THE "BIOS" QUESTION

FRED W. TANNER

Department of Bacteriology, University of Illinois, Urbana, Illinois

In his famous reply to Hayne, Daniel Webster suggested that before floating further on the waves of that debate, they imitate the wise custom of the mariner who, after a storm avails himself of the first glimpse of the sun to take his bearings and to determine how far he has driven from his true course. The suggestion seems not amiss for the subject of this review although in this case we may be unable to determine how far we have drifted in the controversies which have centered around the relation of hypothetical substances to yeast growth for the storm of experiment and controversy has not yet subsided. It is hoped, however, that a review of the subject may point out some of the inadequacies of the methods which have been used and also indicate in what further directions research may be necessary.

Since the early attempts of biologists to propagate yeasts in pure cultures under controlled conditions in the laboratory, many nutrient solutions have been used. Important among these are the so-called synthetic media or mineral salt-sugar nutrient solutions. Such media have many advantages over chemically indefinite solutions such as peptone media or sterilized fruit decoctions. During the early days arguments centered around the assimilation of organic and inorganic nitrogen but later and more recently, the work has centered around a mysterious organic substance or substances essential for the proliferation of yeast cells, to which Wildiers provisionally gave the name of "bios."

Largely because of failures to isolate "bios" or to determine its chemical identity within the time immediately following Wildiers' announcement, the subject seems to have lost interest and it was not until a similarity between the properties of "bios" and those

of vitamin B suggested a possible relationship between the two, nearly eighteen years after Wildiers' observations, that the interest in the subject was again revived. The multiplication of yeast cells as it may be influenced by "bios" is not only an interesting problem but a fundamental one, the solution of which will undoubtedly aid in the study of animal nutrition and growth. Thus it was that the work of Neuberg and his colleagues with single celled organisms greatly increased our knowledge of the decomposition of glucose and gave support to views advanced to explain its decomposition in the animal body.

An effort has been made to review most of the available literature bearing directly or indirectly on the influence of "bios" or of vitamines on yeast, keeping in mind the biological significance of the question. Attempt has not been made to include a discussion of certain nutrient solutions which have great industrial value (Hayduck's, etc.,) because these solutions may not have been prepared from chemically pure salts and sugars. The subject has been treated chronologically as far as possible since it was believed that such a method would better show the development of the various phases of the question. Although the period covered in the papers which form the basis of this review extends from 1860 to 1924, few papers relative to the subject were published immediately after the controversy between Pasteur and Liebig until 1901.

Pasteur (1860) was one of the first to propagate yeast in nutrient solutions containing mineral salts and sugar. A medium consisting of 100 grams of sucrose (rock candy) and 700 grams of pure water inoculated with 6.254 grams of yeast was later abandoned for one containing 10 grams sucrose, 100 cc. water, 0.1 gram ammonium tartrate and 1 gram yeast ash, inoculated with a bit of yeast the size of a pin head. A small inoculation was followed by a slow onset of fermentation which extended over a long period. A heavier inoculation insured a more active evolution of gas bubbles, Pasteur's criterion for judging the course of fermentation. Inasmuch as fermentation must have progressed for some time before its products were present in sufficient concentration for detection, too broad an interpretation of his results

should not be made. When applying his data to the question under discussion it will be well to remember that Pasteur was laying the foundation stones rather than putting the towers on the structure of yeast metabolism. Pasteur's experiments have been analyzed and used as evidence for supporting arguments for which they were probably never intended. What he demonstrated to his own satisfaction and to that of many others was that yeast cells were able to function normally with an inorganic source of nitrogen, converting inorganic into organic material. It should be pointed out in light of more recent work that Pasteur used heavy seedings for inoculation.

Although he published full details of his experiments in 1860, Pasteur wrote another confirmatory report on the growth of yeasts in mineral salt-sugar media in 1874.

Duclaux (1864) confirmed Pasteur's work that yeast could utilize the nitrogen in ammonium tartrate and refuted a statement by Millon that Pasteur's yeast did not utilize the nitrogen in Pasteur's solution but that this nitrogen evaporated into the air.

Eleven years after the publication of Pasteur's paper, Liebig (1871) who supported an older theory of alcoholic fermentation, contested the statements of Pasteur in regard to the multiplication and fermentation of yeast in a medium free from nitrogenous organic matter. He failed to get the same results when he repeated Pasteur's experiments and vigorously denied the possibility of obtaining either growth or fermentation in a mineral salt-sugar solution.

For the following quarter of a century, Pasteur's statements that a solution containing an ammonium salt, the mineral salts found in yeast ash and a fermentable sugar, constituted a complete culture medium for yeast, seem to have been very generally accepted. During this time much valuable work was done with synthetic media based on the medium of Pasteur.

Mayer (1869) studied the nitrogen metabolism of yeast but probably did not use pure cultures and consequently his work loses much of its value in the present discussion. A little later Nägeli (1879) reported that the weight of beer yeast from sugar

and ammonium tartrate could be increased 12 times by the passage of air through the medium during incubation for twenty-four hours. Such cells were richer in fat and poorer in nitrogen, but were weak as far as fermentation was concerned. Nägeli suggested that this lack of aeration might have influenced Mayer's results. According to Kossowicz, Nägeli was one of the first to question Pasteur's results.

In 1894 Beijerinck found that Schizosaccharomyces octosporus under the best conditions showed only slight growth with ammonium salts and asparagin. Peptone alone, which for beer yeast furnished an exceptionally good source of nitrogen, permitted only scant growth. Only such natural nitrogen compounds as are found in malt and grapes functioned as the most useful source of nitrogen.

In an attempted study of the synthesis of the phosphorus-containing organic compounds of yeast, Wildiers' (1901) used a synthetic medium and a small inoculum of yeast cells, thus reducing to a minimum the addition of preformed compounds to the medium. Such cultures either grew very slowly or did not grow at all. Similar cultures in sterile wort grew well and fermented vigorously. The significance of Wildiers' paper justifies a little more of our attention than can be given to succeeding contributions.

Wildiers' medium had the following composition:

Water	200 grams
Sucrose	20 grams
Magnesium sulfate, Potassium chloride, Ammonium chloride, Disodium phosphate	50 ctgr.
Calcium carbonate	10 ctgr.

Fermentation flasks containing 125 grams of well aerated medium, with 10 grams of sugar, were inoculated with varying amounts of a culture of Saccharomyces cerevisiae I Hansen, from sterile beer wort, and were weighed and kept at 28°. The flasks were weighed every day and the loss in weight due to CO_2 was determined from which the amount of sugar fermented could be

calculated. At the end of five days the flasks inoculated with 2 drops had lost nothing in weight, while those receiving 5 drops had lost 5 to 5.5 grams. It was evident from many experiments of this kind that with small seeding there was no fermentation in mineral salt-sugar solutions such as Pasteur had used, while with sufficiently large seeding there was fermentation of the sugar. This same observation has been reported by many of those who worked after Wildiers. By further experiments Wildiers showed that the effect of the larger seedings was not due to the increased number of living cells, but to the influence of a chemical substance or substances provided by the filtrate from boiled yeast cells. To this unknown and hitherto unrecognized constituent of yeast water, which he considered indispensable to the growth of yeast, Wildiers gave the name "bios." Wildiers proposed the name "bios" because it was not known chemically and because this name would be satisfactory until the factor was better understood. This discovery has been the center of extensive investigations since 1901.

Wildiers described "bios" as follows:

1. Soluble in water.
2. Insoluble in absolute alcohol and ether; 80 per cent, however, permits a good extraction of "bios."
3. Not present in yeast ash. It is, then, not an inorganic substance.
4. Not destroyed by boiling for a half hour in a 5 percent solution of sulfuric acid. To destroy it a 20 per cent solution is necessary.
5. "Bios" seemed to be changed by one half hour boiling in 1 per cent solutions of NaOH.
6. Not precipitated by lead acetate.
7. Dialyzable.
8. Contained in Liebig's meat extract, commercial peptone and in beer wort.
9. "Bios" is not present in such substances as urea, asparagin, aniline, tyrosine, nuclein bases, adenine, guanine, thymus nucleic acid, creatine, peptic and tryptic digestion products of albumin.

Wildiers explained the differences between Pasteur and Liebig on the basis that Pasteur used a larger inoculum (a portion the size of a pin head) while Liebig used a smaller bit of yeast.

While Wildiers' work has certain weaknesses in light of present-day methods it stimulated much discussion and experiment. He did not use adequate criteria of growth. His conclusions were based upon losses in weight assumably due to carbon dioxide from fermentation. There is a good basis for criticizing such a criterion for it is known that cells may ferment and form CO_2 after they have stopped growing. The number of cells in his inoculum could probably have been controlled a little better.

Wildiers concluded his paper with a very pertinent discussion of the Pasteur-Liebig controversy. He thought that the differences in experimental results may have arisen because of different methods of inoculation. Pasteur used large bits of yeast; these were visible after days in the bottom of the culture flask on account of their dark color amongst the white young cells. When Wildiers repeated Pasteur's experiments, he found that there was a minimum limit to the number of cells which would give growth and that much depended upon the nutrient medium from which the cells were taken. Liebig was thought to have used too few cells or they came from a medium too poor in "bios." He claimed that Pasteur had added, besides the living yeast cells, an unknown but necessary chemical substance. Wildiers mentioned the work of Laurent, who stated that yeast could grow without "bios" but explained it on the basis that only after weeks and months did he weigh his cultures. Wildiers studied the growth of yeasts from small seedings. The small amount of "bios" contained in the inoculation allowed only slow and scant growth; after some months much sugar was destroyed and the yeast was visible at the bottom of the flask. Such yeast, however, is quite different from that propagated in a medium allowing rapid growth. Cells from the former medium were not healthy, their growth seeming to have been gradual, one cell waiting for the disintegration of another before multiplying.

