, R., H. D. Branion, & W. R. Graham, "Studies in the nutrition of the chick. II. Effect of purification of casein in simplified diet." Poultry Sci. 14:285 (1935).

18. Nestler, R. B., T. C. Byerly, N. R. Ellis, & H. W. Titus, "A new factor, not vitamin G, necessary for hatchability", Poultry Sci. 15:67 (1936).

19. Christiansen, J. B., H. J. Deobald, J. G. Halpin, & E. B. Hart, "Further studies on the nature of the effective supplements for soybean oil meal in chick rations", Poultry Sci. 18:481 (1939).

20. Christiansen, J. B., H. J. Deobold, J. G. Halpin, & E. B. Hart, "Practical supplements for soybean oil meal in chick rations", Poultry Sci. 19:18 (1940).

21. Hammond, J. C. and H. R. Bird, "Effects of nutrition on variability in the growth of chickens", Poultry Sci. 21:230 (1942).

22. Hammond, J. C. & H. W. Titus, "The use of soybean meal in the diet of growing chicks", Poultry Sci. 23:49 (1944).

23. McGinnis, J., P. T. Hsu, and W. D. Graham, "Studies on an unidentified factor required by chicks for growth and protein utilization", Poultry Sci. 27:674 (1948).

24. Whitson, D., J. C. Hammond, H. W. Titus, & H. R. Bird, "The use of soybean meal in the diet of growing chicks. II. Effect of different grains", Poultry Sci. 24:406 (1945).

25. Rubin, M. and H. R. Bird, "Chick growth factor in cow manure. I. Its nonidentity with chick growth factors previously described", J. Biol. Chem. 163:387 (1946).

26. Rubin, M. & H. R. Bird, "A chick growth factor in cow manure. II. The precipitation of concentrates and properties of the factor", J. Biol. Chem. 163:393 (1946).

27. Rubin, M. & H. R. Bird, "A chick growth factor in cow manure. III. Its occurrence in eggs", Poultry Sci. 26:309 (1947).

28. Rubin, M., A. C. Groschke, & H. R. Bird, "Chick growth factor in cow manure. VI. Effect on hatchability and storage in hens", Proc. Soc. Exp. Biol. Med. 66:36 (1947).

29. Bird, H. R., M. Rubin, & A. C. Groschke, "A chick growth factor in cow manure. VII. Its stability and solubility", J. Biol. Chem. 174:611 (1948).

30. Lillie, R. J., A. C. Dentow, & H. R. Bird, "Relationship of vitamin B_{12} to the growth factor present in cow manure", J. Biol. Chem. 176:1477 (1948).

31. Mapson, L. W., "Evidence of the existence of a dietary principle stimulating general growth and lactation", Biochem. J. 26:970 (1932).

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

33. Jaffe, W. G., "The growth stimulating effect of a heat-labile factor in liver extract on rats fed a natural diet", J. Biol. Chem. 165:387 (1946).

34. Jaffe, W. G. & C. A. Elvehjem, "Fractionation of growth stimulating factor in liver", J. Biol. Chem. 169:287 (1947).

35. Sporn, E. M., W. R. Ruegamer, and C. A. Elvehjem, "Growth and reproduction in rats on synthetic rations", Proc. Soc. Exp. Biol. Med. 65:5 (1947).

36. Bowland, J. P., W. E. Ensminger, & T. J. Cunha, "Need for an unidentified factor or factors for growth, reproduction, and lactation of rats fed purified rations", Arch. Biochem. 16:257 (1948).

37. Hartman, A. M. and C. A. Cary, "Occurrence in food of an unidentified factor essential for rat growth", Federation Proc. 5:137 (1946).

38. Bird, H. R., M. Rubin, D. Whitson, & S. K. Haynes, "The effectiveness of dietary supplements in increasing hatchability of eggs and viability of progeny of hens fed a diet containing a high level of soybean oil meal", Poultry Sci. 25:285 (1946).

39. Bethke, R. M., P. R. Record, D. C. Kennard, & V. D. Chamberlin, "The relation of nutrition to production and hatchability of chicken eggs. I. Effect of protein supplements and alfalfa meal", Poultry Sci. 25:570 (1946).

