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Microbial growth factors in commercial products of bacterial and fungal origin

Jacob Julius Pruzansky
Iowa State College

MICROBIAL GROWTH FACTORS IN COMMERCIAL PRODUCTS OF BACTERIAL AND FUNGAL ORIGIN

by

Jacob Julius Pruzansky

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Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

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I. INTRODUCTION

Growth factors and microorganisms have had an interesting history of fruitful interrelationships. Attention was focused at first on specific growth substances required by microbes. This dates back to the period when synthetic media were first sought for the culture of organisms. It was found that successful growth could be obtained only when small amounts of naturally occurring materials were added to the media. These materials were early recognized to provide unknown but essential metabolites. Interesting relics of this early phase remain in the form of voluminous compilations of media for culturing bacteria. Later when emphasis in growth factors shifted because of their significance in animal and human nutrition, microbiology again became involved by providing convenient assay methods.

These developments served to emphasize the fact that growth substances for one organism are also required by others. By this token plant materials contain vitamins for animals, microorganisms and other plants. Many organisms themselves provide stimulating materials for other members of the same group. In this manner the growth promoting effect of large inocula in synthetic media can be explained since the carry over of filtrate and autolyzed cells provide growth

factors for the succeeding generations.

Observations illustrating the interrelationships already mentioned are numerous. One such case is the increased alcohol yields obtained when mold amylase was used instead of malt as the saccharifying agent for grain mashes subjected to yeast fermentations. In all likelihood such results were due in part to growth factors present in the unfractionated mold enzyme preparations. For this reason it was considered interesting to determine the vitamin content of mold bran which was the source of the amylase. In order to distinguish between the effects of the factors produced by the growth of the mold on the substrate and that of the substrate itself, wheat bran was used as a control.

Another instance in which microbiology has played a vital role in nutrition has been the work on animal protein factor (APF). Plant proteins alone were found insufficient to sustain normal chick growth. Materials of animal origin such as fish meal and meat scraps were needed. Before long, however, certain fermentation products were found to supply APF. With this reaffirmation of the interdependence of growth substances for different organisms it was thought likely that a microbiological assay could be developed for APF. A further useful application of such a method would be to follow the fractionation of materials containing the active agent. A major purpose of this investigation was, therefore, to develop a microbiological assay procedure and to apply it

in following the fractionation of materials exhibiting APF activity.

II. REVIEW OF THE LITERATURE

As with so many other aspects of fermentation chemistry, bacteriology or related subjects, the discussion of microbial growth factors properly starts with the work of Louis Pasteur (1860). In the course of a comprehensive examination of the alcoholic fermentation Pasteur described the first attempt to use a completely synthetic medium. He had been struck by the simplicity of the ultimate analysis of yeast and decided that it would be reasonable to try to grow yeast on a medium composed of sucrose, yeast ash obtained from muffle furnace ignition and dextrorotary ammonium tartrate. The inoculum was very precisely stated to be a "pin head of yeast". In 24 to 36 hours bubbles of carbon dioxide gave visible evidence that fermentation had begun. Gradually a deposit of yeast cells formed on the bottom of the flask. Using the same technique, addition of small amounts of raisin juice, filtrates of yeast fermentations and other similar materials gave the same results but the action was swifter and much more vigorous. His conclusion was that yeast would grow on a simple medium consisting of sugar, an ammonium salt and inorganic ash. In this very small part of the total work of Pasteur he opened a new field. As a result of this investigation a polemic developed between Liebig and Pasteur because

the former was unable to repeat Pasteur's experiments.

In time Pasteur's opinion was generally accepted but Wildiers (1901) reopened the question of synthetic media. In studying the synthesis of phosphorous compounds such as phosphatides, nucleic acids and lecithin in yeast he decided to use the smallest inoculum possible to avoid carry over of such compounds into the medium. Under these conditions no growth was obtained in the synthetic medium. Aeration or the addition of invert sugar or asparagine did not help growth. Only the increase in size of the inoculum over a definite minimal amount would allow growth and fermentation. In order to show that the effect was due to a chemical substance and not just live yeast cells the suspension was boiled and incorporated into the medium. A very small inoculum was then able to produce good growth. Filtration through a Chamberland filter gave a filtrate containing the active agent which was named bios. The properties of bios were also investigated. It was found to be soluble in water and 80 per cent ethanol but not in absolute ethyl alcohol or ether. It was not lecithin, urea, asparagine, aniline, tyrosine, adenine, guanine, nucleic acid, creatine or products of proteolysis. Wildiers' contribution to the Pasteur-Liebig controversy was that both were right but Liebig was more parsimonious than Pasteur and used a smaller pin head of inoculum with insufficient bios to allow visible growth.

Wildiers did not receive unanimous approval either and

quickly several other explanations were advanced. It was proposed on one hand that the lack of growth or poor growth with small inocula was due to the presence of metallic poisons in the distilled water used. On the other hand Pringsheim (1906) attributed everything to adaptation of the yeast to use the simple compounds present. The function of the large inoculum in the poison theory was for some of the excess cells to tie up the metals and allow the remainder to develop normally. In the second explanation the nutrients carried over in the large inoculum helped supply the added energy required for some of the yeasts to synthesize their complex constituents from the simple chemicals supplied thus allowing adaptation to the new metabolic conditions. Kossowicz (1903) generally supported Wildiers but questioned his lack of precision in several respects. He objected to the use of 2 or 5 drops of culture instead of seeding to a definite cell count, to lumping growth and fermentation together and to estimating carbon dioxide evolution instead of alcohol production as a measure of fermentation. Interestingly enough he added Penicillium glaucum along with a small yeast inoculum and got a stimulation of growth similar to that obtained by using a larger amount of yeast. This was due to water soluble substances present and not to the cells. Another worker upholding Wildiers was Ide (1907) who found bios essential, with no evidence to support the adaptation or poison theories. He isolated a basic substance which he named biosin and which

was not lecithin but was closely related to it.

At about this time a more practical approach to growth substances in microbiology was developed in the medical field where numerous investigators were trying to get parasitic organisms to grow on laboratory media. Previous to Cantani (1902) it had been thought that hemoglobin was necessary for in vitro culture of the influenza bacillus. In some earlier work and in the reference cited he obtained growth with such materials as bull sperm, serum albumin, serum globulin, egg yolk and egg albumin. Dead or living cells of diphtheria, staphylococci, sarcina, typhus and yeast produced heavy growth of the bacillus. A brief review of the earlier work along these lines was presented by Ghon and von Preyss (1902) in the introduction to their experimental findings. In further studies on influenza bacillus culture, Neisser (1903) found that Corynebacterium xerose, Pfeiffer's bacillus or others of not too different morphology and cultural characteristics could supply the needed growth factors when grown together with the influenza organism. He found no adaptation of the bacillus even after 8 generations of mixed culture. Although he termed the relationship symbiosis the influenza bacillus grew largely at the expense of the other organism. Intimately mixed cultures did not thrive as well as those in which streaks of the individual cultures were crossed on agar plates.

A new and very stimulating concept was applied by Twort and Ingram (1911). They noted the close relationship between Johne's bacillus and the T.B. organism and decided that they must have come from a common ancestor. Since the tuberculosis organism could grow on some media on which Johne's bacillus could not they assumed the former could synthesize those factors which Johne's bacillus required but which were absent in the laboratory medium. Previously they had cultivated lepra bacillus of man utilizing this concept. They succeeded in this instance of doing the same for Johne's bacillus by adding dried and powdered acid fast organisms to the medium. Timothy grass bacillus was the best of the group tested and this coincided with their views since this organism was the least parasitic and therefore most able to synthesize all the needed factors. The required substances were extractable in hot ethanol.

After this time many papers appeared to show that growth factors from various sources were needed for a large number of organisms. Lloyd (1916) declared that accessory growth substances were necessary for the <u>in vitro</u> cultivation of the meningococcus. These were to be found in blood, serum, milk and other animal secretions, fluids or extracts. They were moderately stable to heat, soluble in both water and alcohol and adsorbed on filter paper. An inverse relationship between vitamins and amino acids in the medium was found indicating

to Lloyd that the required substances regulated protein metabolism. Recognition that two factors were necessary for hemophilic bacteria was granted by Davis (1917) in work on the influenza bacillus. He cited the practice of inoculating blood agar with Bacillus influenzae and then streaking with staphylococcus, pneumococcus, meningococcus or yeast. The blood obviously supplied one factor and the second organism another. He also found that fresh animal tissue, potatoes or carrots supplied the second factor which was shown to be organic since the ash was inactive. With clear foresight Davis stated that specific body fluids and tissues because of their specific vitamin contents were invaded by specific microorganisms. Thjötta (1921) who is usually credited with differentiating the X and V factors for hemophiles believed vitamins to be the cause of growth of Bacillus influenzae on non-hemoglobin containing media. Growth was obtained on ordinary broth when extracts of mucoid bacilli or Bacillus proteus were added.

The evolutionary scale of nutrition was the theoretical and experimental concern of Loeb and Northrup (1916). They felt that as living organisms became more and more differentiated they became more and more parasitic. This was a similar concept to that of Twort and Ingram (1911) mentioned above. They were quite surprised to find that the same media which was used for the cultivation of yeast was also capable of supporting

the growth of Drosophila. Such a result would be contrary to their theory unless the bacteria present were synthesizing materials required by the fly. By use of sterile technique and mercuric chloride treatment of the eggs and larvae they claim to have gotten sterile flies which could not survive on the yeast medium. Although the experimental results are open to criticism on the grounds of the harsh treatment used, the idea that microbes produced growth factors was valid.

An imaginative application of symbiotic relationships was made by Hammerschmidt (1921). This worker found that Koch-Weeks bacillus was copiously produced in media not ordinarily supporting its growth if diphtheria organisms, staphylococci, Friedländers bacillus or pneumococci were simultaneously introduced. Dogs which were never known to be infected with Koch-Weeks conjunctivitis were made to come down with typical symptoms if pneumococci were also smeared on the eye. Ayers and Mudge (1922) found that autolyzed yeast contained growth factors for pathogenic streptococci which they decided was not vitamin B. The blood of scorbutic animals did not support the growth of Bacillus influenzae as well as the blood of slightly deficient or normal animals in studies by Kollath and Leichtentritt (1925). Curing the animal of scurvy also improved the growth of influenza bacillus on its blood. Reactivation of the blood of such animals was obtained by the addition of potato extract.

Another tributary joined the main stream of microbial growth factors when vitamin work in animals and man led Funk and Macallum (1916) to the discovery that yeast fractions had anti-beriberi activity for rats. The fractions had been obtained by precipitation with phosphotungstic acid. Pacini and Russell (1918) showed that an acidic ethanol extract of typhoid bacilli stimulated rat growth. Choice of this organism was made because of the known fact that typhoid patients might grow 1 to 7 inches in height during their illness. Further animal work on growth factors of bacterial origin was performed by Damon (1923). Dried Pfeiffer's bacillus added to a diet deficient in vitamin B kept the test animals at constant weight or even induced rapid and continuous growth. Azotobacter cells were reported by Hunter (1923) to replace water soluble vitamins in a ration for white rats and also to cure polyneuritic pigeons. Allison, Hoover and Burk (1933) suggested that the contribution of leguminous plants to Rhizobia in their symbiosis was provision of coenzyme R which was later identified as biotin. Numerous other investigations were carried out along similar lines which proved that some microorganisms at least were good sources of accessory growth substances.