Wildiers could not admit the differences in the character of growth and multiplication, if the medium was adequate, on the basis of the use of 10,000 cells or 50,000 cells. If nothing was lacking the 10,000 cells should be only a few hours behind the 50,000 cells. The situation, however, is different. On the one

hand growth and fermentation continue until the sugar is gone, on the other hand growth and fermentation go on at a very slow rate for weeks and months, even though the sugar is not used up. A medium allowing such slow development is not a good medium.

There are several points in Wildiers' paper which need comment. *First,* he grants that yeast is able to grow in mineral salt-sugar media but that the growth is slow and the cells resulting from such growth are not healthy cells. He stated that much of the sugar disappeared when small inoculations were incubated over a long period and that yeast is visible at the bottom of the flask. He does not give the data on which he based this statement; but the duration of his recorded experiments does not, in any case, exceed 15 days. This statement, we believe, is often overlooked by those who quote Wildiers' work.

Second, he used drops of a rich wort culture of the yeast as the inoculum. This is open to criticism since even in a drop of such a culture there may be in addition to "bios" sufficient food material to start growth. After growth has once been started, "bios" may be formed by the cells, or the old cells may break up, rendering food materials available for the growth of living cells. *Third,* yeast growth was greatly accelerated by the addition of certain substances in malt and yeast water of more or less known composition, or by some special hypothetical substance to which he gave the name "bios." We are told that Wildiers found it a painful duty to publish data which were not in accord with data reported by Pasteur.

It is not at all surprising that a claim like Wildiers' should stimulate opposition. One of the first to negate Wildiers' conclusions was Fernbach (1901) who suggested that the conclusions of Wildiers were due to the presence of toxic substances in the culture media. According to Windisch, Fernbach stated that the only organism which is known to grow better in mineral media than in natural media is Aspergillus niger, and that it may safely be assumed that yeast is at a considerable disadvantage under these conditions. If Wildiers had sown only single cells, the explanation would have been clear, namely, that those cultures

which failed to develop had been sown with cells deficient in vigor. But since the mineral sowings must have been made with at least some hundreds of cells, this explanation is not valid, since some at any rate must have been vigorous. In Fernbach's opinion, the failure to develop must be put down to the presence of some antiseptic introduced as an impurity in the salts or in the form of traces of copper in the distilled water. The action of antiseptics is to react with the protein of the cells, and the result of the presence of minute traces would be to kill a small number of cells whilst allowing a larger seeding to develop.

Henry (1902) was also unable to confirm Wildiers' conclusions. According to Pringsheim, Henry added 3 drops of wort cultures to 500 cc. of Wildiers' mineral salt-sugar medium and secured rich growth of the yeast. His results were apparently in direct confliction with those of Wildiers who also used drops of wort cultures as the inoculum. This makes it probable that "bios" was introduced into the synthetic nutrient solution from the wort cultures or perhaps a sufficient amount of organic material to allow the inception of growth. Henry used a greater number of yeasts than heretofore had been used, about five in number, cane sugar yeast from the Pasteur Institute, Logos yeast, Burton yeast, Berlin yeast race II, and S. Ludwigii. The yeast cells were thought to produce no "bios" but used it up when it was in the medium. If this were so, it might be expected that if minute quantities of the above cultures were seeded into fresh mineral nutrient solutions, there would not be sufficient "bios" to permit development. But it was found on the contrary that when 5 drops of these cultures were seeded again into 500 c.c. of the inorganic salt-sugar nutrient solution, the seedings corresponded to only 1/666 of a drop of the original yeast, the second cultures developing as well as the first. Naumann interpreted Henry's experiments to support Pringsheim's acclimatization theory.

In 1901 Krieger also criticized Wildiers' conclusions and stated that it was necessary to take into consideration the whole physiology of the yeast cell. Krieger pointed out contradictory statements in Wildiers' paper which made it difficult to know just what Wildiers intended to say about the formation of new "bios"

and its disappearance from the medium. Krieger's advice to consider the physiology of the cell more should be kept in mind by many of those who are working in this field today, especially those who are trained in other fields than microbiology. Krieger believed that cells which had been grown in a medium rich in "bios" stored it for future needs.

Another ardent but open-minded opponent of Wildiers' conclusions was Windisch (1902) who explained Wildiers' conclusions on the basis of toxic materials such as copper salts in the media. Windisch became one of Wildiers' active opponents publishing several papers on the subject.

Amand (1902) defended Wildiers and refuted the statements of the several German investigators that toxic substances in the media explained Wildiers' results. Amand showed that the water was without effect. This work was discussed by Windisch which caused Amand to continue his work. Windisch suggested that the sugar used by Amand contained a little poison. According to Naumann, Amand made the subject more confused because he said that "bios" was consumed by the yeast without the yeast producing any new "bios." Amand grew yeast in mineral salt-sugar solutions containing "bios." He showed that the yeast used up the "bios" since a filtered yeast culture showed no "bios." Naumann, however, claimed that if Amand had prolonged his experiments somewhat, he could have shown the presence of a "Beautiful Bios" in the form of soluble protein nitrogen.

Windisch (1902) replied to Amand's defense of Wildiers in a long discussion. He did not agree with Amand. Windisch stated that while he could confirm the fundamental observation of Wildiers' as to the inability of minute quantities of yeast to develop in mineral media, he preferred to keep an open mind as to the explanation. Windisch gave much weight to Fernbach's objection to Wildiers' hypothesis since minute quantities of copper are frequently present in distilled water. This, according to Windisch seemed to be supported by the fact that the introduction of yeast extract enabled the yeast cells to develop. It was stated that these yeast proteins fixed the copper. On the other hand, the copper precipitating power of most proteins would be

destroyed by boiling for half an hour with 10 per cent sulfuric acid, an operation which does not—according to Wildiers—destroy "bios." Again, Wildiers introduced boiled, dead yeast-cells, and failed to get development of minute sowings, but the operation of boiling would not destroy the power of the cells to react with metallic salts. Windisch considered that the question is important, and called for further investigation. Windisch (1902) continued his remarks on Wildiers' "bios" and recalled his own experiments when it was found that small seeding of yeast failed to develop in cane sugar solutions owing to the presence of traces of ultramarine which had been used for blueing the sugar. Windisch used the experiments of Nägeli on the poisoning effect of very small amounts of metals on living cells. One type of reaction reported by Nägeli was the death of living cells in the presence of large amount of the poisonous salt such as 1 gram of salt in a few liters of water. This reaction was explained on the basis of a chemical reaction. Another type of reaction reported by Nägeli was the death of living cells when but a small amount of poisonous salt was present such as 1 part in several millions of medium. To the latter reaction which Nägeli did not explain on the chemical basis, he gave the name *oligodynamic reaction.* Windisch used this experiment to show how small traces of metals might cause an inhibition in the development of microorganisms. He believed that Wildiers' conclusions should be held in abeyance but was willing to believe that they might be substantiated by experiment in the future.

In 1902 Stern reported some observations on the growth of yeasts in pure solutions. While his work was not concerned with "bios" and therefore he may not have given great care to determine the purity of his chemicals, his data are pertinent to this discussion. After a considerable amount of work with wort, Stern used an artificial medium composed of dextrose, asparagin, and certain inorganic salts dissolved in water. Stern stated that this medium was a very suitable one for yeast growth and fermentation but that it did not allow such large crops of yeast as wort yielded when used under the same conditions and seeded with the same amount of yeast. In order to increase the growth

of yeast Stern finally substituted a yeast extract in place of the asparagin. We may leave his investigation at this place since Stern did not use inorganic salt-sugar solutions in relation to the subject under discussion.

Amand (1904) then showed that "bios" was removed from the medium by the yeast. According to the richness of the medium upon which the yeast cells have grown, they may contain much or little of this "bios." The cells were said to contain a large amount of "bios" or only traces. Amand suggested a number of questions. Are cells poor in "bios" able to multiply as well as cells rich in this substance? What are the exact contents of brewery worts? Is "bios" destroyed like a sugar molecule or is it used synthetically as are the nitrogen compounds? Amand's interpretation of his data is noticeably influenced by the fact that he accepts the existence of "bios" as well established. Consequently, when he interprets either the growth or lack of growth of the yeast, it is usually explained on the basis of the presence or absence of "bios."

Lindner (1905) at this time, came into the argument and gave a new explanation. He contested the statement of Wildiers that substances such as urea and peptone did not accelerate the growth as did "bios." Lindner stated that these substances would stimulate the growth of yeast.

Kossowicz (1903) published a valuable contribution to the subject. He worked with wine yeasts Saccharomyces ellipsoideus I, Hansen in mineral salt-sugar solutions. He followed growth not only by means of CO_2 estimations by loss of weight but also by means of alcohol determinations. Kossowicz paid great attention to the amount of inoculum which he used and therefore may, perhaps, be looked upon as originating some of the more careful work on this phase of the subject.

His first experiment dealt with the multiplication of yeasts in mineral salt-sugar solutions. He placed 100 cc. of Wildiers' nutrient solution in a cotton stoppered bottle and seeded it with 500 cells of Sacch. ellipsoideus I, Hansen. After 12 days there was no development, after fourteen days there was a scant growth but after thirty-one days there had been an increase in cells to 140

million per 100 cc. of medium. The temperature of incubation during the experiment was between 23 and 26°.

His second experiment was carried out like the first with the exception that 1000 cells were used instead of 500. After 10 days, there was a slight development and after 27 days an increase to 392 million cells. A parallel experiment yielded 340 million cells.

Kossowicz's third experiment consisted of using 100 cc. of the following nutrient solution.

Water	100 cc.
KCl	0.2 gram
$(NH_4)_2HPO_4$	0.1 gram
$MgSO_4$	0.02 gram
$Ca_2H_2(PO_4)_2$	0.02 gram
Commercial refined sugar	5 grams

There was no development during the first two weeks but after forty days there were 364 million cells in 100 cc. of the nutrient solution.

The fourth experiment was identical with the third except that pure saccharose was used. There was no development during the first three weeks but after sixty days, 220 million cells per 100 cc. of medium had developed. A duplicate test gave 180 million cells per 100 cc. of media.