40. Bethke, R. M., J. M. Pensack, & D. C. Kennard, "The influence of hen's diet on growth of progeny", Poultry Sci. 26:128 (1947).

41. Nichol, C. A., A. R. Robblee, W. W. Cravens, & C. A. Elvehjem, "Distribution of an unidentified chick growth factor", Poultry Sci. 27:438 (1948).

42. Cravens, W. W., J. G. Halpin, & W. H. McGibbon, "The use of various vitamin supplements in rations for laying and breeding hens", Poultry Sci. 25:99 (1946).

43. McGinnis, J. & J. S. Carver, "The storage of an unidentified growth factor or factors in the egg, and its relation to chick growth and mortality", Poultry Sci. 26:457 (1947).

44. Rubin, M., H. R. Bird, & I. Rothchild, "A growth promoting factor for chicks in the feces of hens", Poultry Sci. 25:526 (1946).

45. McGinnis, J., J. M. Stevens, & K. Groves, "The in vitro synthesis of a chick growth promoting factor in hen feces", Poultry Sci. 26:432 (1947).

46. McGinnis, J., J. M. Stevens, & K. Groves, "Studies on an unidentified factor required for chick growth and livability", Poultry Sci. 26:550 (1947).

47. Stokstad, E. L. R., A. Page, J. Pierce, A. L. Franklin, T. H. Jukes, R. W. Heinle, H. Epstein, & A. D. Welsch, "Activity of microbial animal protein factor concentrates in pernicious anemia", J. Lab. Clin. Med. 33:860 (1948).

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

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

50. Shorb, M. S., "Activity of vitamin B_{12} for the growth of Lactobacillus lactis", Science 107:397 (1948).

51. Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, & K. Folkers, "Crystalline vitamin B_{12}", Science 107:396 (1948).

52. Koditschek, L. K., D. Henlin, & H. B. Woodruff, "Investigations on the nutrition of Lactobacillus lactis Dorner", J. Biol. Chem. 179:1093 (1949).

53. Caswell, M. C., L. K. Koditschek, & D. Hendlin, "The microbiological estimation of L. lactis Dorner activity with vitamin B_{12} as a standard", J. Biol. Chem. 180:125 (1949).

54. West, R., "Activity of vitamin B_{12} in addisonian pernicious anemia", Science 107:398 (1948).

55. Smith, E. L., "Purification of anti-pernicious anemia factors from liver", Nature 161:638 (1948).

56. Ungley, C. C., "Anti-anemic substances from liver", Lancet 1:771 (1948).

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

58. Hall, B. E. and D. C. Campbell, "Effect of vitamin B_{12} on the hematopoietic and nervous systems in addisonian pernicious anemia", J. Lab. Clin. Med. 33:1646 (1948).

59. Spies, T. D., R. M. Suarez, G. G. Lopez, F. Milanes, R. E. Stone, R. L. Toca, T. Aramburu, & Sam Kartus, "Tentative appraisal of vitamin B_{12} as a therapeutic agent", J. Am. Med. Assoc. 139:521 (1949).

60. Bethell, F. H., M. C. Meyers, & R. B. Heligh, "Vitamin B_{12} in pernicious anemia and puerperal macrocytic anemia", J. Lab. Clin. Med. 33:1477 (1948).

61. Spies, T. D., R. E. Stone, G. L. Garcia, F. Milanes, R. T. Lopez, & T. Aramburu, "Thymidine, folic acid, and vitamin B_{12} in nutritional macrocytic anemia, tropical sprue, and pernicious anemia", Lancet 2:519 (1946).

62. Bethell, F., "Treatment of macrocytic anemias with vitamin B_{12}", J. Am. Dietet. Assoc. 25:89 (1950).

63. Wetzel, N. C., W. C. Fargo, I. H. Smith, & J. Helikson, "Growth failure in school children as associated with vitamin B_{12} deficiency - response to oral therapy", Science 110:651 (1949).

64. Ibid., 50.

65. Ibid., 55.

66. Smith, E. L. & L. F. J. Parker, "Vitamin B_{12} isolated in England", Chem. Eng. News, 26:2218 (1948).

67. Ott, W. H., E. L. Rickes, & L. R. Wood, "Crystalline vitamin B_{12} activity for chick growth", J. Biol. Chem., 174:1047 (1948).