Throughout the period of interest in vitamins the examination of microbial extracts for growth promoting properties in the same and other groups continued. Robertson (1924) reported that 68th generation B. coli filtrates added to yeast media

which were suboptimum allowed luxuriant growth of the yeasts. The organisms associated with Cellulomonas folia in nature were found by Sanborn (1926) to enhance the growth of that organism in vitro. In some cases the relationships were truly symbiotic and in others the Cellulomonas grew at the expense of the second culture. A meat extract medium which was repeatedly extracted with charcoal and no longer supported the growth of Streptothrix corallinus was shown by Orr-Ewing and Reader (1928b) to regain this ability after a strain of meningococcus was grown on the medium. According to Goy (1925) Amylomucor delemar contained a stimulant for yeast and microbes in general. The factor was different from vitamin B but was associated with it in many natural sources. He considered that animal vitamins were heat labile and could in this manner be distinguished from vegetable vitamins. Schopmeyer and Fulmer (1931) noticed that yeast media which became contaminated with molds gave tremendously increased growth. Several different molds were grown on sucrose or glycerol substrates and proved to be highly stimulatory for Saccharomyces cerevisiae. Two kinds of growth substances were discovered in Rhizopus suinus cultures by Nielsen and Hartelius (1932). One was ether soluble, readily destroyed by oxidation and had an effect on Avena coleoptyles but not bacteria. The other stimulated Aspergillus niger with no effect on Avena, was insoluble in ether and was not readily destroyed by oxidation. It was not determined whether these were single

substances or groups of substances. Bünning (1934) showed that materials designated as growth substances A and B from Aspergillus niger and vitamin B_1 and vitamin B_2 hastened nitrate absorption and retarded ammonia uptake in Aspergillus niger. Extracts of Phycomyces were reported by Schopfer (1934) to make an otherwise insufficient medium complete for the culture of Phycomyces. Many other molds also produced the requisite factors.

Thus far it has been seen that microorganisms are more or less dependent on external supplies of growth factors. These may be furnished by the organism itself or its filtrates, by other organisms or by plant or animal materials. Similarly, microorganisms may supply vitamins and stimulatory substances for plants and animals. The convergence of all of the preceding lines of endeavor served to emphasize the identity of metabolism of living cells regardless of the source. The idea that any growth factor found was required by all living organisms however difficult the demonstration of its essentiality might prove to be was the logical conclusion to be drawn. Essentiality in the restricted sense is meaningless and only serves to differentiate organisms with respect to their synthetic abilities.

The question as to the identity of bios began to reach a solution with the work of Fulmer, Duecker and Nelson (1924) who succeeded in separating bios into four components on the basis of ethanol precipitation. Of these fractions at least

two were suspected of being different since both together were more effective than either alone. Miller (1924) separated bios into two fractions by precipitation with barium hydroxide in alcoholic solution. Each alone had no effect on yeast but both together showed bios activity. The precipitate was designated Bios I and the filtrate factor which could be adsorbed on charcoal was called Bios II. As late as 1930 Peskett and O'Brien (1930) concentrated bios from a cotton extract and claimed that there was no evidence that it was a complex body although i-inositol had already been identified as one of the bios fractions. Following this early work a period of rapid isolation and identification of many water soluble factors took place. The story of this phase although important and interesting can be found for example in a review by Peterson (1941). This activity was then reflected in the studies on bacterial nutrition which followed providing pure compounds for the first time.

The effect of some pure vitamins on growth of yeast was studied by Williams and Saunders (1934). Inositol was found to be unimportant when used alone but along with B_1 or other vitamins it had a definite effect. Thiamin was stimulatory for only the Old Process and another yeast strain. A new factor was described which was named pantothenic acid because of its widespread distribution. Here the availability of some pure factors made possible the detection of a new stimulatory substance. This illustrated the catalytic effect

of fundamental findings on progress in a relatively new field. Crystalline vitamins also allowed further study of nutrition by Orla-Jensen, Otte and Snog-Kjaer (1936) on the lactic acid bacteria. They concluded that lactics require bios, lactoflavin and B_6 with bios further characterized as B_1, inositol and pantothenic acid. Janke and Sargo (1939) found B_1 to be necessary for Phycomyces, slightly stimulatory for Rhizopus, and inhibitory toward Aspergillus niger while yeast was unaffected. Meso inositol was active only for yeast.

In the beginning biological assays using pigeons were the only means of determining the vitamin B_1 content of various materials. Previous to a complete structure determination of a factor this is the only way in which a quantitative determination can be made. Even after characterization the biological method is usually best because of the difficulty of quantitatively handling and isolating such minute amounts of material. Williams (1919) noted that vitamins and yeast growth factors were prepared from the same sources and had very similar properties. He proposed therefore, that a single cell yeast growth test could serve as a qualitative method for identifying "vitamine" with the hope that it could probably be made quantitative with refinement of the technique. Development of the yeast assay in a quantitative manner by Eddy, Heft, Stevenson and Johnson (1921) was found to give only approximate agreement with rat feeding tests. They did recognize the basic requirements of a good method, namely

that the true vitamin content could only be found microbiologically if the basal medium were complete in all factors but the test component. Orr-Ewing and Reader (1928a) desired to get around the laborious process involved in the pigeon test for vitamin B_1. They developed a Streptothrix corallinus growth method. In completely impure extracts anomalous results were often obtained but in most cases reasonable values were procured. The method was soon invalidated by the finding of Peters, Kinnersley, Orr-Ewing and Reader (1928) that B_1 and the Streptothrix factor were not identical because the heat stabilities and other properties of their concentrates were quite different. This seemed to discourage attempts for some time to come.

As a result of comprehensive studies of the nutrition of the lactobacilli and the availability of many members of the B complex in pure form, Snell and Strong (1939) developed the first practical assay method for riboflavin. This work laid the foundation for all of the microbiological assays now used. This was the first rapid, simple and accurate method to be found. A tremendous amount of work followed the initial success and it is now possible to determine members of the B complex by microbiological methods with an error of about 10 per cent. Some references to specific methods will be made in the experimental sections below. The literature in this field is voluminous and could not and will not be covered here.

Commercially the production of vitamins by microorganisms has been carried out for many years. Yeast feeds have long been used and some of the commercial vitamin concentrates are obtained from brewer's yeast. Riboflavin production in the United States was for a time largely a by product of the butyl-acetonic fermentation. Peltier and Borchers (1947) studied the riboflavin yields from 240 mold species grown on wheat bran. They concluded that molds warranted further investigation as a commercial source of riboflavin. Enzyme free supernatant liquid after the precipitation of mold amylase were examined by Rao, Mistry and Sreenivasaya (1948) as a potential vitamin source. They reported that the content of biotin, pyridoxine, folic acid and nicotinic acid were sufficient to warrant possible utilization of this by product in the yeast fermentation.

The history of growth factors thus far has been the recognition that certain crude materials have activity for certain organisms. By means of a biological assay utilizing this activity the factors are concentrated, crystallized and then characterized. This then permits new stimulants to be recognized in natural sources and the cycle repeats itself. With respect to many new factors we are still in the crude extract stage as is to be expected in the beginning. Many unidentified factors have been described in the more recent literature some of which will probably prove to be identical and some of which may be just mixtures of known accessory

substances. In the discussion that follows only a selected number of unknown factors will be described with no attempt at comprehensiveness. Special emphasis will be given animal protein factor (APF) since some of the experimental work to be reported has been done in this field.

Kuiken, Norman, Lyman, Hale and Blotter (1943) found that tomato juice contained a factor for Lactobacillus arabinosus which could not be replaced completely by p-aminobenzoic acid. It was adsorbed by Norit A from a solution at pH 3.0 and could be eluted with a pyridine-ethanol-water mixture. The "unknown" substance stimulating yeast growth that was present in yeast extract was determined by Leonian and Lilly (1943) to be a mixture of inositol, biotin and pantothenic acid. This emphasizes the need of eliminating interference by known factors present in extracts to be assayed, which is usually accomplished by saturating the basal medium with respect to all the known factors.

Sprince and Woolley (1945) found a new growth promoting substance in tryptic digests of purified proteins and named it strepogenin. The nature of the substance was inferred to be a peptide from an examination of its properties. The activity could be estimated by a microbiological method using Lactobacillus casei. An assay for a substance believed identical with strepogenin was developed by Scott, Norris and Heuser (1946) also using Lactobacillus casei. This factor was believed to be the same as chick factor S. Peeler,

Daniel, Norris and Heuser (1949) using paper chromatography isolated l-glutamine from strepogenin concentrates. They were of the opinion that strepogenin and l-glutamine might be identical since they found the same activity for both fractions using Lactobacillus casei. This does not appear to be generally accepted to be the case, however, and the identity of strepogenin still seems uncertain. A new factor was described by Sauberlich and Baumann (1948) for Leuconostoc citrovorum. Antipernicious anemia concentrates promoted similar growth but not in proportion to the known activity of the concentrates. It was not identical with the chick factor and could be found in liver, yeast extract and peptone.

A chick growth substance was discovered by Novak, Hague and Carrick (1947) in distiller's dried solubles and condensed fish solubles. It was shown to be different from vitamins A, B_1, B_2, B_6, pantothenic acid, nicotinic acid, folic acid and other known factors. It was differentiated from the cow manure factor of Whitson, Hammond, Titus and Bird (1945) by its solubility in ether. Novak and Hague (1948a) made further studies of the properties of their new growth substance. The material was heat stable in both acid and alkaline solution and soluble in ether, ethanol and water. It was precipitated by phosphotungstic acid and lead acetate and adsorbed from acid solution on Florisil, Lloyd reagent, norit and Decalso. Fuller's earth and Darco did not adsorb it from solution. The new factor was named B_{13} after

isolation by Novak and Hague (1948b). The fractionation consisted of extraction with 0.1 normal hydrochloric acid, precipitation of protein with ethanol, chromatographic adsorption of the impurities on fullers earth, precipitation of the active material with phosphotungstic acid and extraction with chloroform. This series of papers illustrates the process involved in the early stages of purification of a new factor. Even after processing large amounts of materials and obtaining a very active concentrate, it is not always possible to get a crystalline compound.