The fifth experiment involved the use of a medium consisting of

Water	100 cc.
Sugar	2 grams
KCl	0.40 gram
$(NH_4)_2HPO_4$	0.40 gram
$MgSO_4$	0.20 gram
$Ca_2H_2(PO_4)_2$	0.10 gram

This was inoculated with 200 cells of Sacch. ellipsoideus. After fifty days, there were 140 million cells per 100 cc. of nutrient medium.

These data led Kossowicz to the conclusion that with small numbers of yeast cells, a slow development takes place. After a month or two the number of cells was quite large. The forma-

tion of carbon dioxide in such amounts as to be discernible with the naked eye did not take place. This seemed to be the case also when greater numbers of cells were used. Kossowicz's data caused him to state that the mass of yeast crop seemed to depend somewhat on the presence of some organic compounds. He stated that the addition of from 1 to 2 cc. of beer wort which had been sterilized 3 times, accelerated the development of the yeast; without this wort, other conditions being equal a weaker development resulted.

He also attempted to determine whether the organic compounds essential for yeast growth could be produced by other fungi. To determine this he inoculated nutrient solutions simultaneously with small amounts of yeast and Penicillium glaucum. Yeast cultures inoculated with the mold showed an active normal fermentation. The stimulation, according to Kossowicz, was not to be looked upon as a mere symbiosis because dead mold mycelium would also cause the same stimulation in growth. It was due to substances which are water soluble. In 21 of 22 experiments he could not get growth in mineral salt-sugar solutions when only one cell was used.

Kossowicz concluded his paper as follows:

> We have seen that commercial sugar stimulates growth and fermentation. Also in these cases the slight traces of organic impurities in the crude sugar may be responsible for the stimulation. The tests mentioned above do not justify the assertion that for the multiplication of yeast certain organic compounds aside from sugar, are necessary, although it must be admitted that traces of organic compounds are of vast influence on the rapidity of yeast growth. This work emphasizes the saprophytic nature of the yeasts.

Kossowicz apparently connected the action of "bios" with Harden's co-enzyme.

Kossowicz (1906) later published another paper which showed that Mycoderma exerted a favorable influence on the growth and fermentation of yeast. This was also found to be the case by Amand (1904) and Ide (1907) who found that bacteria exerted a favorable effect on the growth of yeasts in mineral salt-sugar

media. Martinand (1908) also reported the favorable effect on the fermentation of yeast of Penicillium glaucum. However, in good media such as grape must, according to Müller-Thurgau (1892–93) and Behrens (1902) this Penicillium may exert an inhibiting effect. (Kossowicz, 1911.) These investigations are of importance in connection with the investigations which are being carried out at the present time.

In 1907 Henneberg reported an extensive study of the development of yeasts in synthetic salt solutions. He used the cultivated yeasts which were used in the Instituts für Gärungsgewerbe in Berlin. Seven such top and bottom yeasts were grown in synthetic solutions. Besides such observations as deposit, acid formation, etc., Henneberg studied the fermentation and growth of the yeasts. The solutions were sterilized in 50 cc. bottles to which were added counted drops of the thick yeast suspension. The cells were previously washed in water. Henneberg noticed that the first cultures were always more luxuriant and explained this on the basis that food materials were carried into the nutrient solutions along with the yeast cells In order to avoid this, Henneberg reinoculated several times to remove these food materials and also to reduce the food supplies in the cells themselves. While Henneberg was not directly concerned with the presence of accessory food materials, his data have indirect bearing on the question. He stated his position on "bios" as follows: "In conclusion it may be remembered that 'bios' can possibly be added in the form of soda, etc., to a solution."

Another explanation of the "bios" question was offered by Delbrücks (1908). This investigator stated that he had succeeded in demonstrating a poisonous substance in yeast cells which inhibited the growth of yeasts. This substance was demonstrable in distilled water containing salts. Delbrücks believed that the conditions in the mineral salt-sugar nutrient solutions were favorable for the action of this poison and that lack of growth and dying of the cells was better explained in this manner than on the basis of "bios." A year later Dzierzbicki (1909) reported that humus had a favorable action on growth and fermentation of small amounts of yeast.

Quite another explanation was given by Chrzaszcz (1904) who repeated Wildiers' work. Chrzaszcz prepared Wildiers' synthetic medium with salts of the best known purity and with water redistilled several times, the last time from apparatus of Jena glass This investigator chose four varieties of yeast, a brewery yeast, a distillery yeast, a wine yeast and a wild yeast. All of these yeasts developed in this medium and Chrzaszcz was led to regard his conclusions as confirming those of Windisch—that failure to secure growth by Wildiers was due to the presence of toxic metals in the medium. In order to prove this Chrzaszcz prepared Wildiers' medium with the ordinary distilled water and secured no growth as against good growth in the medium prepared from the carefully redistilled water. Chrzaszcz then concluded that "bios" did not exist and that yeast would grow well in synthetic media.

It is not easy to understand the results which Chrzaszcz reported after using the two different distilled waters. Besides Wildiers' solution Chrzaszcz also used several other synthetic solutions; two of them were prepared from the same inorganic salts with the difference that asparagin was the source of nitrogen in one and peptone in the other. While the growth was better in the peptone medium, it was not as good as in beer wort.

In 1906, after reviewing the work of Chrzaszcz, Pringsheim placed another explanation on Wildiers' work. He stated that yeasts may acclimatize themselves to mineral salt-sugar solutions and develop therein, whether one used a heavy inoculation or just one cell. A greater expenditure of energy was involved on the part of the yeast when the yeast was supplied with nitrogen, sulfur, phosphorus, etc., from mineral salts rather than from organic compounds. If only a few cells are transferred from a medium rich in organic matter, to the organic-free synthetic solution, it might find the latter medium a poor one. A heavy inoculation will contain a few cells which can adapt themselves to the salt-sugar medium and these will develop slowly. According to Pringsheim, "bios" was probably protein in nature and a protein which was especially available to the yeast. Pringsheim explained the growth of yeast in cases where larger amounts of

organisms were used in inoculation, by the liberation of food substances from dead cells. This was a fact which Henneberg had already reported. With small inoculations this food for the cells is absent. Through repeated transplanting, the yeast cells became accustomed to the solution so that also single cells showed multiplication.

Even in cases where a large amount of yeast constituted the inoculum the fermentation was slow in starting, while transfers from this showed results in a few days. Such cells acclimatized through numerous transplanting multiplied later, even when the inoculum consisted of very few cells. The acclimatization of the yeast was one of Pringsheim's important contributions.

This explanation of Pringsheim was denied by Ide (1907). He cultivated yeast cells in mineral salt-sugar media and even after weeks and months could demonstrate no change in characteristics nor acclimatization to ammonium salts. At no time could he prove that a yeast could become so adapted to ammonium salts that it could do without "bios." No substance could be substituted for "bios." "Bios" was said to be an organic nitrogen compound. Ide also stated the yeast became devitalized in a medium devoid of "bios" to such an extent that it could not recover even when "bios" was added. Devlo (1906) also endeavored to confirm Wildiers' conclusions. He inoculated 125 grams of mineral salt-sugar solution as did Wildiers but did not state how many cells were used. Two or three weeks later he observed a small daily loss of 0.05 to 0.01 gm. of CO_2. Intensive fermentation occurred in 48 hours in this solution if a sterile "bios" containing extract was added. This was reported in daily loss in weight varying 0.1 and 0.3 gram. He said that this substance was a molecule contained in lecithin. It was said to be a nitrogenous base without any relation to choline and probably an amine. It was precipitable by mercuric chloride and barium hydroxide and was soluble in a solution of phosphomolybdic acid. Its chemical characteristics were: (1) solubility in water, (2) not distillable, (3) the corresponding chloride, sulfate, and oxalate soluble in water and in 75 per cent alcohol, (4) precipitable by mercuric chloride neutralized by $Ba(OH)_2$, in the form of

a white mercurial compound from which the mercury is removed with difficulty, (5) alcohol below 80°, neutral or basic lead acetate silver in acid solution, phosphomolybdic acid, phosphotungstic acid, platinic chloride, double iodide of mercury and potassium, do not precipitate "bios." (6) It forms a molecule common in nature and it is none of the alkaloid or glucosides that have been isolated in the pure state.

Pringsheim (1907) in a second paper gave further opinions on the subject. He gave the ammonium salts great weight in yeast metabolism. Pringsheim found that peptone as N source increased yeast growth with increasing peptone concentration. Leucine, asparagin and ammonium sulfate did not seem to act in this way. Pringsheim's methods may be open to criticism. Pringsheim believed that the yeast used some of the organic nitrogen compounds for energy. About this time the literature contains numerous publications on the nitrogen metabolism of yeast from the laboratory of this author.

Bokorny (1907) also stated that small amounts of metallic poisons like copper might be poisonous to a single yeast cell while when many cells were used, a few might survive and grow; or the explanation might be that with many cells a little copper would be distributed in all of the cells and be too small to cause death. The presence of the organic matter might also use up the metallic poison. In the light of work since then, the metallic poison explanation seems very improbable.

After the vitamin question had been opened up this early work by Wildiers took on new significance. It was suggested that the substance "bios" might be a vitamin-like substance and consequently the literature, since 1917, is replete with publications on this question. Even after several years of experimentation by different ones in different laboratories, the question seems to be just as intricate if not more so, than ever. Some pertinent facts were reported by Kurono in 1915. He found that the addition of oryzanin (a constituent of the outer coating of polished rice) to fermenting solutions in very small amounts caused the activities of the yeast to be greatly speeded up. This acceleration in growth was especially noticeable in such synthetic mediums as

Nägeli's and Hayduck's solutions. The amounts used were 0.01 to 0.1 per cent. Kurono also reported that the growth of yeasts was stimulated by this substance in wort and koji extracts and especially in the artificial media.