68. Lillie, R. J., C. A. Denton, & H. R. Bird, "Relation of vitamin B_{12} to the growth factor present in cow manure", J. Biol. Chem. 176:1477 (1948).

69. Nichol, C. A., A. R. Robblee, W. W. Cravens, & C. A. Elvehjem, "The growth response of chicks to anti-pernicious anemia preparations", J. Biol. Chem. 177:631 (1949).

70. Lindstrom, R. G., P. R. Moore, C. F. Peterson, & A. C. Wiese, "Activity of a vitamin B_{12} concentrate for chick growth and hatchability", Poultry Sci. 28:464 (1949).

71. Lillie, R. J., S. J. Marsden, A. C. Groschke, & H. R. Bird, "Relative requirements and source of vitamin B_{12} for turkeys and chickens during the later stages of growth", Poultry Sci. 28:541 (1949).

72. Ibid., 67.

73. Lillie, R. J., M. W. Olson, & H. R. Bird, "Role of vitamin B_{12} in reproduction of poultry", Proc. Soc. Exp. Biol. Med. 72:598 (1949).

74. Hartman, A. M., L. P. Dryden, & C. A. Cary, "A role of vitamin B_{12} in the normal mammal", Arch. Biochem. 23:165 (1949).

75. Lewis, U. J., U. D. Register, & C. A. Elvehjem, "Vitamin B_{12} content of various organs and tissues of the rat", Proc. Soc. Exp. Biol. Med. 71:509 (1949).

76. Luecke, R. W., W. N. McMillen, F. Thorp, & J. R. Boniece, "The effect of vitamin B_{12} concentrates on the growth of weanling pigs fed corn-soybean diets", Science 110:139 (1949).

77. Neumann, A. L., B. C. Johnson, & J. B. Thiersch, "Crystalline vitamin B_{12} in the nutrition of the baby pig", J. Nutrition 40:403 (1950).

78. Anderson, G. C. and A. G. Hogen "Requirement of the pig for vitamin B_{12}", J. Nutrition 40:243 (1950).

79. Cunha, T. J., J. E. Burnside, D. M. Buschman, R. S. Glasscock, A. M. Pearson, & A. L. Shealy, "Effect of vitamin B_{12}, animal protein factor, and soil for pig growth", Arch. Biochem. 23:324 (1949).

80. Hogan, A. G. & O. C. Anderson, "Vitamin B_{12} in swine nutrition", Federation Proc. 8:385 (1949).

81. Johnson, B. C. & A. L. Neumann, "Crystalline vitamin B_{12} compared to antipernicious anemia liver extract for pig growth", J. Biol. Chem. 178:1001 (1949).

82. Johnson, B. C. & A. L. Neumann, "Crystalline vitamin B12 compared to anti-pernicious anemia liver extract for pig growth", Proc. Soc. Exp. Biol. Med. 69:513 (1948).

83. Stokstad, E. L. R., T. H. Jukes, J. Pierce, A. C. Page, & A. L. Franklin, "The multiple nature of the animal protein factor", J. Biol. Chem. 180:647 (1949).

84. Hill, F. W. "The multiple nature of the deficiency of unidentified nutrients in crude all-vegetable protein chick starter rations", Poultry Sci. 27:536 (1948).

85. Ershoff, B. H., "An anti-thyrotoxic factor for the rat not identified with vitamin B_{12}", Proc. Soc. Exp. Biol. Med. 71:209 (1949).

86. Ershoff, B. H., "Beneficial effects of liver on growth and survival in the immature hyperthyroid mouse", Proc. Soc. Exp. Biol. Med. 70:398 (1949).

87. Ibid., 83.

88. Zucker, T. F. & L. M. Zucker, "The identity of vitamin B_{12} and the animal protein factor", Abstracts of papers, Am. Chem. Soc. 117th meeting.

89. Hartman, A. M., L. P. Dryden, and C. A. Cary, "Role and sources of vitamin B_{12} in the normal mammal", U. S. Dept. of Agr. BDIM-Inf-76 (1949).