Cow manure was suggested by Hammond (1942) as a good source of vitamins for chickens. Whitson, Hammond, Titus and Bird (1945) then found that 3 per cent sardine meal or 8 per cent dried cow manure supplied factors making soybean meal sufficient as a protein source for chicks. Wheat apparently contained this material since cow manure did not enhance the value of wheat as a feed. Sardine meal did increase the efficacy of wheat but this may have been due to larger amounts of the same factor. The fact that cow manure factor, later designated as APF, was different from the previously known stimulatory substances was demonstrated by Rubin and Bird (1946a). The manure factor caused improved growth in chicks when added to the ration whereas the addition of all known factors caused no such effect. In a subsequent paper Rubin and Bird (1946b) described the properties of APF. It

was soluble in 50 or 90 per cent alcohol, insoluble in ether or chloroform, stable one hour to dry heat or autoclaving for 15 minutes and could be dialyzed. Concentrates were made by extracting the cow manure with boiling water or 50 per cent alcohol at 50° C. Nichol, Robblee, Cravens and Elvehjem (1947) using a typical APF assay ration found that antipernicious anemia concentrates of liver were active for chicks. A fish solubles material of similar activity was prepared which was soluble in 70 per cent methanol but precipitated in 95 per cent ethanol. The activity of these extracts was greater if injected than if given orally. Lactobacillus lactis Dorner was found by Shorb (1947) to require a factor from tomato juice and another which was present in antipernicious anemia concentrates. The response of the organism seemed to be directly related to anti anemia activity and this substance was designated as Lactobacillus lactis Dorner (LLD) factor. Here again is a concrete example of the merging of factors for animals and bacteria. The same type materials are required by both groups of organisms and since microorganisms are so much easier to handle giving faster assays, they are preferred in isolating or quantitatively determining factors whose primary economic importance may be centered on animals.

A report on the chick growth factor in condensed fish solubles was made by Robblee, Nichol, Cravens, Elvehjem and Halpin (1948). It was soluble in water, 70 per cent ethanol

or methanol but insoluble in ether or acetone and was strongly adsorbed on charcoal. The concentrated material was acid and alkali sensitive with no loss on enzymic digestion and it could be dialyzed through cellophane. The latter property distinguished it from cow manure factor but this difference may only have been in the mode of combination in the two sources. Using a completely synthetic basal medium, Daniel, Scott, Heuser and Norris (1948) had to supply two different factors to get good growth of Lactobacillus casei. Strepogenin and APF were the required substances. They concluded that APF and antipernicious anemia factor were not alike since Lactobacillus casei did not respond equally to both concentrates. Animal protein factor was adsorbed from acid solution by norit. Bird, Rubin and Groschke (1948) told further of the properties of their cow manure factor. It was soluble in 80 per cent acetone or ammoniacal alcohol and stable to autoclaving at neutral pH for 2 hours. There must have been a protein combined form of the factor because it was no longer precipitated at pH 3.0 after papain digestion whereas before digestion such precipitation did take place. The activity was found to be incompletely adsorbed from the papain digest and incompletely eluted by ammoniacal ethanol. Zucker, Zucker, Babcock and Hollister (1948) described a factor for weaned rats which was present in crude casein, liver extract and fish solubles. Successful production of a deficiency

depended on depleting the parent in order to get results in the offspring. Symptoms were high mortality in the young although pregnancy and lactation in the mother were normal. High protein in the diet accentuated the difficulty. The authors felt that their substance which was called zoopherin was identical with APF. Such was the status of APF in the early stages of work. Evidence from several fields pointed to the presence of a new growth substance or group of similar substances effective in animals and certain microorganisms.

Liver fractionation by Smith (1948) resulted in the isolation of two rather pure substances having red pigmentation. One could be derived from the other by proteolytic digestion. Four tons of ox liver were processed to produce one gram of final product. The latter was soluble in water and in nearly anhydrous alcohol, acetone and acetic acid. Non-polar solvents were not able to dissolve the material. Precipitates were obtained from a solution of the factor when Reinecke acid, rhodanilic acid or phosphotungstic acid were added. Boiling for one hour in normal hydrochloric acid rendered the pigment soluble in butanol. Shortly after this, crystalline material was obtained. Independently and at the same time Rickes, Brink, Koniuszy, Wood and Folkers (1948a) announced the isolation of crystalline vitamin B_{12}. Very few details other than some physical properties were given. The same group of Rickes, Brink, Koniuszy, Wood and Folkers

(1948b) reported some further findings on the nature of vitamin B_{12}. Cobalt, nitrogen and phosphorous were detected in the crystals. Lactobacillus lactis Dorner was not responsive to cobaltous ion but crystalline B_{12} was very active. The conclusion was that vitamin B_{12} is a cobalt coordination complex containing six groups one or more of which is organic. Rickes, Brink, Koniuszy, Wood and Folkers (1948c) isolated crystals from Streptomyces griseus that compared in all ways with those obtained from liver and designated as vitamin B_{12}. More data on the properties of vitamin B_{12} was given by Brink, Wolf, Kaczka, Rickes, Koniuszy, Wood and Folkers (1949). An approximate molecular formula was calculated from the quantitative results. Vitamin B_{12} was found to be an optically active weak polyacidic base and no amino acids or pteridine components could be detected. Pyrrole and porphyrin tests were positive and the crystalline material reacted like known pyrroles when degraded by sodium hydroxide fusion and tested with Ehrlich reagent.

Crystalline vitamin B_{12} was added to a soybean meal diet for chicks by Ott, Rickes and Wood (1948) and it exhibited animal protein factor activity. Six to thirty micrograms per kilo of ration were needed to produce satisfactory growth. On the basis of this evidence it was decided that vitamin B_{12} was APF or closely related to it. Lillie, Denton and Bird (1948) found that crystalline B_{12} was completely effective in replacing cow manure, fish meal or meat scraps. The action

in the chicks was direct since injection gave the same results as feeding.

Microbiological aspects of APF have been mentioned. The cited need of Lactobacillus lactis Dorner for B_{12} was made the basis of an assay. Difficulties were encountered with this method, however. Dissociation was thought to be the reason for the erratic non-reproducible results. Shorb and Briggs (1948) found that the use of ordinary laboratory carrying media induced such cultural instability. The addition of skim milk and tomato juice tended to stabilize the culture. Skeggs, Huff, Wright and Bosshardt (1948) used Lactobacillus leichmannii for the assay of APF and described the medium and methods used. No sooner did some of the difficulties in the assay disappear than new ones arose. Hoffman, Stokstad, Franklin and Jukes (1948) found that Lactobacillus leichmannii also responded to thymidine. The presence in extracts of this desoxyriboside would interfere with determinations of B_{12}. Apparently other materials also interfered since five different zones were found on strip chromatograms by Winsten and Eigen (1949). Two of the zones were identified as vitamin B_{12} and thymidine, respectively. Possible characterization of the unknown components of the B_{12} group was indicated by the work of Kitay, McNutt and Snell (1949). They found that thymine, adenine, cytosine and hypoxanthine desoxyribosides as well as desoxyribonucleic acid and ascorbic acid were all effective in promoting growth of

Lactobacillus leichmannii 313.

Speculation as to the significance of the multiple stimulation observed resulted in the general belief that B_{12} was a cofactor involved in the synthesis of nucleic acids or their components. Tomarelli, Norris and György (1949) found that B_{12} could not replace the desoxyriboside requirement of Lactobacillus bifidus. This then casts doubt on the proposed mode of action of vitamin B_{12}. To say the least the true status of these factors is still very much in doubt. The relationships between B_{12}, desoxyribosides and APF are similarly not clear. In view of the additional presence of vitamin B_{13} in the whole picture it seems possible that animal protein factor activity is really a group activity even though a good deal of it may be accounted for by the action of vitamin B_{12}.

III. MATERIALS

A. Microbial Preparations

1. Mold bran

Four samples designated as #7, 8, 9 and 10 of mold bran, which is a culture of Aspergillus oryzae grown on wheat bran as a substrate, were obtained through the courtesy of Enzymes Inc., Eagle Grove, Iowa.

2. Bran

Wheat bran which is the solid substrate used in the production of mold bran was made available by Enzymes Inc., Eagle Grove, Iowa.

3. Feed concentrate No. 44

Adequate supplies of a feed concentrate designated as No. 44 (hereafter referred to as #44) were also supplied by the courtesy of Enzymes Inc., Eagle Grove, Iowa. This material was prepared by culture of a selected strain of aerobic spore forming bacteria on a medium of undisclosed composition containing soybean cake, alfalfa meal and other ingredients. No. 44 was reported to completely replace meat scraps and fish meal in poultry rations according to chick

feeding tests.

B. Organisms

The following organisms were utilized in this investigation: Lactobacillus casei (American Type Culture Collection Number 7469); Lactobacillus arabinosus 17/5 (American Type Culture Collection Number 8014); Lactobacillus fermentum 36 (American Type Culture Collection Number 9338); Saccharomyces cerevisiae (Gebrüder Mayer strain); Lactobacillus leichmannii (American Type Culture Collection Number 4797); and Lactobacillus lactis Dorner.

C. Chemicals

1. Constituents of the synthetic media

Dextrose of C. P. grade from Pfanstiehl Chemical Company, Waukegan, Illinois was used. Amino acids used were those of Merck and Company, Rahway, New Jersey and Dow Chemical Corporation, Midland, Michigan. The acids from Dow were supplied through the courtesy of Professor S. W. Fox of Iowa State College and were recrystallized from boiling water before use. The purines and vitamins used were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

2. Enzymes

Papain and trypsin were supplied by Nutritional Biochemicals Corporation, Cleveland, Ohio. Takadiastase was obtained from Parke, Davis and Company, Detroit, Michigan.

3. Special nutrients

Tween 80 was donated by Atlas Powder Company, Wilmington, Delaware. Difco Bacto-Peptone, Difco Bacto Yeast Extract and Difco Bacto Peptonized Milk were obtained from Difco Laboratories Inc., Detroit, Michigan. Edible grade purified and dried whey was donated by Kraft Foods Company Research Laboratories, Glenview, Illinois. Labco Vitamin Free Casein was supplied by Casein Company of America, New York, New York. Canned tomato juice was purchased on the open market as required.

D. Apparatus

A KWSZ photocell filter photometer distributed by Wilkens-Anderson Company, Chicago, Illinois was used for turbidity measurements. Filter No. 7 having a wavelength of 650 millimicrons was used. Kimble lipless 19 by 150 mm. test tubes were used in the fermentations.

IV. METHODS

A. Amino Acid Mixture

The following amino acids in the designated quantities were dissolved in 500 ml. of boiling water: 400 mg. dl leucine, 400 mg. dl isoleucine, 400 mg. dl valine, 400 mg. dl methionine, 400 mg. l(-) tyrosine, 400 mg. phenylalanine, 800 mg. glutamic acid, 400 mg. dl threonine, 400 mg. dl alanine, 800 mg. aspartic acid, 400 mg. l(+) lysine, 400 mg. l(+) arginine, 400 mg. l(+) histidine and 400 mg. dl serine. Four hundred mg. l(-) cystine was dissolved separately by using a minimum amount of conc. HCl and added to the above solution and 400 mg. of dl tryptophan was treated in the same manner. The solution was made to a volume of 720 ml. and stored in the refrigerator under toluene.