Ehrlich (1911) has also reported that yeasts could be grown in pure solutions containing only mineral salts, sugar and amino acids. He found that the amino acids were deaminized and only the ammonia was used. The rest of the molecule was excreted as fatty acid or corresponding alcohol. The sugar was said to be the sole source of the carbon from which the yeast protoplasm was made. Ehrlich further studied the carbohydrate decomposition products which were necessary for protein synthesis. The greater portion of Ehrlich's work is not pertinent to this review and will not therefore, be discussed here. He was especially interested in an elucidation of certain phases of yeast metabolism and probably worked with pure solutions. The same problem interested Lindet (1917) who placed yeast in a solution of pure sugar and ammonium sulfate; these materials were then the sole source of carbon and nitrogen. The fermentation was very slow and only a small amount of yeast was formed by weight. Synthesis of proteins by yeast fed with amino or ammoniacal nitrogen in the presence of pure sugars alone proved very difficult. Addition of not more than 2 per cent of other carbohydrates such as gum arabic, tannin, or brown sugar or peat, caused the fermentation to proceed more rapidly with 3 times the formation of yeast. These data, of course, do not necessarily indicate that the increased crop was due to hypothetical substances.

Susuki's work with oryzanin stimulated Kita (1914) also, to investigate the relation of this substance to the assimilability of maltose by yeast. He used a synthetic medium with the following composition: 1000 grams of water; 0.25 gram magnesium sulfate; 5 grams KH_2PO_4; 5 grams of ammonium nitrate; and a few drops of ferric chloride. To 100 cc. portions of this medium were added the following types of maltose: 5 grams of maltose which had been extracted with absolute alcohol, 5 grams of unextracted maltose, and to a third flask 2.5 grams of each kind of maltose. These culture flasks were inoculated with loop-fuls of

yeast culture. Data presented in the paper indicate that the purified maltose was less readily assimilated by yeast (Saccharomyces saké) than impure maltose. Kita believed that this was explained on the presence of an oryzanin substance in the unpurified maltose. Such reasoning has also been characteristic of quite a little of the work in this field. Why should he explain his data on that basis rather than on the presence or absence of a chemically definite substance? Kita probably added very little of value to the discussion.

Volk (1915) experienced no difficulty in using yeasts grown in synthetic solutions in animal feeding. Williams (1919) was one of the first to study this question with the idea in mind that vitamins were essential for vigorous growth of yeasts just as certain other substances (vitamins) are necessary for animals. He grew his yeasts in synthetic salt solutions to which small amounts of the materials to be tested were added. From this work it was stated that the anti beri-beri vitamin was essential in yeast growth. When a very small amount of the alcoholic extract of "protein-free" milk, wheat germ, lactose, or fullers earth activated by wort, were added to the synthetic media, growth of yeast cells was hastened. This, according to Williams indicated the identity of the water-soluble vitamin in different materials. This work was carried out with single cells. Williams did his work with a pure culture of yeast secured from the Fleischmann Company which would indicate that it was probably a culture of Saccharomyces cerevisiae. The correctness of Williams' hypothesis rests, then, on the absolute identity of vitamin B. necessary for the growth of mammals and birds, and the accessory substance which stimulates the growth of yeast. Williams' medium had the following composition.

Cane sugar	20	grams
Ammonium sulfate	3	grams
KH_2PO_4	2	grams
$CaCl_2$	0.25	gram
$MgSO_4$	0.25	gram

Williams stated that the phosphate buffer served to control the hydrogen ion concentration of the medium. This medium was

sterilized. He made the interesting statement that if a medium became contaminated, yeast cells grew in the solution better than before contamination although the solution was sterilized before being seeded with yeast. Others had also reported this some years before Williams worked. In the discussion following this paper Williams stated that it was apparently possible to cause single yeast cells to produce from 20 to several thousand cells in twenty-four hours by varying the vitamin content of the culture medium. Williams (1920) later changed this method so that a large number of cells could be used. The changes in technic which Williams proposed in his second paper, were significant. He changed from the use of drop cultures to a method of weighing the yeast mass. Williams apparently believed that the data secured by any method could be compared and that a method based on estimation of the multiplication rate could be replaced by a method based on estimation of yeast mass by weighing. A vitamin number was secured. His vitamin number was defined as the number of milligrams of yeast producted by the addition of its extract minus that produced in a control solution under given conditions and within certain limits computed to 1 gram of the original material tested. A solution of ammonium sulfate and asparagin was not improved by the addition of amino acids but was by a small amount of antineuritic vitamin. Williams was probably the first to assume that "bios" and vitamin B were identical.

From Williams' description one may be quite certain that he did not have pure cultures. He described his technic as follows:

> A yeast suspension is made by weighing out 0.300 gram of fresh Fleischmann's yeast (small cake in tin foil) taken from the center of the cake; this is made into a paste with a very small amount of water and suspended finally in 1 liter of sterile water. One cubic centimeter of this suspension well shaken and freshly made is introduced into the culture medium with a sterile pipette.

A bacteriologist knows that cakes of Fleishmann's yeast contain many bacteria which according to Williams' technic would

appear in his media. As stated above, Williams claimed that the presence of bacteria greatly stimulated the growth of the yeast. Even though he tried to avoid contamination by the use of sterile water and sterile apparatus, his experiments were probably contaminated at the very beginning. Furthermore, he stated that his medium was "sterilized or pasteurized to kill all vegetative organisms," etc. This idea was well emphasized by Ide who called attention to the possibility of small cocci contaminating the flasks. Numerous bacteria are known which produce very resistant spores. Some have been known to withstand seventeen hours continuous boiling. This method for detecting the presence of vitamins has been criticized by many in America and in foreign countries.

Wright (1922) freed lemon juice from citric acid according to Harden and Zilva's (1918) method and reported that small quantities enabled a yeast to grow which would not grow in its absence. Wright then, by means of Williams' nutrient solution, attempted to ascertain the smallest amount of lemon juice capable of producing growth. The yeast used was baker's yeast which was washed three times with sterile distilled water. One loopful of the yeast suspension was used in each tube; the tubes also received increasing amounts of the lemon juice. No growth took place in the tubes until 5 per cent of the lemon juice had been added. Finally Wright used the hemacytometer method for counting the cells. Wright stated that it was necessary to determine whether "bios" was a vitamin, and, if so, its identity with one of the known vitamins. He reported that "bios" did not enable a yeast to assimilate ammonium sulfate simply by its presence or by being consumed at the same time, but that the yeast grew solely at the expense of the "bios" until it reached a certain concentration, after which it was able to use the ammonium sulfate.

Bachmann (1919) studied the problem using a strain of yeast isolated from "Yeast Foam". Two sizes of inoculum were used. A loop of the yeast growth from solid media was used for heavy inoculation and a loop of a water suspension for a lighter inoculation.

Two types of media were used as follows:

A

Distilled water	100 cc.
Ammonium tartrate	1 gram
Yeast ash	1 gram
Dextrose (dextrose)	10 grams

B

Distilled water	838 cc.
Ammonium tartrate	10 grams
Magnesium sulfate	0.2 gram
Calcium Phosphate	2 grams
Potassium acid phosphate	2 grams
Dextrose	150 grams

Bachmann used fermentation tests (gas formation) as criteria of growth. Vitamin extracts prepared from orange pulp were added to the media. Addition of the vitamin extract seemed to favor the growth and Bachmann concluded that the media were not entirely favorable for fermentation with these yeasts and that the addition of a vitamin extract accelerated growth. Bachmann also proposed that in heavy inoculations, some of the cells might be dead and that these cells could contribute the growth stimulating factor. In the rest of her work Bachmann used two other strains of yeast designated under the names of No. 49 and 51. The first came from "Yeast Foam"[1] while the latter came from canned pears. The medium in each case was Nägeli's Nutrient Solution. Nägeli's medium was not a good medium for yeast 51. It became a good one only after some yeast water was added.

This was due to either a little organic matter or to vitamins. Very large numbers of cells had to be introduced to get growth without the hypothetical substances. Bachmann was then led to conclude that this strain could be used for detecting the presence of vitamins in unknown materials with the heavy inoculations which made the medium more favorable for the growth of yeasts. This substance was postulated to be vitamin B and when vitamin B prepared from orange peel was added to the

[1] "Yeast Foam" is a commercial yeast preparation for leavening. The yeast cells are incorporated in starch such as corn meal, etc.

medium, fermentation took place much more quickly. The tubes which received heavy inoculations and vitamin required just half as long to show gas as did those with no vitamin. With the tubes receiving light seedings, it required almost 4 times as long to show gas. From this and other data, Bachmann concluded that Pasteur and Wildiers were both right. Bachmann's use of fermentation methods as criteria of growth and multiplication is open to criticism. It is known that yeast cells may ferment after they have ceased growing. Euler and Petterson stated that in general increased fermentation and growth did not parallel each other.

About this time Lindner, whose work on yeast in Germany should make his opinions on "bios" of interest, entered the field of discussion, with a unique theory. Lindner (1920) reviewed the various opinions which had been advanced to explain the action of yeast in synthetic solutions but did not regard any one of them with much favor. Lindner did not agree with Naumann that weak or dead cells gave off a soluble nitrogen compound in the presence of much sugar. He stated that a cell rich in plasma budded in 5 per cent solution of pure sugar, developing in clumps of from 6 to 8 cells and became at the same time extraordinarily rich in fat. The fat appeared only when substances high in oxygen were present; without oxygen the buds and colonies were smaller but the cells were richer in protein, than if grown in presence of oxygen; the cells rich in protein, which are easily distinguished through the large size of cells and vacuoles are responsible for the acidity, which results from removing of NH_3 from the NH_4 compounds. In the absence of air, the alcohol formed diffused out the cells very rapidly.