90. Ibid., 69.

91. Ibid., 83.

92. Zucker, L. M. & T. F. Zucker, "Zoopherin: A nutritional factor for rats associated with animal protein sources", Arch. Biochem. 16:115 (1948).

93. Zucker, T. F. & L. M. Zucker, "The occurrence of zoopherin in lower animal farms", Arch. Biochem. 18:513 (1948).

94. Zucker, L. M. & T. F. Zucker, "Does 'Animal protein factor' occur in green plants?" Proc. Soc. Exp. Biol. Med. 68:432 (1948).

95. Stokstad, E. L. R., A. Page, J. Pierce, A. L. Franklin, T. H. Jukes, R. W. Heinle, M. Epstein, & A. D. Welch, "Activity of microbial animal protein factor concentrates in pernicious anemia", J. Lab. Clin. Med. 33:860 (1948).

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

97. Bethell, J. J. & H. A. Lordy, "Comparative effectiveness of vitamin B_{12}, whole liver substance, and extracts high in anti-pernicious anemia activity as growth promoting materials for hyperthyroid animals", J. Nutrition 37:495 (1949).

98. Emerson, G. A., "Growth-promoting activity of vitamin B_{12} in rats receiving thyroid substance", Proc. Soc. Exp. Biol. Med. 70:392 (1949).

99. Nichol, C. A., L. S. Dietrich, W. W. Cravens, & C. A. Elvehjem, "Activity of vitamin B_{12} in the growth of chicks", Proc. Soc. Exp. Biol. Med. 70:40 (1949).

100. Personal communication, Dr. A. C. Groschke.

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

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

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

104. Smith, E. L., "Presence of cobalt in the anti-pernicious anemia factor", Nature 162:144 (1948).

105. Ellis, B., V. Petrow, & G. F. Snook, "Chemistry of anti-pernicious anemia factors. I. Liberation of phosphorus as phosphate from vitamin B_{12} by acid hydrolysis", J. Pharm. Pharmacol. 1:287 (1949). Abstracted Chem. Abstracts 43, 5432b.

106. Folkers, K., "Research on vitamin B_{12}", Chem. Eng. News 28; 1634 (1950).

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

108. Lichtman, H., J. Watson, V. Ginsberg, J. V. Pierce, E. L. R. Stokstad, & T. H. Jukes", "Vitamin B_{12b}: Some properties and its therapeutic use", Proc. Soc. Exper. Biol. Med. 72:643 (1949).

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

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

111. Kaczka, E. A., D. E. Wolf, F. A. Kuehl, Jr., & K. Folkers, "Vitamin B_{12}: Reactions of cyano-cobalamin and related compounds", Science 112; 354 (1940).

112. Ibid., 108.

113. Ibid., 96.

114. McGinnis, J., E. L. Stephenson, B. T. H. Levadie, J. S. Carver, J. A. Garibaldi, N. Ijiche, N. S. Suell, & J. C. Lewis, "Response of chicks and turkey poults to vitamin B_{12} supplements produced by fermentation with different organisms. Abstracts of papers, Amer. Chem. Soc. 116th meeting.

115. Ansbacher, S. & H. H. Hill, "Selective synthesis of animal protein factor complex by fermentation", Abstracts of papers, Amer. Chem. Soc., 116th meeting.

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

117. Hoffmann, C. E., E. L. R. Stokstad, A. L. Franklin, & T. H. Jukes, "Response of Lactobacillus leichmannii 313 to the anti-pernicious anemia factor", J. Biol. Chem. 176, 1465 (1948).

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

119. Stokstad, E. L. R., A. C. Dornbush, A. L. Franklin, C. E. Hoffmann, B. L. Hutchings, & T. H. Jukes, "Microbiological Assay of vitamin B_{12} by Lactobacillus leichmannii", Federation Proc. 8:257 (1949).

120. Skeggs, H. R., J. M. Huff, L. D. Wright, & D. K. Bosshart, "Lactobacillus leichmannii in the microbiological assay of the "animal protein factor", J. Biol. Chem. 176;1459 (1948).