B. Vitamin Mixture

A solution of 25 ug. of biotin was made to a volume of 200 ml. Ten mg. of calcium pantothenate was dissolved in 50 ml. of neutral 25 per cent neutral ethanol. Ten mg. of p-aminobenzoic acid was dissolved in 50 ml. of 25 per cent neutral ethanol. Twenty mg. of pyridoxine was dissolved in 50 ml. neutral 25 per cent ethyl alcohol. Twenty mg. of pyridoxal was dissolved in 50 ml. neutral 25 per cent alcohol. Ten mg. vitamin B_1 was dissolved

in 50 ml. alcohol which had been adjusted to pH 3.0 with HCl. Ten mg. of riboflavin was dissolved in 200 ml. of 0.02 N acetic acid. Ten mg. of nicotinic acid was dissolved in 100 ml. of distilled water. All of the separate solutions were combined and 5 mg. of folic acid dissolved in the mixture. The solution was stored under toluene in the refrigerator.

C. Purines

One hundred mg. of uracil, 124 mg. of guanine hydrochloride, 174 mg. of adenine sulfate and 100 mg. of xanthine were suspended in a small volume of water. Two ml. of conc. HCl was added and the mixture heated until solution took place. The volume of the solution was made to 100 ml. and it was stored under toluene.

D. Dische Reagent

Three grams of diphenylamine was dissolved with stirring in 6 ml. of concentrated sulfuric acid and then 294 ml. of glacial acetic acid was added. The solution was stored in a dark bottle and kept in a dark place.

E. Preparation of Assay Inocula

Stock cultures of the organisms used were maintained on the appropriate nutrient agar; stabs for lactobacilli and

slants for yeast. Inoculation was made from these cultures to an appropriate liquid medium and incubated until the organism was in its logarithmic growth phase. The tube was then centrifuged and the supernatant poured off with due regard for sterile technique. The precipitated cells were resuspended in sterile physiological saline and again centrifuged. The cells were washed a total of two times and the suspension then diluted 10 to 20 times. One drop of the final suspension was used for inoculating each test tube culture.

F. Final Determinations

The end point of the test tube assays was read either by titration of the lactic acid produced with bromthymol blue indicator or by turbidity measurements.

V. EXPERIMENTAL

A. Known Factors

1. Riboflavin

The method and medium used were those of Barton-Wright (1945). One g. each of mold bran samples #7, 8, 9 and 10 and of bran were placed in 250 ml. Erlenmeyer flasks and 40 ml. of 0.1N HCl added to each. The flasks were autoclaved for 30 minutes at 15 lb. steam pressure, cooled, the solutions adjusted to pH 5.8 with 0.5N NaOH and then filtered. The pH was reduced to 4.5 and the mixtures again filtered. The pH was adjusted to 6.7 with 1N NaOH and made up to 500 ml. From each solution duplicate amounts of 1, 2 and 5 ml. were pipetted into test tubes, made to a total of 5 ml. with distilled water and 5 ml. of double strength basal medium added. Duplicate standards containing 0, 0.03, 0.06, 0.12, 0.18, 0.24 and 0.30 µg. of riboflavin were prepared in analogous manner to the samples. All of the tubes were plugged with cotton, autoclaved for 10 minutes at 10 lb. steam pressure and cooled quickly with protection from the light. One drop of a dilute suspension of Lactobacillus casei was added to each tube and the series was incubated at 37° C. for 72 hours after which the acid produced was

titrated with 0.1N NaOH. Two runs had to be made since the original bran solutions were too dilute. Data for the establishment of the standard curves are given in Table 1.

Table 1

Titration Values for Riboflavin Standards

Run	Riboflavin (μg.)	ml. 0.1N NaOH (average)
I-1	0.00	0.64
I-2	0.03	1.51
I-3	0.06	2.44
I-4	0.12	4.80
I-5	0.18	6.30
I-6	0.24	6.91
I-7	0.30	7.58
II-1	0.00	1.39
II-2	0.03	2.34
II-3	0.06	2.79
II-4	0.12	4.12

Riboflavin values were plotted against ml. NaOH on fine line graph paper and the curve obtained was of typical shape. The assay values for the samples were calculated by referring the titers to the standard curve and multiplying by the proper dilution. The final results are shown in Table 2.

Table 2

Riboflavin Content of Samples

Sample	ml. 0.1N NaOH (average)	Curve reading (µg.)	Dilution factor	µg. Riboflavin per g.
Bran	2.69	0.048	100	4.8
#7	3.11	0.078	250	19.5
#8	2.97	0.074	250	18.5
#9	2.94	0.073	250	18.2
#10	3.96	0.100	250	25.0

2. Nicotinic acid

The method and medium were those of Barton-Wright (1945). One g. each of samples #7, 8, 9, 10 and wheat bran were autoclaved for 20 minutes at 15 lb. pressure with 50 ml. 1N HCl in 250 ml. Erlenmeyer flasks. After cooling, the solutions were adjusted to pH 6.7, filtered and made up to 1 liter of which 50 ml. was further diluted to 100 ml. Duplicate amounts of 1, 2 and 5 ml. of the solutions were pipetted into test tubes and sufficient distilled water was added to make a total of 5 ml. per tube. Five ml. of double strength basal medium was then added to each. Standards in duplicate containing 0, 0.20, 0.40, 0.60, 0.70 and 0.80 µg. of nicotinic acid per tube were prepared similarly. All of the tubes were plugged, autoclaved 15 minutes at 15 lb. pressure and cooled.

They were inoculated with Lactobacillus arabinosus and incubated for 72 hours at 30° C. following which the lactic acid produced was titrated with 0.1N NaOH using bromthymol blue indicator. The results obtained for the standards appear in Table 3. Utilizing this data a standard curve was

Table 3

Titration Values for Nicotinic Acid Standards

Run	Nicotinic Acid (ug.)	ml. 0.1N NaOH (average)
1	0.00	1.28
2	0.20	4.59
3	0.40	6.88
4	0.60	8.62
5	0.70	9.30
6	0.80	9.73

drawn and the usual type curve was obtained. By reference of the titers of the samples to the curve and taking the dilution into account, the values of nicotinic acid present in the samples were calculated. The final results are shown in Table 4.

Table 4

Nicotinic Acid Content of Samples

Sample	ml. 0.1N NaOH (average)	Curve Reading (µg.)	Dilution Factor	µg. Niacin per g.
Bran	8.60	0.60	500	300
#7	7.27	0.45	250	112
#8	7.25	0.44	250	110
#9	5.05	0.23	500	115
#10	5.25	0.24	500	120

3. Biotin

The method and medium used were those of Barton-Wright (1945). Samples were prepared by autoclaving one gram of #7, 8, 9, 10 and bran in 250 ml. Erlenmeyer flasks with 50 ml. of 1N sulfuric acid for 2 hours at 15 lb. pressure. Upon cooling the solutions were adjusted to pH 6.7, filtered, diluted to 500 ml. and then 10 ml. was further diluted to 100 ml. Duplicate tubes received 1, 2 and 5 ml. of this solution after which each was brought to a total of 5 ml. and 5 ml. of double strength basal medium was added. In like manner standards containing 0, 0.10, 0.20, 0.40 and 0.80 millimicrograms of biotin were prepared. The tubes were plugged with cotton, sterilized at 15 lb. pressure for 15 minutes and cooled. A drop of inoculum of Lactobacillus arabinosus was

added to each tube and the series was incubated for 72 hours at 37° C. Upon completion of the incubation period, the tubes were titrated with 0.1N NaOH using bromthymol blue as indicator. The data for the standard series is presented in Table 5. From this information a standard curve of typical

Table 5

Titration Values for Biotin Standards

Run	millimicrograms Biotin	ml. 0.1N NaOH (average)
1	0.00	1.92
2	0.10	5.26
3	0.20	7.13
4	0.40	9.25
5	0.80	11.60

shape was drawn on fine line graph paper and this curve then used to determine the assay values for the samples tested. These values are recorded in Table 6.

Table 6

Biotin Content of Samples

Sample	ml. 0.1N NaOH (average)	Curve reading (µg.)	Dilution factor	µg. Biotin per g.
Bran	4.86	70	5000	0.35
#7	5.20	84	5000	0.42
#8	5.70	108	5000	0.54
#9	6.42	152	5000	0.76
#10	6.05	126	5000	0.63

4. Pantothenic acid

The method and medium used were those of Barton-Wright (1945). One g. each of mold bran #7, 8, 9 and 10 and of bran in a finely ground state was digested at 37° C. with 25 mg. papain and 25 mg. takadiastase in 40 ml. of 0.5 per cent acetate buffer under toluene for 24 hours. The digest was adjusted to pH 6.7, filtered and diluted to 1 liter. In duplicate tubes 0.5, 1 and 2 ml. of bran solution and 2, 3 and 5 ml. of the mold bran solutions were made to a total of 5 ml. with distilled water and 5 ml. of the double strength basal medium added. A series of standards containing 0, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.10 µg. of calcium pantothenate was set up in the same manner as the samples. The tubes were fitted with cotton plugs, autoclaved 15 minutes at 15 lb.

steam pressure and cooled. A drop of inoculum of Lactobacillus arabinosus was added to each of the tubes which were then incubated at 30° C. for 72 hours. At the end of this time the lactic acid was titrated with 0.1N NaOH using bromthymol blue as indicator. The results for the standard series are shown in Table 7. A smooth curve was drawn for these values having the usual shape. Using the titration values for the samples, the standard curve and the dilution, the pantothenic acid contents expressed in terms of calcium pantothenate were obtained. This information is given in Table 8.

Table 7

Titration Values for Calcium Pantothenate Standards

Run	Calcium Pantothenate (µg.)	ml. 0.1N NaOH (average)
1	0.00	1.60
2	0.01	3.15
3	0.02	5.10
4	0.04	7.43
5	0.06	8.98
6	0.08	9.80
7	0.10	10.90

Table 8

Pantothenic Acid Content (as Calcium Pantothenate) of Samples

Sample	ml. 0.1N NaOH (average)	Curve Reading (µg.)	Dilution Factor	µg. Calcium Pantothenate per g.
Bran	6.80	0.033	2000	66
#7	6.50	0.031	500	15
#8	5.42	0.022	500	11
#9	4.60	0.017	500	8.5
#10	6.35	0.029	500	15

5. Thiamin

The method and medium used in this determination were those of Sarett and Cheldelin (1944). One g. each of mold bran #7, 8, 9 and 10 and of bran in a finely ground state was digested at 37° C. for 24 hours with 25 mg. papain and 25 mg. takadiastase in 40 ml. 0.5% acetate buffer. After digestion the flasks were steamed 15 minutes, filtered, adjusted to pH 6.7 and made up to 1 liter of which 20 ml. was made up to 100 ml. Duplicate tubes containing 0.5, 2 and 5 ml. of the prepared solutions were set in a rack, enough distilled water added to make the total volume in each 5 ml. and 5 ml. of double strength basal medium was added. Duplicate standards of 0, 0.005, 0.010 and 0.020 µg. of thiamin hydrochloride were similarly made up to a total of 5 ml. each and 5 ml. of

double strength basal medium added. All of the tubes were then plugged, steamed 15 minutes, allowed to cool and inoculated with 1 drop of a dilute suspension of Lactobacillus fermentum. They were incubated at 30° C. for 23 hours at the end of which time the turbidities were measured in the KWSZ photometer. The values obtained are given in Table 9. These figures were plotted to produce the typical standard curve which was then used to calculate the assay values for the samples. The final results are shown in Table 10.