In presence of oxygen the cell retained the alcohol and transformed it into fat. In this case the alcohol was not excreted but served as nourishment for the cell. Without oxygen there was no fat building, even in presence of rich sugars and alcohol. Therefore, when a cell showed numerous granulations it proves that it was in contact with oxygen. Where strongly granular yeast forms are seen we cannot expect the alcohol output to be proportional to the fermentation. Where the cells are rich in fat, as on

the top of gelatin culture, it was assumed that this was obtained from the alcohol that came from the underlying strata. Alcohol is also transformed into fat in cases where it is in vapor-form. Whether alcohol is a poison for the cell can be grasped in the fundamental sense of the question, only after following the processes under the microscope. The cell may also be immoderate in the use of the alcohol, becoming fattened with from 40 to 50 per cent of fat. It then loses the ability to bud further. The more scanty the nitrogen supply, the slower the budding, but the more pronounced, however, the fat production. This situation exists in the nutrient mineral salt-sugar solution. The NH_4 salts are altogether suitable only for plasma synthesis, but when the fat formation out of alcohol sets in, then the process of budding is inhibited. Where the cells are separated in aerated solution, they can provide themselves with oxygen and so instead of giving off the alcohol, they transform it immediately into fat.

It is another story where the cells are close together. Here because of competition the oxygen is immediately divided and possibly used for budding, but the supply seems limited for budding and for fat making also, because enveloping the cells is carbonic acid.

According to Lindner, the controversy, whether Liebig used an inoculating mass the size of a pin-head, and whether Pasteur, in way of illustration, used a larger one, appears very insignificant today. Of more importance is the amount of air in the solutions which they both used; how much was absorbed during inoculation. Still more important in the final solution of the "bios" question is the established fact, that alcohol in an excellent way serves as the fat builder for the cell, and can be used in such capacity especially by our cultured yeasts.

This interesting explanation of the "bios" question on the basis that yeast cells undergo a "fatty degeneration" in mineral salt-sugar solutions which are well aerated, was carried further by Lindner (1919, 1921). He stated that Liebig may have worked with such a solution when he tried to repeat Pasteur's experiments. Lindner suggested that Pasteur may have used freshly sterilized solutions in which the "fatty degeneration" would

not occur since without oxygen neither sugar nor alcohol could be changed into fat. Lindner thus places the explanation of the "bios" question on the methods of experimentation. If Lindner's explanation is true, we then have a more satisfactory explanation for the controversies which have arisen in this field.

Fleming's (1921) work seemed to indicate that the addition of organic nitrogen to the inorganic media might accelerate yeast growth. This organic nitrogen probably came from the vitamin extracts. Fleming followed the technic of Fulmer, Nelson, and Sherwood and determined the "Count," i.e., the number of cells seen in 16 small squares of the hemacytometer. Fleming used an extract of Fleischmann's yeast in 0.1 per cent of acetic acid as a standard source of vitamin B. The yeast method was given up as a means for estimating vitamin content of materials. When the stimulating extracts were treated with alkali to destroy any water soluble B vitamin, the effect on yeast growth was as great as before treatment and in some cases greater. This led Fleming to discredit the yeast test as a means of testing for the presence of vitamin B.

In order to satisfy themselves with regard to the availability and accuracy of the Williams' method for testing the presence of antineuritic substances (water soluble B vitamin), Souza and McCollum (1920) undertook some work in the field. On account of considerable difficulty in carrying out the test, as outlined by Williams, Souza and McCollum introduced a modification in which a platinum syringe needle, having an opening at right angles to its axis, was used for depositing the droplets on the cover-slip. The needle was attached to a piece of rubber tubing and with this instrument the suspension of yeast could be uniformly distributed. Other modifications were introduced which need not be reproduced here. Souza and McCollum made no statements in their paper concerning the yeast used other than that it was Fleischmann's yeast. Was it a pure culture? These workers then went on to examine, by the modified Williams' procedure, several substances known to be rich or poor in the dietary factor water soluble B. They first studied the effects

of addition of hot-water extracts of wheat germ to the basic synthetic medium. They did find a stimulating effect of the water extracts on the rate of multiplication of the yeast and that in general the greater the amount of extract added, the more pronounced the multiplication. The same was found for the alcoholic extracts. In further work with other materials, Souza and McCollum were led to conclude that the yeast test was complicated by so many factors that it was probably of little or no value. They further stated that whenever extracts of natural food were to be tested, it was inevitable that food substances of one kind or another which greatly stimulate the growth of yeast must be added simultaneously with the unidentified dietary essential for which the test was designed. Glucose and amino acids caused such a stimulation. They did not wish to commit themselves on whether the antineuritic substance was necessary for the development of yeast, or that it did not stimulate the growth of yeast.

Linossier (1919, 1920) used an organism closely related to the yeasts Oidium lactis and also Mycoderma vini. In this contribution, he reported that although Oidium lactis could grow in media composed of pure materials, it was sensitive to the action of vitamins. With a very small inoculation vitamins are indispensable. The addition of vitamin containing extracts increased the crop markedly. Linossier claimed that certain fungi could get along without vitamins while others required them. From the data presented by Linossier one may conclude that there is a marked difference in the crop of Oidium lactis with and without vitamin during the first few days, but later the amounts are more nearly alike.

Lumiere (1921) working with some of the common molds reported that they did not need vitamins. Even in media which are not good ones for abundant and rapid development the increased growth resulting from the addition of organic extracts containing vitamins did not seem to be due to the vitamins. The addition of organic extracts to poor media may be advantageously replaced by chemically definite mineral salts. These data, then, with molds agree very well with those reported for the yeasts.

Sammartino (1921) reported a study which bears perhaps indirectly on the subject and which probably should be included. He found that both the rapidity of reproduction and the rapidity of fermentation of the yeast cells are affected in a positive manner by vitamins. He then sought to determine whether the vitamin stimulated the cell or possibly the cell membrane or whether the zymase itself was acted upon. The question whether it was the cell or the cell enzyme which was stimulated was investigated on the cell-free fermentation (on the zymase separated from the cell); it is known that zymase consists of the true zymase and the coferment; as the zymase without coferment is in itself ineffective and unable to split sugar; and as coferment by itself is a promoter and hastener, the question arose whether the vitamin acted upon the true zymase or upon the coferment; and, also whether the vitamin action was purely upon the zymase or whether it acted in a similar manner upon enzymes of other kinds.

Sammartino first investigated the behavior of other enzymes in the presence of added vitamin. Proteolytic and amylolytic enzymes and catalase were examined. In the albuminous digestion with pepsin no effect upon the pepsin digestion was noted from the presence of vitamin; probably for the reason that vitamin was destroyed in the presence of a mineral acid or that the high pH inhibited the vitamin in its action upon the enzyme. When the effect of vitamin upon the tryptic digestion was examined in the usual alkaline solution, it was seen that the vitamin hastened the trypsin enzyme in the alkaline medium. Vitamins showed only a slight effect upon the influence of amylase. The experiments with catalase showed that the effect varied markedly according to the reaction of the medium and in the presence of various substances which acted in a favorable or inhibitory manner. It was therefore shown that the vitamin just as it promotes yeast fermentation, also promoted the cell-free zymase fermentation, but that the effect on other splitting enzymes was by no means marked if it occurred at all.

According to the experiments of Hardin and Young zymase can be separated into 2 parts: if the zymase is dialyzed or filtered through a colloid filter, a substance is left on the filter which is

unable by itself to split sugar into alcohol and carbonic acid; the dialysate is also unable to split alcohol and carbonic acid; but if both are reunited the splitting of the sugar results with formation of carbon dioxide and alcohol; if, however, the substance found on the filter is previously heated and then combined with the unheated filtrate, no fermentation occurs, but if the substance found on the filter is not heated but combined with the heated filtrate, fermentation occurs. From this the conclusion can be drawn that in the yeast juice there is an enzyme and co-enzyme and that the enzyme is active only in the presence of the co-enzyme that will stand boiling. The same line of experiment was carried out by Miller (1921). She found that an alcoholic extract of yeast did not accelerate the action of invertase but did stimulate the growth of the cells. The amount of invertase, however, in the cells was increased. This stimulation in growth and the increase in invertase was said to be caused by two different substances. An ethyl alcohol extract of wheat germ stimulated the growth of the yeast but had no effect on the amount of invertase. We may also mention Villaroel's (1923) statement that vitamin B stimulated the action of lipase.

Euler and Petterson (1921) reported a study of the effect of lemon juice, extract of wheat embryo, and extract of yeast on the fermentation of yeast as measured by carbon dioxide production and on the growth of yeast as measured by cell count. The fermentation of the yeast was generally increased by the addition of these extracts, but the amount of carbon dioxide evolved was not proportional to the vitamin concentration, an excess of the extract having an inhibitory effect. An extract of yeast from which the protein had been removed by precipitation with alcohol showed proportionality between the amount of extract and increase in fermentation.

In general, increased fermentation and cell growth did not parallel each other, sometimes an increase of 100 per cent in the fermentation being accompanied by only 10 per cent increase in the cell count. By altering the amount of vitamin preparation it was found possible to obtain a proportionality in the fermentation and cell growth. In another article which gave further results

from the same study, Euler and Myrbäck (1921) described a method of determining the relative amounts of water-soluble vitamin (biocatalyzers) in different materials through the fermentation of yeast.[2] In the nomenclature employed a distinction is made between the antineuritic and the growth-promoting vitamin B. For the former the name vitamin B was retained. The term biocatalyzer B was used to include biocatalyzer B I, the growth-promoting factor of yeast; and biocatalyzers B II and B III, both of which influence fermentation. B III was considered identical with Harden's co-enzyme. The solution used in the tests reported consisted of 25 cc. of a 2 per cent phosphate solution of a hydrogen ion concentration, pH = 4.5. In this were dissolved 2 grams of sucrose and 1 gram of finely pulverized dried yeast, and the solution was made up to 50 cc. with water or a solution of the material to be tested for vitamin activity. Fermentation was allowed to continue at a given temperature for six or seven hours, and the amount of carbon dioxide evolved was measured at the end of given periods of time. Extracts of wheat embryo and blood serum tested in this way showed an increase in the amount of carbon dioxide evolved per hour up to a given concentration of the extract, after which there was a decrease, thus indicating the presence of an inhibiting factor in the extract as reported above. To determine the amount of biocatalyzers II and III in any material, the amount necessary to add to the standard solution described in the first paper to bring about half the maximum fermentation was determined. From this amount of vitamin biocatalyzer B II distinguished from the co-enzyme biocatalyzer B III could be determined by the method of Tholin described below. The yeast used in the experiment should

[2] Mention should be made of the investigations of Fränkel and his colleagues in Germany who used the fermentation of yeast as measured by carbon dioxide determinations for estimating the content of vitamin B. Standard conditions were adopted for carrying out the test. Fränkel also attempted to isolate the active substance from yeast and rice polishings. The methods used will not be repeated here although they are of interest in connection with similar attempts to isolate pure "bios" by Lucas (1924) and Eddy, Kerr and Williams (1924). Using his yeast method Fränkel tested the vitamin content of a number of different animal organs. All of them gave stimulation except bone marrow. Cholin, cholamin and -amino ethyl alcohol showed an inhibiting effect.

first be standardized to determine its fermenting power alone. Data were reported on the vitamin B content of human blood serum, feces, and urine. These indicated a considerable daily excretion of vitamins.