121. Capps, B. F., N. L. Hobbs, & S. H. Fox, "A method for the microbiological assay of vitamin B_{12}", J. Biol. Chem. 178:517 (1949).

122. Foster, J. W. & H. B. Woodruff, "Microbiological aspects of penicillin. VI. Procedure for the cup assay for penicillin", J. of Bact. 47:43 (1944).

123. Schmidt, W. H. & Moyer, A. J., "Penicillin. I. Methods of assay", J. of Bact. 47:199 (1944).

124. Foster, John C., J. A. Lolly, & H. B. Woodruff, "Cup assay with vitamin B_{12} as a standard", Science 110;507 (1949).

125. Yacowitz, H., L. C. Norris, & C. F. Heuser, "An improved method for the microbiolgical assay of growth factors on paper chromatograms", Proc. Soc. Exp. Biol. Med. 71:372 (1949).

126. Peeler, H. T., M. Yacowitz, & L. C. Norris, "A microbiological assay for vitamin B_{12} using Lactobacillus leichmannii", Proc. Soc. Exp. Biol. Med. 72:515 (1949).

127. Register, U. D., W. R. Ruegamer, & C. A. Elvehjem, "An improved assay for a growth factor in liver extracts", J. Biol. Chem. 177:129 (1949).

128. Frost, D. V., H. H. Fricke, & E. C. Spruth, "Rat-growth assay for vitamin B_{12}", Proc. Soc. Exp. Biol. Med. 72:102 (1949).

129. Shorb, M. S. & G. M. Briggs, "Effects of dissociation in Lactobacillus lactis cultures on the requirement for vitamin B_{12}", J. Biol. Chem. 176:1463 (1948).

130. Shive, W., J. M. Ravel, & R. E. Eaken, "An interrelationship of thymidine and vitamin B_{12}", J. Am. Chem. Soc. 70:2614 (1948).

131. Green, R. D., A. J. Brook, & R. B. McCormack, "Some conditions which affect the assay of vitamin B_{12} with Lactobacillus lactis Dorner", J. Biol. Chem. 178:999 (1949).

132. Caswell, M. C., L. K. Koditschek, & D. Hendlin, "The microbiological estimation of Lactobacillus lactis activity with vitamin B_{12} as a standard", J. Biol. Chem. 180:125 (1949).

133. Koditschek, L. K., D. Hendlin, & H. B. Woodruff, "Investigations on the nutrition of Lactobacillus lactis Dorner", J. Biol. Chem. 179:1093 (1949).

134. Shaw, G. E., "Lactobacillus lactis for the assay of vitamin B_{12}", Nature 164:186 (1949).

135. Welch, A. D. & M. F. Wilson, "Mechanism of growth-promoting effect of ascorbic acid on Lactobacillus leichmannii and the reduction of osidation products of vitamin B_{12}", Arch. Biochem. 22:486 (1949).

136. Ibid., 35.

137. Ibid., 31

138. Bosshardt, D. K., W. J. Paul, K. O'Doherty, J. W. Huff, & R. H. Barnes, "House growth assay procedures for the 'animal protein factor'", J. Nutrition 37:21 (1949).

139. Ibid., 31.

140. Angier, R. B., J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow, C. W. Waller, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels, and J. M. Smith, Jr. "Synthesis of a compound identical with the L. casei factor isolated from liver", Science 102:227 (1945).

141. Anglier, R. B., J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow, C. W. Waller, D. B. Cosulich, M. J. Fahrenbach, M. E. Huttquist, E. Kuh, E. H. Northy, D. R. Seeger, J. P. Sickels, and J. M. Smith, Jr. "The structure and synthesis of the liver L. casei factor", Science 103:667 (1946).

142. Shive, W., R. E. Eakin, W. M. Harding, J. M. Ravel, & J. E. Sutherland, "A crystalline factor functionally related to folic acid", J. Am. Chem. Soc. 70, 2299 (1948).

143. Stokes, J. L., "The substitution of thymine for folic acid in the nutrition of lactic acid bacteria", J. Bact. 48:201 (1944).

144. Spies, T. D., C. F. Vilter, J. K. Cline, & W. B. Frommeyer, "The substitution of thymine for folic acid in the treatment of macrocytic anemias in relapse", Southern Med. J. 39:269 (1946). Borden's Rev. of Nutrition Research, X, 9 (1949).