Table 9

Turbidity Values for Thiamin Standards

Run	Thiamin (µg.)	Photometer Reading (average)
1	0.00	100
2	0.005	91.4
3	0.01	89.0
4	0.02	84.4

Table 10

Thiamin Content (as Hydrochloride) of Samples

Sample	Photometer Reading (average)	Curve Reading (µg.)	Dilution Factor	µg. Thiamin per g.
Bran	91.6	0.0047	1000	4.7
#7	92.4	0.0037	1000	3.7
#8	93.1	0.0030	1000	3.0
#9	93.3	0.0028	1000	2.8
#10	94.0	0.0022	1000	2.2

6. Folic acid

The procedure used has been that of Tepley and Elvehjem (1945). One g. samples of mold bran #7, 8, 9 and 10 and of bran in a finely ground state were digested for 24 hours in 250 ml. Erlenmeyer flasks at 37° C. with 25 mg. papain and 25 mg. takadiastase in 40 ml. of 0.5 per cent acetate buffer. The solutions were adjusted to pH 6.7, made up to 1 liter and 20 ml. further diluted to 100 ml. Duplicate amounts of 1, 2 and 3 ml. of the solutions were made to 5 ml. total volume in test tubes to which 5 ml. of double strength basal medium was added. Standards, also in duplicate, were prepared in an analogous manner containing 0, 0.50, 0.75, 1.0, 1.5, 2.0 and 3.0 millimicrograms of folic acid per tube. All were then sterilized for 15 minutes at 15 lb. steam pressure and allowed

to cool. One drop of an inoculum of Lactobacillus casei was added to each tube and the whole set incubated at 37° C. for 72 hours at the end of which time the lactic acid formed was titrated with 0.1N NaOH using bromthymol blue indicator. The results for the standards are given in Table 11. From this information a standard curve was drawn and used together with sample titers and dilutions to calculate the folic acid content of the samples. The final results are shown in Table 12.

Table 11

Titration Values for Folic Acid Standards

Run	millimicrograms folic acid	ml. 0.1N NaOH (average)
1	0.00	1.85
2	0.50	4.65
3	0.75	6.00
4	1.00	6.70
5	1.50	7.50
6	2.00	9.30
7	3.00	10.70

Table 12

Folic Acid Content of Samples

Sample	ml. 0.1N NaOH (average)	Curve Reading (µg.)	Dilution Factor	µg. folic acid per g.
Bran	3.80	0.32	1667	0.53
#7	4.62	0.48	5000	2.40
#8	5.00	0.58	2500	1.45
#9	4.00	0.34	1667	0.57
#10	5.27	0.65	2500	1.60

7. Pyridoxine

The method and medium used were those of Williams, Eakin and McMahan (1941). Samples were prepared by hydrolyzing 1 g. each of mold bran #7, 8, 9 and 10 and bran in 250 ml. Erlenmeyer flasks with 80 ml. of 0.44N sulfuric acid for 1 hour at 20 lb. steam pressure. Upon cooling, the solutions were adjusted to pH 5.0, diluted to 1 liter and a 2 ml. aliquot further diluted to 100 ml. Duplicate amounts of 1, 2 and 5 ml. of the solutions were brought to a total volume of 5 ml. in test tubes and then 5 ml. of double strength basal medium was added to each. In the same way duplicate standards of 0, 0.10, 0.20 and 0.40 millimicrograms of pyridoxine hydrochloride were prepared. The complete set was then sterilized by steaming for 15 minutes, allowed to cool and then each

tube was inoculated with 1 drop of a suspension of Saccharomyces cerevisiae. The tubes were incubated for 16 hours at 30° C. and at the conclusion of this time the turbidities were read in the KWSZ photometer. The readings for the standard series are listed in Table 13. The smooth curve obtained by plotting the standard values was typical and was used along with the titers of the samples and their dilutions to calculate the final assay figures. The results of the calculations are shown in Table 14.

Table 13

Turbidities of Pyridoxine Standards

Run	millimicrograms pyridoxine	Photometer Reading (average)
1	0.00	100
2	0.10	93.9
3	0.20	90.3
4	0.40	87.6

Table 14

Pyridoxine Content (as Hydrochloride) of Samples

Sample	Photometer Reading (average)	Curve Reading (µg.)	Dilution Factor	µg. pyridoxine per g.
Bran	92.0	0.14	25,000	3.6
#7	92.3	0.13	5,000	0.67
#8	93.8	0.098	2,500	0.25
#9	90.6	0.19	2,500	0.47
#10	90.1	0.21	2,500	0.53

8. Inositol

The procedure and medium used were those of Williams, Stout, Mitchell and McMahan (1941). One g. of mold bran #7, 8, 9 and 10 and bran was placed in separate 250 Erlenmeyer flasks and 50 ml. of 1N sulfuric acid was added to each. The flasks were plugged, covered with small beakers and placed in the autoclave for 30 minutes at 15 lb. pressure. The solutions were then diluted to 1 liter and of this 50 ml. was made up to 1 liter. Aliquots amounting to 1, 2 and 5 ml. of the final dilutions were placed in duplicate test tubes, each made to a total of 5 ml. and 5 ml. of double strength basal medium added. The same general procedure was employed in making up a standard series containing 0, 0.40, 0.80, 1.20, 1.60 and 2.00 µg. of inositol. The tubes were plugged and sterilized by treatment

at 15 lb. steam pressure for 15 minutes after which they were allowed to cool. Inoculation was made by adding a drop of a suspension of Saccharomyces cerevisiae to each tube and incubation at 30° C. was carried out for 16 hours. The turbidities were read in the photometer and the standard values are shown in Table 15. A typical growth curve was obtained upon plotting the standard data and it was used to calculate the inositol content of the samples from the sample titers and dilutions. The final results are presented in Table 16.

Table 15

Turbidities of Inositol Standards

Run	Inositol (ug.)	Photometer Reading (average)
1	0.00	100
2	0.20	90.0
3	0.40	86.5
4	0.80	81.4
5	1.20	78.0
6	1.60	75.5
7	2.00	74.4

Table 16

Inositol Content of Samples

Sample	Photometer Reading (average)	Curve Reading (µg.)	Dilution Factor	µg. Inositol per g.
Bran	77.2	1.28	200	256
#7	89.7	0.22	100	22
#8	77.2	1.28	100	128
#9	81.8	0.75	250	188
#10	82.7	0.66	250	165

9. Composite growth effects

In order to determine the composite growth promoting effect of the preparations chosen for study, test tube fermentations were conducted on their extracts as the sole source of accessory growth factors.

Extracts were prepared by digesting 1 g. each of wheat bran, mold bran #8 and feed concentrate #4 with 25 mg. papain and 25 mg. takadiastase in about 50 ml. 0.5 per cent acetate buffer at 37° C. for 24 hours. The extracts were then adjusted to pH 6.8, made to 100 ml., filtered and 5 ml. of each added to 5 ml. of 2 per cent glucose solution in duplicate test tubes. Three comparative sets of tubes containing 1 per cent yeast extract (Difco), 0.5 per cent yeast extract and a synthetic nutrient solution were prepared exactly as

the samples. The composition of the double strength synthetic basal medium used in this run and the following runs employing Lactobacillus arabinosus or Lactobacillus casei is shown in Table 17. The test tubes were sterilized for 15 minutes at

Table 17

Medium for Lactobacillus casei or Lactobacillus arabinosus

Component	Quantity
acid hydrolyzed casein	20 g.
glucose	20 g.
sodium acetate	12 g.
sodium chloride	10 g.
ammonium sulfate	6 g.
dl tryptophane	200 mg.
l cystine	400 mg.
adenine, guanine, xanthine and uracil	20 mg.
biotin	0.8 µg.
calcium pantothenate	200 µg.
thiamin	200 µg.
riboflavin	400 µg.
pyridoxine	200 µg.
p-aminobenzoic acid	200 µg.
nicotinic acid	400 µg.
salts A* and salts B**, each	10 ml.
Adjust to pH 6.7 and dilute to 1 liter	

*salts A: 25 g. KH_2PO_4, 25 g. K_2HPO_4 in 250 ml. distilled water

**salts B: 10 gm. $MgSO_4 \cdot 7H_2O$, 0.5 g. $MnSO_4 \cdot 4H_2O$, 0.1 g. $FeCl_3$ in 250 ml. distilled water.

15 lb. pressure, cooled and inoculated with a drop of a suspension of Lactobacillus arabinosus. After incubating for 16 hours at 30° C. the visual order of decreasing growth was found to be: synthetic, #8, bran, #44, 1 per cent yeast extract, 0.5 per cent yeast extract and blank. Titration of the lactic acid after 72 hours' incubation followed the same relative order and the values are shown in Table 18. The poor showing of yeast extract was attributed to the lack of minerals since salts B (Mn, Fe and Mg) brought the titer of yeast extract up to the same level as the complete synthetic in another experiment.

Table 18

Extracts as Sole Source of Growth Factors

Material Tested	ml. 0.1N NaOH (average)
complete synthetic	11.10
#8 extract	7.95
bran extract	5.60
#44 extract	3.80
1% exast extract	2.05
0.5% yeast extract	0.90
blank	0.25

The significance of these findings is very limited since there were so many uncontrolled variables. Each of the

extracts contained varying amounts of several vitamins. A poor over-all growth effect may have been due to lack of only one substance and again it may have been due to lack of several factors. Since Lactobacillus arabinosus is a fairly fastidious organism failure in a fermentation test using this organism would not necessarily give any information as to the growth promoting properties of the material tested in a different fermentation. In order to determine the composite growth effect of an extract on any particular microorganism the extract in question would have to be tested directly with that microorganism.

B. Unknown Factors

1. Studies using Lactobacillus casei or Lactobacillus arabinosus

It was necessary to test the extracts used above in a medium containing all the known growth factors in order to determine if any new factors were present that were required by lactobacilli. Greater growth in the extract tubes than in the complete synthetic medium as a blank would indicate the presence of new factors.