Tholin (1921) also compared the propteties of co-enzyme with those of vitamins. In this combined study of the co-enzyme and vitamins of yeast, the author used as the foundation, yeast material finely pulverized, dried yeast of constant fermenting power. This was rendered inactive by shaking a mixture of 1 gram of the yeast and 50 cc. of the water for one and one-half hours on a shaking machine and centrifuging for ten minutes at 1700 revolutions per minute, after which the opalescent liquid was decanted and the treatment repeated. The resulting yeast was almost completely inactive when tested with 5 per cent glucose solution. The co-enzyme preparation was made by adding a suspension of 150 grams of pulverized dried yeast in 300 cc. of water to 1200 cc. of boiling water, heating the mixture to boiling, and filtering after two minutes. The filtrate was concentrated in a vacuum at 25° to a volume of 50 cc. and the extract precipitated with 96 per cent alcohol. The precipitate, which consisted principally of proteins, higher carbohydrates, and phosphates, had no co-enzyme activity. The filtrate, which contained all the co-enzyme, was treated with 300 cc. pure acetone. The yellowish-white milky precipitate formed yielded on trituration with absolute alcohol a yellowish-white sandy hygroscopic powder rich in co-enzyme. The powder was easily soluble in water, forming a clear light yellow solution with an acid reaction (pH = 5.6). On analysis it gave an ash content of 26.3 per cent and P_2O_5 content of 14 per cent calculated on the dry basis. The substance could be further purified by adding to the water solution lead acetate, filtering and adding acetone to the filtrate after removing the excess of lead with hygrogen sulphid. On further precipitation with lead acetate and magnesium mixture a precipitate was formed, showing the persistance with which the phosphoric acid clings to the co-enzyme. Tholin was of the opinion that the function of the co-enzyme in alcoholic fermentation was intimately bound up in the phosphoric acid and also that the

co-enzyme was of the nature of an ester. In describing the preparation of the vitamin, attention was called to the similarity between the method described above and the method described by Osborne and Wakeman (1919) for the preparation of vitamin B from yeast, thus showing that the vitamin is present in the co-enzyme preparation. The essential difference in preparation of the co-enzyme and the vitamin in the present study is that in the latter case the material was sterilized by boiling for forty-five minutes or more at 100° before precipitation. Other materials used in the study were a 20 per cent glucose solution sterilized for two hours at 100° and a solution of NaH_2PO_4 of an H-ion concentration of pH = 5.1. The basal solution employed consisted of 1 gram of washed yeast, 5 cc. of the phosphate solution, and 10 cc. of the glucose solution, the whole made to a volume of 20 cc. To this was added the material to be tested for co-enzyme or vitamin and the amount of carbon dioxide evolved in a given time determined. The thermostability of the co-enzyme preparation was found to be a function of the temperature and the acidity. At 96° and an acidity of pH = 5.6, the co-enzyme activity was destroyed to the extent of 50 per cent in one hour, and at 100° in thirty-seven minutes. While the co-enzyme was thus destroyed by heat, the vitamin preparations from yeast and cabbage were active even after heating for an hour at 107 and 127°. The essential difference between co-enzyme and the vitamin preparations was thus shown to be the difference in thermostability.

Harden and Zilva (1921) sought an answer to two questions, whether a yeast grown in a medium devoid of vitamin B could produce it and whether different species could produce this vitamin. This paper was merely a preliminary note and was prompted by Nelson, Fulmer and Cessna's paper. Harden and Zilva grew their yeast on a synthetic medium containing ammonium phosphate and chloride as sources of nitrogen together with the necessary mineral salts and cane sugar. The cane sugar was fractionally precipitated from aqueous solution with alcohol, and the solution of the dried purified material then shaken three times with fuller's earth to remove any possible trace of vitamin

B. The yeasts thus obtained were washed, pressed, and dried and compared with a parallel culture of Saccharomyces ellipsoideus grown on unhopped brewer's wort and tested as to their curative effect on polyneuritic pigeons. Harden and Zilva concluded that yeasts grown on synthetic media contain vitamin B but not to such an extent as yeast grown in wort.

A short time elapsed after the appearance of the "Bachmann Test" before it received critical study. One of the first reports of such study was published by Eddy and Stevenson (1919). Their experiments were pointed toward confirming Bachmann's results to determine whether the method gave promise of use quantitatively and whether it might be used to detect the "B" vitamin qualitatively. Without repeating the details of these experiments which were patterned closely after the Bachmann method, we may record the conclusions which Eddy and Stevenson reached. They were unable to check the gas in the various fermentation tubes. For quantitative work, then, the test was believed to need further standardization. Another difficulty was said to be the great variability of loopfuls of yeast. Eddy and Stevenson, however, concluded this short report by saying that the test offered such marked advantages in sensitiveness and in speed of observation over rat feeding methods that it seemed worth while to devote more time to its improvement.

In their next paper Eddy and Stevenson reported data secured by using the Williams method. As a result of their studies with both of these methods, they derived a method for measuring the vitamin B content of food materials by means of yeast cells. The Bachmann method measured vitamin content in terms of gas generated. This method, then, is based on enzyme (zymase) activity and may not measure the phenomena in terms of growth. The Williams method tested the vitamin content in terms of growth and therefore seemed to be more valuable according to Eddy and Stevenson. The latter investigators then reported a method of their own, using Nägeli's solution, the exact details of which need not be mentioned here. This method is based on growth as determined by counting the number of cells which appear in the medium after incubation in sealed tubes. They

really measured the multiplication rate and not growth. Eddy and Stevenson first of all studied the specificity of their test. They interpreted their data to indicate that the test was specific for the so-called antineuritic vitamin or water soluble B. They studied some of Funk's purified antineuritic vitamin and some navy bean extracts prepared according to a method of McCollum and Simmonds (1918). They stated that what Funk called antineuritic vitamin as prepared by him and what they called water soluble B as prepared according to McCollum and Simmonds both responded to the test whether they were identical or not. Eddy and Stevenson ruled out the "impurities" in their vitamin extracts as factors stimulating growth, and concluded that they were studying the stimulating effect of the vitamin. They next studied the possibility of measuring quantitatively the vitamin content of various materials. They were not quite so successful in this respect but hoped that by further work the technic could be developed to accomplish this. In 1920 Eddy and Stevenson made a more comprehensive report of their earlier work on the yeast test as an indication of vitamin content. No new data seem to have been secured but their modified method based on the Bachmann and Williams' methods was applied to other materials. They reiterated from experimental data that their test was sensitive to small amounts of vitamin extracts. They stated,

> From these results it seems fair to conclude that the test works with small quantities of the Funk antineuritic vitamin as prepared by him in the purest form we have studied to date and tends to strengthen the conviction that the antineuritic or B vitamin is the responsible causative agent in stimulating the growth of yeast cells.

The next experiment was to determine by means of their test method whether Lloyd's reagent would absorb vitamin B from navy bean extract as stated by Seidell and Williams. Using their yeast test they found that the "cause of the stimulus" was almost quantitatively removed from the navy bean extract and orange juice. They also attempted the confirmation of Osborne and Mendell's classification of the various foods on

their vitamin B content by rat feeding. They argued that if the yeast test confirmed Osborne and Mendell's data with animals it would thus gain much support. Eddy and Stevenson did not wish to draw conclusions from their data on this question. In another place, however, when apparently referring to the same work, they stated that the results with the yeast test confirmed animal feeding.

Emmett and Stockholm (1920) sought to find out by means of the yeast test whether the antineuritic and water soluble B vitamins were different. They used the Williams micro method. They did not state what yeast was used but apparently worked under sterile conditions with a pure culture. They made the interesting statement that the Williams method can be used with definiteness for measuring the rate of growth of yeast. The next point studied was whether the yeast growth promoting factor was the antineuritic vitamin. These two substances were found to be different. The yeast growth promoting factor also failed to stimulate the growth of young rats which would indicate that the yeast growth promoting factor was different from water soluble B. In the last sentence of their conclusions, they stated that the yeast method should not be employed with too much definiteness until further study was made.