145. Snell, E. E., E. Kitay, & W. S. McNutt, "Thymine desoxyreboside as an essential growth factor for lactic acid bacteria", J. Biol. Chem. 175:473 (1948).

146. Wright, L. D., H. R. Skeggs, & J. W. Huff, "The ability of thymidine to replace vitamin B_{12} as a growth factor for certain lactobacilli", J. Biol. Chem. 175:475 (1948).

147. Shive, W., J. M. Ravel, & R. E. Eakin, "An interrelationship of thymidine and vitamin B_{12}", J. Am. Chem. Soc. 70:2614 (1948).

148. Kitay, E., W. S. McNutt, & E. E. Snell, "The nonspecificity of thymidine as a growth factor for lactic acid bacteria", J. Biol. Chem. 177:993 (1949).

149. Wright, L. H., "Thymidine and vitamin B_{12}", Science 110:257 (1949).

150. Ibid., 149.

151. Tomarelli, R. M., R. F. Norris, & P. Gyorgy "Inability of vitamin B_{12} to replace the desoxyriboside requirement of lactobacillus bifidus", J. Biol. Chem. 179:485 (1949).

152. Hoff-Jorgensen, E., "Difference in growth-promoting effect of desoxyribosides and vitamin B_{12} on three strains of lactic acid bacteria", J. Biol. Chem. 178:525 (1949).

153. Snell, E. E., E. Kitay, & W. S. McNutt, "Thymine desoxyriboside as an essential growth factor for lactic acid bacteria", J. Biol. Chem. 175:473 (1948).

154. Skeggs, H. R., J. W. Huff, & L. D. Wright, "The ability of thymidine to replace vitamin B_{12} as a growth factor for certain Lactobacilli", J. Biol. Chem. 175:475 (1948).

155. Shive, W., J. M. Ravel, & W. M. Harding, "An interrelationship of purines and vitamin B_{12}", J. Biol. Chem. 176;991 (1948).

156. Skeggs, H. R., J. W. Huff, & L. D. Wright, "The use of Lactobacillus leichmannii in the microbiological assay of the "animal protein factor", J. Biol. Chem. 176, 1459 (1948).

157. Shive, W., J. M. Ravel, & W. M. Harding, "Interrelationship of purines and vitamin B_{12}", J. Biol. Chem. 176:991 (1948).

158. Roberts, I. Z., R. B. Roberts, & P. H. Abelson, "Effect of vitamin B_{12} on the phosphorus metabolism of Lactobacillus leichmannii", J. Bact. 58:709 (1949).

159. Jones, J. H., C. S. Rogers, & C. H. Stone III, "Succinylsulfathiazole and a rat growth factor in liver", J. Nutrition 39:579 (1949).

160. Hall, C. A. & V. A. Drill, "Lipotropic effect of liver extract on dietary hepatic injury in rats", Proc. Soc. Exp. Biol. Med. 69:3(1948).

161. Drill, V. A. & H. M. McCormick, "Lipotropic effects of vitamin B_{12} concentrate", Proc. Soc. Exp. Biol. Med. 72:388 (1949).

162. Schaefer, A. E., W. D. Salmon, & D. R. Strength, "Interrelationship of vitamin B_{12} and choline. I. Effect on hemorrhagic kidney syndrome in the rat", Proc. Soc. Exp. Biol. Med. 71:193 (1949).

163. Schaefer, A. E., W. D. Salmon, & D. R. Strength, "Relation of vitamin B_{12} to the choline requirement of the rat and chick", Federation Proc. 8:395 (1949).

164. Schaefer, A. E., W. D. Salmon, & D. R. Strength, "Interrelationship of vitamin B_{12} & choline. II. Effect on growth of the chick", Proc. Soc. Exp. Biol. Med. 71:202 (1949).

165. Gillis, M. B. & L. C. Norris, "Effect of the animal protein factor on the requirement for methylating compounds", J. Biol. Chem. 179:487 (1949).