Extracts of mold bran #8 and feed concentrate #44 were prepared in three different ways: digestion with takadiastase, digestion with papain and takadiastase and distilled water extraction in a Whizmix. The final solutions in each case

were made up so that 100 ml. of solution represented 1 g. of dry material. Five ml. of basal medium (Table 17) and 5 ml. of each extract were pipetted in duplicate into test tubes. The blank consisted of 5 ml. of basal medium and 5 ml. of distilled water. The tubes were sterilized, cooled, inoculated with 1 drop of a suspension of Lactobacillus arabinosus and incubated at 30° C. In 17 hours there was a definite difference in the growths attained and the visual order of decreasing growth was: 1 per cent yeast extract, #44 (takadiastase), #44 (takadiastase and papain), #44 (distilled water), #8 (takadiastase and papain), #8 (takadiastase), #8 (distilled water) and the blank. At the end of 43 hours the acid production in all the tubes was nearly the same. The evidence obtained was that some growth factor or factors were present in the extracts which stimulated the growth of Lactobacillus arabinosus in its early stages. Since the 72 hour values were about equal for all tubes including the blank it seemed likely that the factor or factors were slowly synthesized by the organism.

Distilled water extracts of bran, #8 and #44 were made in a Whizmix so that 1 g. was equivalent to 100 ml. of extract solution. Five ml. of each extract was added in duplicate to 5 ml. of basal medium in test tubes. A blank and 1 per cent yeast extract were run for comparison. The tubes were sterilized, cooled and inoculated with Lactobacillus casei. After incubation for 27 hours the data in Table 19 were obtained.

Table 19

Effect of Extracts on Growth of *L. casei*

Material Tested	ml. 0.1N NaOH (average)
1% yeast extract	7.10
#8 extract	2.40
#44 extract	4.85
bran extract	2.75
blank	0.60

At the end of 72 hours the growth appeared the same in all of the tubes. This supported the findings of the previous run.

The previous experiments indicated the possible presence of factors in the extracts which stimulated lactobacilli in the early growth period. In order to check the identity of these factors with two described by Daniel, Scott, Norris and Heuser (1948) it was first necessary to obtain standards for comparison. One of their preparations was a charcoal treated tryptic digest of casein, considered to be identical with strepogenin. The other was an alcoholic extract of dried whey treated with permanganate, considered to be similar to or identical with APF. Since dried whey was not readily available and also since it was known that peptone was a good source of microbial growth factors, this material was first used to prepare both the fractions described. The directions

in the reference cited were used except for the mentioned substitution of starting material.

The activity of the extracts was determined using Lactobacillus casei. Amounts of 0.2 ml. of each of the extracts, singly and combined, were made to a total of 5 ml. in duplicate test tubes and 5 ml. of basal medium added. The tubes were plugged, autoclaved 15 minutes at 15 lb., cooled and inoculated. After an incubation of 40 hours the acid was titrated and the results obtained are shown in Table 20. For the sake of convenience the tryptic digest will be called extract A and the alcoholic extract, extract B. Repetition

Table 20

Effect of Concentrates on Growth of L. casei

Material tested	ml. 0.1N NaOH (average)
0.2 ml. extract A	5.88
0.2 ml. extract B	8.62
0.2 ml. each, extract A and extract B	9.05
Blank	5.83

of the same series using double the amounts of the extracts gave the same results when growth was determined photometrically. Further study of the activity of the extracts using still larger amounts led to the results shown in Table 21.

Table 21

Further Effect of Concentrates on Growth of L. casei

Material tested	Photometer reading (average)
0.5 ml. extract A	87.8
1 ml. extract A	76.6
2 ml. extract A	66.1
0.5 ml. extract B	93.3
1 ml. extract B	92.9
2 ml. extract B	95.0
2 ml. extract A and 1 ml. extract B	57.3
Blank	100.0

From these experiments it was concluded that there was a definite stimulation of growth when both extracts were used together. Extract A may have been contaminated with some of the factor present in extract B since amounts of extract A of 0.4 ml. and larger were highly stimulatory for L. casei even when extract B was not added. Another possible explanation for these results was that the medium itself supplied the contaminating factor. The only component of the medium that was not chemically defined and therefore a likely source of contamination was the casein hydrolyzate. Since the purpose of the hydrolyzate was to satisfy the amino acid requirement of the organisms, it was replaced by a synthetic amino acid

mixture the composition of which is given in the section on methods (page 30).

Repeated purification of the extracts did not substantially reduce the interfering substances. New extracts were therefore made using vitamin free casein and whey concentrate following the procedure of Scott, Norris and Heuser (1946) and Daniel, Scott, Norris and Heuser (1948). After several purifications of the new extracts the activities were tested with Lactobacillus casei in the usual manner. The data in Table 22 were obtained from the test fermentations. The

Table 22

Effect of New Extracts on Growth of L. casei

Material tested	Photometer reading (average)
0.6 ml. extract D	49.5
0.6 ml. extract C	81.0
0.6 ml. each, extract C and extract D	58.5
1% yeast extract	76.3
Blank	99.0

alcoholic whey extract was designated as extract C and the trypsin digest of casein as extract D. Examination of the results revealed the presence of an inhibitor in extract C since extract C added to extract D produced less growth than extract D alone. It was thought probable that some

intermediate oxidation product resulting from the permanganate treatment was the cause of the observed inhibition. To see if the inhibitor might not be destroyed by a mild treatment, while preserving the growth factor, extract C was autoclaved for 1 hour at 15 lb. The extracts were then tested with L. casei and L. arabinosus in the usual way and the results obtained are presented in Table 23. Some interesting facts

Table 23

Effect of Extracts on Growth of Two Lactobacilli

Material tested	Photometer Reading (average)	
	L. arabinosus	L. casei
0.6 ml. extract D	85.1	77.3
0.6 ml. extract C	54.5	74.7
0.6 ml. each, extract C and extract D	49.3	42.7
1% yeast extract	14.0	78.0
Blank	97.5	100.0

were revealed by an examination of the data. L. arabinosus grew much better in the medium containing extract C than it did in the one containing extract D whereas L. casei which is the assay organism for strepogenin produced about the same growth in both media. This would lead to the conclusion that L. arabinosus does not require an external source of strepogenin since this is considered to be the factor present in extract D. The autoclaving apparently destroyed the

inhibitor since extract C and extract D together produced better growth than extract D alone.

A review of the work done with Lactobacillus casei and Lactobacillus arabinosus up to this point disclosed that there was evidence that at least two factors could be partially fractionated using these organisms. The separation accomplished in this way was by no means clear cut or even satisfactory and the probability of developing a microbiological assay for animal protein factor (APF) using either of these organisms was not good. What was really required for the job was an organism much more fastidious than these two which would not grow at all in the absence of APF.

2. Studies using Lactobacillus leichmannii

The stab culture of Lactobacillus leichmannii received from the American Type Culture Collection gave evidence of very poor growth. Attempts to transfer to yeast extract medium which was used to carry the previously studied lactic organisms were unsuccessful. Peptonized milk was tried next as a carrying medium but it too was unsatisfactory. By a process of adding materials that seemed likely to contain necessary growth factors, a good carrying medium was found. The ingredients of the medium were: 1 per cent peptonized milk, 1 per cent yeast extract, 1 per cent glucose and 0.2 ml. Tween 80 per 100 ml. of medium. All of the ingredients

mentioned were necessary since a run omitting each ingredient systematically produced poor growth in all tubes except those containing all of the listed materials. The basal synthetic medium used for Lactobacillus leichmannii is shown in Table 24.

Table 24

Synthetic Basal Medium for L. leichmannii

Component	Quantity
vitamin mixture	15.0 ml.
amino acid mixture	80.0 ml.
Tween 80	0.2 ml.
salts A and salts B, each	1.0 ml.
adenine, guanine, xanthine and uracil, each	2.0 mg.
sodium acetate	1.2 g.
sodium chloride	1.0 g.
ammonium sulfate	0.6 g.
glucose	4.0 g.
Adjust to pH 6.7 and make to 100 ml.	

A series was set up in duplicate using the following supplements: 1 ml. extract D, 1 ml. extract C, 1 ml. each of extract C and extract D, 1 ml. each of extract D and 1 per cent yeast extract, 1 ml. each of extract C and 1 per cent yeast extract and a blank. All of the tubes were made to a

volume of 5 ml. and 5 ml. of the basal medium added. The tubes were plugged, sterilized and inoculated with a suspension of Lactobacillus leichmannii. After incubation at 37° C. for 16 hours the turbidities were read in the photometer and the values are shown in Table 25. The results

Table 25

Effect of Extracts on Growth of L. leichmannii

Material tested	Photometer reading (average)
1 ml. extract D	16.3
1 ml. extract C	95.0
1 ml. each, extract C and extract D	20.5
1 ml. each, extract D and 1% yeast extract	6.0
1 ml. each, extract C and 1% yeast extract	77.0
Blank	100.0

indicated that extract D contained considerable amounts of material acting like APF and extract C contained some inhibitory substance due presumably to the permanganate treatment.

In order to determine if the permanganate treatment was responsible for the inhibition found, extract C was freshly prepared according to the directions of Scott, Norris and Heuser (1946) omitting the permanganate treatment. For comparison, yeast extract (Difco) was treated in the same way

to produce an extract designated as extract E. The activities of the new extracts were tested with L. leichmannii. Duplicate test tubes containing 0.2 ml. extract D, 0.2 ml. extract C, 0.2 ml. extract E, 0.2 ml. each of extract C and extract D, 0.2 ml. each of extract D and extract E and a blank. The test tube fermentations were carried out in the usual manner and the turbidities obtained are recorded in Table 26. The

Table 26

Further Effect of Extracts on Growth of L. leichmannii

Material tested	Photometer reading (average)
0.2 ml. extract D	51.5
0.2 ml. extract C	96.0
0.2 ml. extract E	91.0
0.2 ml. each, extract C and extract D	53.5
0.2 ml. each, extract D and extract E	26.0
Blank	97.5

results indicated that the inhibition was due to the whey extract itself and not to the permanganate treatment. Activity of the same type as produced by APF was obtained with extract E which was made from yeast extract, a material known not to have APF activity in animal tests. It was evident that substances other than APF were stimulatory for L. leichmannii. Further purification of extract D seemed

necessary since it supported good growth of the test organism when supplied as the sole supplement to the basal medium. After several treatments with norit A at pH 3.0, extract D in amounts necessary to satisfy the strepogenin requirement of L. leichmannii would no longer support its growth without the addition of a source of APF. This was deemed a satisfactory purification of extract D.

At this time some crystalline vitamin B_{12} was made available through the courtesy of Merck and Company. Since APF activity was obtained in feeding tests with crude and crystalline B_{12} it was thought likely that crystalline vitamin B_{12} would be a good standard for an APF microbiological assay. Even if APF and B_{12} were not identical the APF activity could be expressed in terms of crystalline vitamin B_{12}. To determine the useful growth range of the crystalline material duplicate tubes containing 0, 0.001, 0.002, 0.004 and 0.01 µg. of crystalline B_{12} were prepared and 0.2 ml. of extract D added to each. The fermentations were then carried out in the usual way and the results obtained are shown in Table 27. These data gave a good standard curve and this indicated that the concentrations chosen in this run could be used in a microbiological assay.