In 1921 another report was published by Eddy in collaboration with Heft, Stevenson, and Johnson, and was apparently prompted by the many criticisms which had been published against the yeast test. For various reasons these authors did not use the method proposed by Eddy and Stevenson in 1920 but adopted one announced by Funk and Dubin in 1920. This method consisted in growing the yeast in Nägeli's solution to which the vitamin extract may be added and a subsequent sedimentation of the yeast cells in a Hopkins vaccine standardization tube on a centrifuge. More data were first presented by which the yeast test was compared with the rat feeding test for evaluating the vitamin B content of various food materials. Only approximate agreement was secured by the yeast growth procedure and the rat feeding method of Osborne and Mendell. The most interesting statement in this paper, however, may be

that in which they announced that the yeast test was unreliable. They stated that until a basal medium had been worked out which provided all of the factors of a good medium with the exception of vitamin B the yeast test must be considered of little value. One difficulty in comparing data secured by the several methods which Eddy and his colleagues used was overlooked. The same criticism may be applied to other investigators but since Eddy at various times tried out four different methods, the Williams method, the Bachmann method, his own modification of these methods and finally Funk and Dubin's, all of which used different technic for measuring "growth" of the yeast in response to vitamin stimulation, we may use their work as an illustration. The multiplicity of terms such as growth, multiplication, development, proliferation, propagation, etc., which have been used suggests that the problem is not a simple one. Funk and Dubin's method really measured the volume of yeast mass while the other methods measured multiplication. Slator reported during a discussion of this very thing that the volume of yeast mass could increase 20 per cent after multiplication had ceased. It involves some error, then, to compare the data from any of the methods too closely. Data were also reported at this time by Eddy and his colleagues which seemed to invalidate the conclusions of Fulmer, Nelson and Sherwood that it was possible to develop a medium in which yeast will grow indefinitely without added vitamin. Fulmer, Nelson, and Sherwood claimed that their Medium F was such a medium and that it was not improved by the addition of organic extracts and that since the stimulative factor on yeast growth was not affected by alkalies it was not vitamin B. In order to secure data on these points Eddy and his colleagues substituted Medium F for Nägeli's medium. Eddy's data showed that Medium F was a better medium than Nägeli's but that it was improved by the addition of alfalfa extract. Their work in testing the effect of alkali on the yeast growth stimulating factor did not allow them to make definite statements. Their data seemed to show the destruction by the alkali of some factor but the extent of the destruction was not

as great as would be expected if vitamin B formed a large part of the stimulative factors. Although Eddy and his colleagues did not state it, these data may perhaps be regarded as some of the first indicating a possible difference between vitamin B and the yeast growth promoting substance.

Naumann (1920) took up the investigation of the behavior of yeasts in inorganic salt-sugar nutrient solutions. Naumann used the same wine yeast which had been used by Pringsheim. Naumann did his work with Laurent's medium. When 10 cc. of Laurent's solution were inoculated with only five cells, there was no increase in numbers nor evidences of fermentation even after forty days; when 50 cells were introduced into the medium germination was noticed in three days and fermentation after ten days; in forty days the count was 21,000,000 cells per cubic centimeter. Naumann's work suggests that some investigators who used short transfer periods may not have given their yeasts an opportunity to grow. The factor of dilution outstripped multiplication and growth. By increasing the amount of inoculum, Naumann was able to markedly decrease the time required for evidences of growth and multiplication to appear. Naumann believed that the growth in tubes with the heavier inoculations was due to a disintegration of dead cells. In this case the larger the number of cells in the inoculum, the more food would be available after disintegration. When just a trace of organic matter such as peptone to the extent of 0.0005 per cent was added to the cultures, the yeast was helped over the threshold and multiplication took place rapidly. According to Naumann, then, yeasts and other fungi could perhaps form "bios"; however, Naumann's ideas of "bios" were somewhat different from those of certain other workers. He suggested that "bios" might be a group of complicated organic substances such as nucleo-proteins or nuclein. The acclimatization theory of Pringsheim was accepted that a yeast cell accustomed to use organic food materials could be trained to use inorganic materials. The stimulating effect on yeast growth of other organisms such as scum yeasts, and molds, was explained on the basis of commensalism rather than "bios." Naumann believed that these

microörganisms made nitrogenous compounds more readily available to the yeast cells and that it was this factor and not "bios" that stimulated the growth of yeasts. Naumann also selected narrow tubes instead of the wider vessels in which to carry out his experiments. Lindner (1920) pointed out that the use of these tubes by Naumann instead of flasks, made it easier for the materials which Naumann said diffused from dead cells to come in contact with living cells, since the distance would probably be shorter in the tubes than in flasks.

Somewhat the same quest led MacDonald and McCollum (1921) to continue work in this field. They wished further light on the requirements of accessory substances on the part of yeast and also information on the possible identity of the antineuritic substance and "bios." They used a medium called nutrient solution no 2. having the following composition:

Distilled water	1000	cc.
Sucrose, recrystallized (2 per cent)	20	grams
Ammonium sulfate	3	grams
Potassium dihydrogen phosphate	2	grams
Calcium chloride	0.25	gram
Magnesium sulfate	0.25	gram

Another medium spoken of as nutrient solution 3 contained 5 per cent of sucrose. These solutions were heated to boiling on two successive days. The numerous instances in the bacterial world of the formation of very heat resistant spores, should suggest the use of more reliable methods of sterilization. On the third day, 25 cc. portions were measured into sterile Erlenmeyer flasks by means of a sterile pipette. These flasks were then heated to boiling and stored for use. Three strains of yeast were used, two bakers' yeasts "F" and "XII" and a brewers' yeast "K." The cultures were started by using a small loop of material from an agar slant. This was not enough yeast to produce turbidity in the solutions. MacDonald and McCollum seriously questioned whether the growth of yeast was dependent on a supply of the antineuritic principle for its continued multiplication. They grew quantities of yeast in this purified nutrient solution sufficient to furnish from 2 to 5 grams of dry substance.

They stated, "It would seem that one of two conclusions is admissible; i.e., either yeast must grow without "bios" or it must synthesize the substance to meet its own needs (as was believed by Henry)."

Ide (1921) cautioned against accepting MacDonald and McCollum's conclusions. He stated that there were two kinds of proliferation; one was a very slow one without "bios" and the other fast with "bios." There was said to be such a difference between the two that it could not be overlooked by anyone who had seen it. Ide showed that "bios" greatly accelerated the fermentation of sugar. When "bios" was present 30 times as much sucrose was decomposed as when it was absent. MacDonald and McCollum were not contending that "bios" would not stimulate growth but that yeasts could grow in a synthetic medium indefinitely, either getting along without it or making it. Ide (1921) also stated that it was too soon to announce that water soluble B vitamin and "bios" were the same substance. He called attention to the fact that no organic material had been found which caused such a stimulation of growth as "bios." Ide suggested that MacDonald and McCollum's flasks may have been contaminated. He had found small cocci in flasks which had been held for a time. MacDonald and McCollum (1921) replied to Ide with further discussion and data to confirm their former conclusions. They stated that neither the hypothetical "bios" nor the antineuritic or other uncharacterized dietary factor essential in the nutrition of mammals need be supplied in order to enable yeast to develop. The difference in the rate of proliferation may be due to other causes than the presence of accessory factors. MacDonald and McCollum mentioned Fulmer's work showing that viscosity of the medium might be one cause. MacDonald and McCollum stated that it was fully established that yeast did not need vitamin B, "bios," or any other uncharacterized dietary factor. They also defended the purity of their yeasts and the cultures in the flasks. If MacDonald and McCollum's suggestion that increase in growth after the addition of supposedly "bios"-containing substances might be due to other materials in the "bios"

preparate than "bios" or to other factors, had been kept in mind by some investigators since then, we would probably have a field of knowledge less clouded by controversy.

Goy (1921) working with a yeast (Saccharomyces cervisiae) along with other lower forms of plant life reached the conclusions that such accessory substances as vitamins were not necessary. He did state that the addition of a small amount of the inorganic medium in which the same species or different species had grown stimulated their proliferation. By means of ether he extracted this stimulating substance from a culture of mucor. It did not exhibit growth promoting properties until it had been heated to from 85° to 90° and lost it at from 168° to 170°. Such properties excluded it from the group of accessory factors known as vitamins.

As stated above, in 1921 Fulmer, Nelson and Sherwood announced that they had devised a medium which would support the growth of yeasts and which was not improved by the addition of water soluble B vitamin. They stated that vitamins were not necessary constituents of media for the growth of yeasts. They carried out their experiments with a culture of Saccharomyces cerevisiae, Race F., isolated in pure culture from a cake of Fleischmann's compressed yeast. They prepared alcoholic extracts of wheat embryo and alfalfa and studied the effects of adding these to the mineral salt-sugar medium which differed from Williams' medium only in the amount of sugar. These products stimulated the growth of yeasts but the authors showed that the stimulation was not due to vitamin B since it survived treatment with dilute alkali. They also found that extracts of alfalfa and wheat embryo contained sufficient nitrogen and inorganic material to influence the growth of yeast. They stated that the addition of water soluble B did not improve their synthetic medium. The results of Eddy, Heft, Stevenson and Johnson with Medium F have been referred to above.

Fulmer, Nelson and Sherwood (1921) next gave some attention to the relative amounts of various constituents which were necessary for nutrition of yeasts. A medium containing am-

monium chloride 0.188 gram, calcium chloride 0.1 gram, dipotassium phosphate 0.1 gram, precipitated calcium carbonate 0.04 gram, dextrin 0.6 gram, and sucrose 10 gram. in 100 cc. of water was said to be better than Williams' medium. The interesting observation was reported that the optimum concentration of ammonium chloride varied with the temperature. Consequently it is important to use Medium F at the temperature for which the ammonium chloride content is specific. It was also reported that several other ammonium salts had the same optimum concentrations. The preliminary work which Fulmer and his colleagues carried out to find out the optimum concentration of the various ingredients in their several media, should carry considerable weight. They did not put into their medium a number of different ingredients which were assumed to be used by yeast. Many investigators of the nutritional requirements of microörganisms also overlook the fact that their cells may also secure nutrients from the air.

In 1921 further confirmatory data were secured by Nelson, Fulmer, and Cessna to show that yeast can synthesize water soluble B. These authors used Medium F, the composition of which was given above. To prove this they fed albino rats on a ration free from accessory substances. After a decline in weight for from two to three weeks, 2 per cent of the yeast grown on the Medium F was added to the ration. After the yeast was added, there was a rapid gain in weight showing that the yeast formed accessory substances in a synthetic solution.

Funk and Dubin (1920) reported a test for the presence of the antiberi-beri vitamin which, while much like Williams' procedure, was thought to be more easily and quickly manipulated. Their method rested on the use of autolyzed yeast as a standard against which the vitamin content of an unknown substance could be checked. The details need not be reproduced here. Different substances were tested for their antiberi-beri vitamin content. Funk and Paton stated that a positive result was strong evidence of the presence of vitamin B in an unknown substance while a negative result might be due to the presence of inhibitory substances.