166. Cunha, T. J., H. H. Hopper, J. E. Burnside, A. M. Pearson, R. S. Glasscock, & A. L. Shealy, "Effect of vitamin B_{12} and animal protein factor supplements on methionine needs of the pig", Arch. Biochem. 23:510 (1949).

167. Stokstad, E. L. R. & T. H. Jukes, "Vitamin B_{12} and some of its interrelationships", Abstracts of papers, Am. Chem. Soc. 117th meeting.

168. Popper, H., D. Koch-Weser, & P. B. Szanto, "Protective effect of vitamin B_{12} on hepatic injury produced by carbon tetrachloride", Proc. Soc. Exp. Biol. Med. 71:688 (1949).

169. Shaefer, A. E., M. D. Salmon, D. R. Strength, & D. H. Copeland, "Interrelationship of folacin, vitamin B_{12}, and choline. Effect on hemorrhagic kidney syndrome in the rat and on growth of the chick", J. Nutrition 40:95 (1950).

170. Nichol, C. A., A. E. Harper, & C. A. Elvehjem, "Effect of folic acid, liver extract, and vitamin B_{12} on hemoglobin regeneration in chicks", Proc. Soc. Exp. Biol. Med. 71:34 (1949).

171. Roberts, R. B. & M. Sands, "The Influence of vitamin B_{12} on the growth of bacteriophage T4r", J. of Bact. 58:710 (1949).

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

173. Dorrance, S. S., G. W. Thorn, M. Clinton, H. W. Edmonds, and S. Farber, "Effect of cobalt on work performance under conditions of anoxia", Am. J. Physiol. 139:399 (1943).

174. Marston, H. R. "Ruminant Nutrition", Ann. Rev. Biochem. 8:557 (1939).

175. Thompson, J. F. & G. H. Ellis, "Is cobalt a dietary essential for the rabbit?", J. Nutrition, 34:121 (1947).

176. Underwood, E. J. & C. A. Elvehjem, "Is cobalt of any significance in the treatment of milk anemia with iron and copper", J. Biol. Chem. 124:419 (1938).

177. Ibid., 174.

178. National Research Council, A Report of the Committee on Animal Nutrition #4,"Recommended nutrient allowances for beef cattle".

179. McCance, R. A. & E. M. Widdowson, "Mineral Metabolism", Ann. Rev. Biochem. 13:315 (1944).

180. Gall, L. S., S. E. Smith, and D. E. Becker, "Rumen bacteria in cobalt-deficient sheep", Science 109:468 (1949).

181. Comar, C. L., "Cobalt metabolism studies. II. Partition of radioactive cobalt by a rumen fistula cow", J. Nutrition 32:61 (1946).

182. Comar, C. L. & G. K. Davis, "Cobalt metabolism Studies III. Excretion and Tissue distribution of radioactive cobalt administered to cattle. Arch. Biochem. 12:257 (1947).

183. Comar, C. L. & G. K. Davis, "Cobalt metabolism studies. IV. Tissue distribution of radioactive cobalt administered to rabbits, swine, and young calves", J. Biol. Chem. 170:379 (1947).

184. Becker, D. E., S. E. Smith, & S. K. Loosli, "Vitamin B_{12} and cobalt deficiency in sheep", Science 110:71 (1949).

185. Burroughs, W., P. Gerlaugh, & R. M. Bethke, "The use of an artificial rumen in studying roughage digestion with rumen microorganisms under controlled laboratory conditions", Abstracts of papers, Society proceedings of American Society of Animal Production, 41st meeting.

186. Hale, W. H., A. L. Pope, P. H. Phillips, & G. Bohstedt, Abstracts of papers, Society proceedings of American Society of Animal Production, 41st meeting.

187. Henlin, D. & M. L. Ruger, "The effect of cobalt on the microbial synthesis of L. L. D. - active substances", Science 111:541 (1950).

188. Ibid., 124.

189. Ibid., 124.

190. Ibid., 121.

191. Ibid., 124.

192. Personal communication, Dr. C. F. Huffmann.

193. Ibid., 26.

194. Ibid., 181.

195. Winsten, W. A. & E. Eigen, "Paper chromatography of vitamin B_{12} and related bacterial growth factors", J. Biol. Chem. 181:109 (1949).