In view of the suspected activity of substances other than B_{12} in stimulating growth of L. leichmannii several materials were tested with this organism. Duplicate tubes

Table 27

Effect of Crystalline Vitamin B_{12} on L. leichmannii

Run	µg. vitamin B_{12}	Photometer reading (average)
1	0.000	100
2	0.001	68
3	0.002	54
4	0.004	39
5	0.010	35

containing 4.8 ml. of tomato juice filtrate (obtained by filtering commercial canned tomato juice through ordinary filter paper) and 0.2 ml. of extract D, 4.8 ml. of 10 per cent yeast extract and 0.2 ml. of extract D, 0.02 µg. vitamin B_{12} and 0.2 ml. of extract D and a blank were prepared. The solutions were made to a volume of 5 ml. and 5 ml. of basal medium added to each. The fermentations were carried out in the usual manner. Results of the experiment are presented in Table 28. Tomato juice filtrate apparently had no effect on the growth of L. leichmannii, yeast extract had a definite effect but was suboptimum and crystalline vitamin B_{12} produced abundant growth of the organism. Other experiments along the same lines indicated that yeast extract in greater or smaller amounts than 10 per cent, the amount used in this run, did not increase the growth stimulation to the level attained by B_{12}.

Table 28

Effect of Several Substances on the Growth of L. leichmannii

Material tested	Photometer reading (average)
4.8 ml. tomato juice filtrate and 0.2 ml. extract D	101
4.8 ml. 10% yeast extract and 0.2 ml. extract D	63
0.02 ug. vitamin B_{12} and 0.2 ml. extract D	34
Blank	100

This emphasizes the fact that the growth promoting substance in yeast extract is different from vitamin B_{12} and even at optimum concentration can only partially replace B_{12}.

Samples for a new run were prepared by digesting 1 g. each of wheat bran, mold bran #8 and feed concentrate #44 at 37° C. with 25 mg. each of takadiastase and papain in about 50 ml. of 0.5 per cent acetate buffer for 24 hours. The digests were adjusted to pH 6.7, filtered and the bran sample diluted to 500 ml. while the samples of #8 and #44 were diluted to 1,000 ml. In duplicate tubes 1, 3 and 5 ml. of each of the digests were made to a total volume of 5 ml. and 5 ml. of basal medium added to each. The composition of the basal medium was the same as given in Table 24 with the exception that 10 ml. of strepogenin concentrate prepared by the method of Skeggs, Huff, Wright and Bosshardt (1948)

replaced 10 ml. of the amino acid mixture per 100 ml. of medium. This modification has been made in all succeeding experiments. For comparison duplicates containing 0.8 millimicrograms of crystalline vitamin B_{12} and a blank were prepared in an analogous manner to the samples. The test tube fermentations were carried out in the usual way. Results obtained for the series are shown in Table 29. Since the

Table 29

Effect of Extracts on Growth of L. leichmannii

Material tested	Photometer reading (average)
wheat bran	49.2
mold bran #8	89.3
feed concentrate #44	51.7
0.8 millimicrograms vitamin B_{12}	72.4
Blank	100.0

dilution of the bran extract was about half that of #8 or #44 it was really about half as active as is indicated by the figures. This behavior of bran which was known not to have APF activity indicated again that there were stimulatory substances for the growth of L. leichmannii that were neither APF nor vitamin B_{12}.

Since thymidine and other desoxyribosides were found by Kitay, McNutt and Snell (1949) to stimulate L. leichmannii

it was considered possible that the anomolous effects obtained with yeast extract and wheat bran were due to their desoxyribonucleic acid (DNA) contents. If the DNA contents of the test materials could be evaluated independently by some method such as the Dische test and then correlated with the growth effect of known amounts of pure DNA on L. leichmannii then this growth effect could be deducted from the total growth effect due to the material in question leaving the effect due to APF or similar substances. An assay method would then be available for APF if the DNA content of the anomolously behaving extracts could account for the total growth effect in these cases and not account for the effects in the case of materials known to contain APF by animal feeding.

The effect on the growth of L. leichmannii of some nucleic acids and derivatives was tested. Duplicate test tubes were prepared containing 0.5 mg. ribonucleic acid, 0.5 mg. desoxyribonucleic acid, 0.5 mg. adenylic acid and a blank. These tubes were run through the standard fermentation procedure and the results obtained are presented in Table 30. These data show that adenylic acid was inhibitory while ribonucleic acid had no effect and desoxyribonucleic acid definitely stimulated the growth of L. leichmannii. This confirmed the action of desoxyribonucleic acid and showed it to be somewhat specific in that some closely related compounds were inactive.

Table 30

The Effect of Nucleic Acids on Growth of *L. leichmannii*

Material tested	Photometer reading (average)
0.5 mg. ribonucleic acid	85
0.5 mg. desoxyribonucleic acid	51
0.5 mg. adenylic acid	100
Blank	84

One g. samples of bran, #8 and #44 were digested with 25 mg. papain and 25 mg. takadiastase in about 50 ml. of 0.5 per cent acetate buffer for 24 hours at 37° C. The digests were diluted to 100 ml. and made to pH 1.0 with concentrated HCl. Three ml. aliquots of each were pipetted into duplicate test tubes, the tubes placed in boiling water for 15 minutes and cooled. Duplicate tubes containing 0, 0.30, 0.90, 1.20 and 1.80 mg. of desoxyribonucleic acid were also prepared so that the volume of the solution in each tube was 3 ml. at a pH of 1.0. The standards were then heated in boiling water for 15 minutes and cooled. Six ml. of Dische reagent was added to each tube and then all of the tubes were again placed in boiling water. After 3 minutes the tubes were rapidly cooled and allowed to stand for 5 minutes. The solutions were analyzed in the photometer and the results for the standard series were shown in Table 31. The points were plotted to produce a standard curve from which the DNA contents of the

Table 31

Standard Curve for DNA

DNA mg.	Photometer reading (average)
0.00	100.0
0.30	95.7
0.90	88.1
1.20	84.3
1.80	75.8

samples were calculated. The final results are presented in Table 32. There was no correlation between DNA content and

Table 32

DNA Contents of Samples

Material tested	Photometer reading (average)	DNA mg. per g.
bran	97.0	0.21
#8	97.0	0.21
#44	98.2	0.12
Blank	100.0	0.00

the growth effect of the extracts on L. leichmannii. All further attempts along these lines were therefore abandoned.

Another method of eliminating the interferences in the L. leichmannii assay for APF was sought in the stabilities of

the factors involved. Animal protein factor was destroyed by autoclaving alkaline solutions of that material. Interfering substances of an acid nature such as DNA should be stable to such treatment.

A series was prepared by digesting 1 g. each of bran, #8 and #44 with 25 mg. papain and 25 mg. takadiastase in about 50 ml. 0.5 per cent acetate buffer for 24 hours at 37° C. The solutions were diluted to 1,000 ml., filtered and each divided into two portions. One part was made to pH 6.7 and the other portion was treated with 0.2 ml. of saturated NaOH in the autoclave for 30 minutes at 15 lb. pressure. The alkaline solutions were then adjusted to pH 6.7. Five ml. of each of the 6 solutions was added to duplicate test tubes already containing 5 ml. of basal medium. As a comparison a solution of crystalline vitamin B_{12} containing 0.4 millimicrograms per ml. was prepared exactly as the samples and a blank was also included in the run. The fermentations were completed in the usual manner and the turbidities obtained are shown in Table 33. The results show that only #44 extract, containing APF and the vitamin B_{12} solution were affected by the alkaline treatments. This suggests that an assay could be developed on the basis of the NaOH treatments which would eliminate the effect of the interfering factors.

The possibility has been indicated of developing a microbiological assay method that would at least distinguish two groups of factors. One group represented by DNA was stable

Table 33

Effect of Alkali on Activity of Extracts

Material tested	Photometer reading (average)	
	Untreated	NaOH treated
bran	61.5	60.8
#8	87.8	89.0
#44	52.5	88.6
B_{12}	39.4	99.0
Blank	100.0	. . .

to alkali treatment and the other group labile in the presence of alkali. Since there was this complication, however, of interfering substances it was deemed advisable to investigate another organism known to be more fastidious in its requirements than L. leichmannii. The organism chosen for this study was Lactobacillus lactis Dorner and this work is reported in the next section.

3. Studies using Lactobacillus lactis Dorner

Lactobacillus lactis Dorner failed to grow on the carrying medium prepared for L. leichmannii. A milk filtrate was made by coagulating the proteins in milk with acid, heating for 10 minutes on the steam bath and filtering. Addition of 1 ml. of this filtrate per 100 ml. of L. leichmannii carrying medium allowed good growth of L. lactis.

Preliminary experiments showed that the synthetic basal medium used for L. leichmannii could be adapted for use by L. lactis by the addition of tomato juice filtrate and whey extract. The whey extract was made by extracting 20 g. whey with 200 ml. of distilled water for 15 minutes on a steam bath. Four ml. of the whey extract and 2 ml. of tomato juice filtrate were used per 100 ml. of basal medium.

One g. each of wheat bran, mold bran #8 and feed concentrate #44 were digested with 25 mg. of papain and 25 mg. of takadiastase for 24 hours at 37° C. in about 50 ml. of 0.5 per cent acetate buffer. The solutions were all made to a volume of 500 ml., filtered and adjusted to pH 6.7. Duplicate tubes containing 5 ml. of each of the extracts plus a set of blanks were prepared and 5 ml. of basal medium was added to each. Sterilization, inoculation and fermentation were carried out in the usual manner using a culture of L. lactis and incubating at 37° C. The results obtained after incubating for 59 hours are shown in Table 34. These data compare

Table 34

Effect of Extracts on Growth of L. lactis

Material tested	Photometer reading (average)
wheat bran digest	49.3
#8 digest	93.4
#44 digest	13.4
Blank	96.1

favorably with those obtained using L. leichmannii. Similar results were found in several instances. As further experiments were carried out with this organism, erratic behavior of duplicate tubes was found. One tube might grow very well and the duplicate would show no visible turbidity. This tendency became so common that in spite of the promising results obtained in some of the runs, the use of L. lactis was dropped from further consideration in this investigation. In order to follow the isolation of active fractions only a qualitative or semi-quantitative assay method was required and since the previous work on L. leichmannii provided such a method this organism was selected for the fractionation. If the cause of the erratic behavior of L. lactis could be ascertained and corrected it would have value for quantitative work.

4. Procedures for the concentration of animal protein factor

If animal protein factor were typical of most growth factors it could be found both in the uncombined form and conjugated with protein. Both of these forms are known for vitamin B_{12}. Should APF present in feed concentrate #44 be in the conjugated form, digestion with papain would split the combination. Since the properties of the complex might be more suitable for isolation purposes it was necessary to investigate the extraction of active material before papain

digestion as well as after.

One g. each of wheat bran, mold bran #8 and feed concentrate #44 were extracted on the steam bath for 15 minutes in about 50 ml. of 0.5% acetate buffer. The extracts were made to 50 ml. and of these 1 ml. aliquots were made to 20 ml. The remainder of the original extracts were digested for 24 hours with 25 mg. of takadiastase and 25 mg. of papain. The digests were diluted to 1 liter. Five ml. samples of both the undigested extracts and the digested extracts were tested in the usual way with Lactobacillus leichmannii. The results obtained in the test tube fermentations are shown in Table 35.

Table 35

Effect of Extracts on Growth of L. leichmannii

Material tested	Photometer reading (average)
bran extract	98.0
bran extract after digestion	97.9
#8 extract	100.6
#8 extract after digestion	95.8
#44 extract	95.0
#44 extract after digestion	95.6
blank	99.2

The conclusion that can be drawn from the data is that APF was not extracted by the acetate buffer or was destroyed in

the process of extraction since the extract had no activity before or after digestion with papain and takadiastase.

Since APF is destroyed by autoclaving with alkali this method of extraction could not be used. It was possible, however, that the previous treatment did not extract APF because the treatment was not drastic enough to release the factor from its combined form. A new run using 0.1N HCl and autoclaving for 30 minutes at 15 lb. was made and the extracts so obtained were also inactive in growth studies with L. leichmannii.

Five g. of #44 was extracted on the steam bath three times with 100 ml. portions of 95 per cent ethanol adjusted to pH 2.0. The combined extracts were evaporated almost to dryness on the steam bath and the residue taken up in 250 ml. of 0.5 per cent acetate buffer. To 50 ml. of this solution was added 25 mg. of papain and 25 mg. of takadiastase and digestion was carried out at 37° C. for 24 hours. Five ml. of the neutralized extracts both before and after digestion were tested with L. leichmannii in the usual manner. No stimulation with either of the extracts was observed and it was concluded that the active material was not extracted or was destroyed in evaporating off the solvent. In either case alcohol extraction of feed concentrate #44 would not be useful in concentration of the APF present.

One g. of feed concentrate #44 was digested for 24 hours at 37° C. in about 50 ml. of 0.5 per cent acetate buffer. The solution was diluted to 500 ml. and duplicate samples of 1 ml. and 5 ml. were saved for analysis. A 100 ml. aliquot of the extract was treated twice with 1 g. amounts of norit for 15 minutes after adjustment of the extract to pH 2.0 with concentrated HCl. The filtrate was neutralized and duplicate 1 ml. and 5 ml. samples withdrawn for analysis. All of the samples both before and after norit treatment were tested in the usual manner with L. leichmannii. The results obtained after incubation for 19 hours are shown in Table 36. Growth of the organism was stimulated by the digests and in proportion to the size of the sample taken indicating that there were no inhibitors present in the extracts. Norit was effective in removing the active material from solution since the extracts no longer stimulated growth after norit treatment.

The possibility that the active material was vitamin B_{13} or contained some B_{13} was tested by extracting digests of #44 with ether. The water layer which was left had the same activity as the original digest when tested with L. leichmannii.

The properties of the active material in #44 were such that of the extraction procedures tried only enzymic digestion with papain and takadiastase was capable of bringing the active component into solution. The APF activity could then be removed from the solution by adsorption on norit.

Table 36

Effect of Norit Treatment of Digests on Growth of *L. leichmannii*

Material tested	Photometer reading (average)
1 ml. digest	84.4
1 ml. digest, norit treated	98.5
5 ml. digest	64.3
5 ml. digest, norit treated	101.2
blank	98.1

A larger scale digestion was made using 200 g. of #44 in 2 liters of 0.5 per cent acetate buffer. The resultant solution was treated four successive times with 20 g. of norit. The norit was eluted with five successive 100 ml. portions of ammoniacal alcohol (90 ml. 95 per cent alcohol and 10 ml. concentrated ammonia). The eluate was evaporated to near dryness and then taken up in 100 ml. of distilled water. Appropriate amounts of the original digest, the filtrate after norit treatment and the final solution were tested with *L. leichmannii*. The activity present in the original digest was removed by the norit treatment as expected but the eluate was inactive. This could have been due to either a failure to elute the APF or to destruction of APF in evaporating down the basic solution. Repetition on a smaller scale and elution with neutral but hot 65 per cent alcohol

led to recovery of an active concentrate. The scale of operations necessary to recover crystalline material would require several tons of crude material and facilities for processing such quantities. The necessity of using such quantities can be seen from the fact that the activity of #44 expressed in terms of crystalline vitamin B_{12} was roughly 0.2 λg. of B_{12} per g. Assuming complete recovery in all stages of purification this would mean processing about 5.5 tons of #44 to get 1 g. of crystalline material. Such operations were not feasible on a laboratory scale.

VI. DISCUSSION

Evaluation of the growth promoting properties for bacteria of any material can be accomplished in two ways. A complete quantitative survey of the vitamins present in the preparation could be made. In this case knowledge of the requirements of any organism would immediately show whether the material in question was suitable for the fermentation being considered and just how much of it would be necessary. The other alternative is to test the material in the specific fermentation as a composite source of growth factors. This procedure is the simpler but has limitations. Knowledge of only one specific fermentation is obtained and sometimes this may be ambiguous. If the material being tested contains only small amounts of a required vitamin, the quantity needed to give satisfactory results may be extremely large. If complete quantitative information were available a small amount of a secondary supplement might allow much smaller quantities of the material to be used with subsequent economy of operation. The first approach was chosen in the present study because of the advantages mentioned above.

Although it was not necessary for the purposes of this investigation to develop more than a semi-quantitative assay for animal protein factor, sufficient information was obtained

to suggest the basis for a strictly quantitative method. The destruction of vitamin B_{12} and APF by autoclaving with alkali provides the means by which the interference in the assay of desoxyribonucleic acid and similar compounds can be eliminated. The procedure would necessarily be more complex than in the usual microbiological method but would still retain most of the advantages of this technique. First the samples to be analyzed would be autoclaved with NaOH and the extracts so obtained tested with L. leichmannii using pure desoxyribonucleic acid as a standard. This would essentially be a determination of the DNA content of the sample. Next the samples could be tested without alkali treatment using standards which all contain the corresponding amount of DNA in addition to varying amounts of crystalline vitamin B_{12}. Since the standards would all have the same amount of DNA as the samples the differences in the growth of L. leichmannii could in each case be attributed to APF or vitamin B_{12}. Had the colorimetric procedure for determining DNA been satisfactory this would have been able to replace the step involving NaOH treatment with the second step remaining the same. The method suggested really amounts to an independent determination of the desoxyribonucleic acid content of the substance to be tested and incorporation of corresponding amounts of pure DNA in the standards so that B_{12} or APF are the only variables. A critical survey must also be made of the basal medium to make certain that it is optimum and of the alkali

treatment to determine the conditions for the most complete destruction of APF and vitamin B_{12}.

Further work along lines similar to those already suggested could be carried out. It is probable that organisms could be found that require APF or B_{12} but not DNA. This would eliminate the interference of DNA observed using L. leichmannii for microbiological assay of APF and vitamin B_{12}. Since B_{12} is known to be a pyrrole derivative it is possible that some hemophilic organisms require vitamin B_{12} or APF. Aerobic culture of lactobacilli by means of shaking might prove useful since it is known that L. leichmannii does not require B_{12} under strictly anaerobic conditions.

In all of this discussion the identity of APF has not been considered. It is quite probable that APF is either identical with vitamin B_{12} or very similar to it. This statement is made in view of the similarity of properties observed for APF as compared with the known properties of vitamin B_{12}. In any case assay values for APF can be stated in terms of crystalline B_{12} since this is a convenient standard and does not imply anything regarding the structure of APF. A solution to the problem of identification awaits the processing of large amounts of the crude material to obtain pure crystalline material. The magnitude of this job may be judged from the fact that Smith (1948) used four tons of ox liver to produce one gram of a highly active concentrate of vitamin B_{12}.

VII. SUMMARY AND CONCLUSIONS

1. The vitamin content of four samples of mold bran and one sample of bran as a control was determined using microbiological assay methods. The results obtained are summarized in Table 37. The increased yields in the alcoholic fermentation using mold bran as the saccharifying agent instead of malt could be explained on the basis of the vitamin content of the mold bran.

Table 37

Vitamin Content of Mold Bran

Vitamin	µg. per g. (average)	
	bran	mold bran
riboflavin	4.8	20.0
nicotinic acid	300.0	113.0
biotin	0.35	0.59
pantothenic acid	66.0	12.3
thiamin	4.7	2.9
folic acid	0.53	1.51
pyridoxine	3.6	0.48
inositol	256.0	160.0

2. Using Lactobacillus casei and Lactobacillus arabinosus as test organisms, a definite stimulation of growth was obtained with preparations known to have animal protein factor activity. The requirement of these microorganisms for animal protein factor was not absolute and as a consequence considerable growth was obtained in blanks even after incubating for 16 hours. Animal protein factor was apparently synthesized slowly by the organisms since the blanks continued to grow and after 72 hours approached the growth of the tubes containing the most concentrated extracts. These organisms are not recommended for the assay of animal protein factor or similar factors.

3. A simple carrying medium containing yeast extract, peptonized milk, Tween 80 and glucose was developed for Lactobacillus leichmannii. A basal medium for growth factor studies was also described. This organism was found to require animal protein factor or vitamin B_{12} and was later found to be responsive to desoxyribonucleic acid and desoxyribosides as well. The latter group of substances interfered with the use of L. leichmannii in quantitative assays for vitamin B_{12} or animal protein factor. Two methods of eliminating the interference were tried. The first was a colorimetric determination of desoxyribonucleic acid using the Dische reagent but the shades of color varied with the samples and no useful information was obtained. The second

method was successful and involved destruction of animal protein factor or vitamin B_{12} by autoclaving with alkali. Lactobacillus leichmannii is recommended for assay methods to determine animal protein factor and vitamin B_{12}.

4. Lactobacillus lactis Dorner was studied in an effort to find a more fastidious organism than those previously used. It was hoped that the interfering substances might not affect the use of this organism. Although some favorable results were obtained the response was so erratic that the use of this microorganism had to be abandoned.

5. The animal protein factor activity present in feed concentrate #44 could not be extracted by steaming in acetate buffer. Autoclaving with 0.1N HCl failed to extract any active material from the solid feed concentrate. Also unsuccessful was extraction of #44 with 95 per cent ethyl alcohol followed by evaporation to dryness and redissolving in distilled water. The only method by which the active material could be released from its conjugated form was by digestion with papain and takadiastase. The animal protein factor activity was removed from the digests by norit. Elution of the factor from norit was either unsuccessful in ammoniacal alcohol or else the factor was destroyed in evaporating the alkaline solution. The factor could be eluted from norit by hot 65 per cent ethanol. Lactobacillus leichmannii was used to test the activity of all of the extracts made.

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