At about this same time Swoboda (1920) also modified the Williams method to enable one to detect small amounts of water soluble vitamin. A count was made of the number of cells developing from one or two cells in a hanging drop at 30° after eighteen hours. Swoboda reported that glandular and other tissues were rich in water soluble vitamin. Swoboda reported a substance toxic for yeast cells in thyroid extract.

What was probably the first information that "bios" was not a single substance was announced in 1922 by Fulmer and Nelson. They prepared two extracts as follows from alfalfa which had been previously extracted with ether. Extract A was an extract by long extraction with absolute alcohol. Extract B was an extract prepared by long extraction of the absolute-alcohol-extracted material with water. Both extracts showed optimum concentrations for maximum stimulation and were about equally potent. Combinations of the two extracts were much more potent than the optimum concentration of either alone. "Bios" was thus regarded as composed at least of two materials, "Bios" A soluble in absolute alcohol and water and "Bios" B insoluble in absolute alcohol but soluble in water.

Clark (1922) using Fraser's (1921) rocker tube to secure aeration and agitation found that a pure culture of a bottom yeast isolated from Fleischmann's compressed yeast grew in the following synthetic medium: 100 cc. of water, 10 grams potassium dihydrogen phosphate, cryst., 5 grams, magnesium sulfate, cryst., 20 grams of ammonium nitrate, composing solution A, and 100 cc. of water with 1.4 grams of calcium chloride hexahydrate for solution B. These solutions were sterilized separately in order to prevent the formation of a precipitate. Five cubic centimeters of each solution, 10 grams of cane sugar were diluted with enough water to make 100 cc. of medium. Clark reported that actively budding, filtered and washed yeast gave good growth in this medium. It required about four hours to double the number of cells in this artificial medium while in wort the number was doubled in one hour and fifty-three minutes. Clark also reported some experiments on the absorption of "bios" by yeast cells. While he found evidence sup-

porting the absorption of "bios" from wort by yeast cells this part of the work was not done with a pure culture but with a few grams of washed Fleischmann's yeast cake which, as all microbiologists know, contained many other organisms besides yeast.

In 1921 Funk and Dubin announced that yeasts required a different substance for growth than animals since they separated from autolyzed yeast a substance active for yeast and another active for rats and pigeons. They were led to separate from vitamin B a substance active for microörganisms to which they gave the provisional name vitamin D. This substance was said to be specific for the growth of yeasts. Funk and Dubin stated that vitamin D could be separated from vitamin B but that the reverse had not been accomplished.

According to Eijkman (1922) and his colleagues the anti-neuritic factor is taken from the medium and may be regenerated but not synthesized by the yeast cell. Yeasts (Saccharomyces) grown in vitamin free medium would not cure polyneuritic fowls. The same yeast grown in the presence of vitamins did cure the disease. These authors could not demonstrate the formation of vitamin B by Bact. coli, a result not in agreement with the reports of Robertson and Hosoya and Kuroya.

Fulmer and Nelson (1922) published further data in a paper which was prompted by the statement of Eddy, Heft, Stevenson and Johnson mentioned above, to the effect that Medium F could be improved by the addition of certain materials. Fulmer and Nelson claimed that Eddy and his colleagues had made some unjust interpretations when they reported that the stand had been taken by Fulmer that Medium F was not improved by the addition of certain substances and that Medium F allowed maximum growth. Fulmer and Nelson replied that such a stand had never been taken for that would make them state that Medium F was as good a medium as beer wort. However, they did state in a former paper (1921) that Medium F was not improved by the addition of water soluble B. Fulmer and Nelson reiterated their thesis that the addition of alcoholic extracts from alfalfa and wheat embryo, containing water solu-

ble B, would not improve the medium. The denial that Medium F could not be improved urged Fulmer and Nelson to check their former data. They found verification of it in every respect. They called attention to the fact that Eddy and his colleagues had not used Medium F under the conditions required for it. Again, they used a different method for extracting the alfalfa. Fulmer and Nelson concluded their paper by stating that the alcoholic extract contains along with water soluble B, "bios," the yeast growth stimulant.

Eddy and his colleagues (1922) then used Fulmer and Nelson's technic of alcoholic extraction with varying concentrations and 30°. They secured stimulation with the water extract. They were inclined to explain the discrepancies between their data and Fulmer and Nelson's data on different methods of working. Eddy said that he was in substantial agreement with Fulmer except one point, that Medium F was not improved by adding of extract. They stated that further work had served only to increase their distrust in the yeast test as an accurate measure of vitamin B. Willaman and Olsen (1923) explained the controversy between Fulmer and his colleagues on the one hand and Eddy and his colleagues on the other, on the basis that alcohol is a good solvent for vitamin B but a poor one for "bios."

That yeast does have the ability to synthesize vitamin B, was reported by MacDonald (1922). Yeast grown in synthetic mineral salt-sugar solutions promoted the growth of young rats as well as yeast grown in malt. The yeasts used were Saccharomyces cerevisiae, Sacch, ellipsoideus strains XII and K and a strain isolated from commercial yeast. The fact that the yeast cells grown in the synthetic solutions promoted the growth of young rats confirmed the results, mentioned above, of Nelson, Fulmer and Cessna (1921) and Eijkmann, et al. (1922).

In 1922 Funk and Paton[3] published more data on vitamins

[3] McCollum and his colleagues used the term vitamin D for the antirachitic factor which they differentiated from the growth-promoting vitamin A. Drummond (1924) stated that the term vitamin D should be used for McCollum's accessory factor. This position was taken probably because he did not consider "bios" as a vitamin. While the controversy may not be accurately settled

B and D. They stated that alkali had a more destructive action on vitamin B than on vitamin D. Vitamin B was also more susceptible to autoclaving at 25 pounds pressure for three hours than vitamin D. Experiments on pigeons and rats showed that growth of yeast or some other fungus took vitamin D out of solution but left vitamin B. Funk and Paton claimed that this method could be used for separating these two vitamins. The yeast cells were said to hold the vitamin D very tenaciously. These results do not agree with those published by Eijkmann, et al. referred to above. Further work must, therefore, be done on this question.

Funk and Freedman (1922) found that growth stimulating substances for both yeasts and bacteria were present in various substances such as beef, beef-heart infusions, peptone, and autolyzed brewers yeast. These substances were said to be of a vitamin nature. They stated that they were either identical with vitamin D of Funk and Dubin or of a similar nature.

In the light of the contradictions in this field and the probable errors in the Williams method of assaying the amount of vitamin B in substances, Lobeck (1922) carried out some experiments which were reported in 1922. He resorted to a method based upon the counting of cells, for evaluating unknown substances. The method was as follows: The yeast was cultivated on wort agar which was partially neutralized, frequent transfer being made. When it was used, a little of this culture was diluted in sterilized water. In order to mix well, the liquid was vigorously agitated and blown into by means of a sterile pipette containing a bit of cotton. Pipettes from 0.2 to 0.5 mm. in diameter at the delivery end were used. The pipette was filled with a dilution of the yeast which should show the slightest turbidity by the use of a small number of cells. Two drops of the dilution are put into a test tube. The new dilution was shaken

according to present experimental evidence, it is probably best to retain the term "bios" for the yeast growth stimulating factor and not use the system of nomenclature now generally accepted for a series of vitamins known to be necessary in mammalian metabolism. The recent papers on the fractionation of "bios" indicate that the field is already becoming more complex or confused.

and two drops were removed from this and put into the next flask. The hemacytometer was used for counting. When many cells were formed it was often necessary to dilute the suspension so that each square on the hemacytometer contained not more than 3 cells. Before counting, the growth of the yeasts may be stopped by a little 5 per cent formol solution. Since the number of cells added to each flask was not always the same, Lobeck found it desirable to run four tubes and take the average. He stated that beer wort contained all of the necessary materials for the growth of the yeast. He attempted to remove the accessory substance for yeast, which he spoke of as a vitamin, by means of heat. He held wort at 50°, 70°, 95°, and 110°, for one hour and at 135° for forty-five minutes. He experienced a strong decrease in the growth of the yeast in the heated wort. This decrease was especially apparent in media heated at 110° and above. Consequently, Lobeck took wort heated at 135° as the basal medium for this work. Lobeck also tried to determine whether some poisonous substance for the yeast was formed by heating to 135°. He reached the conclusion that a necessary factor for the growth of yeast was inhibited by the high temperature; no poison seemed to be formed. A precipitate was formed and it was shown that it was not accompanied by the "stimulating factor." The precipitate really hindered the growth of yeast because the contact of living and dead cells was less intimate. In this respect, Lobeck like Henneberg, believed that the living cells secured some advantageous substance or substances from the dead cells. Lobeck then added increasing amounts of sodium hydroxide to wort and heated at 120° for twenty minutes. As the alkali increased growth of the yeast decreased. Lobeck titrated his media with phenolphthalein. Wort was neutralized with NaOH and heated to 135° for one-half hour. Then its acidity was restored by means of tartaric acid. Such a wort gave less growth than wort heated to 135°, without alteration of its reaction. Lobeck also stated that hydrolysis greatly influenced the content of stimulating substance. He was able to confirm Ide's statement that "bios" greatly stimulates the growth of yeast

but that the yeast will develop slowly without "bios." Lobeck left the question by stating that there was a difference in the rate of growth of yeasts with and without "bios." "Bios" is taken from dead cells which are always present when more than 100 cells are inoculated into the new medium, or the yeast may synthesize it slowly in solutions which do not contain it in the beginning. His first conclusion stated that "bios" is necessary for the growth of yeast, a far reaching statement not in accord with certain statements in the discussion of results nor data in the paper. He further concluded that there was no "bios" in yeasts. Last of all Lobeck stated that the yeast method was not sufficiently exact for evaluating the vitamin content of substances. Lobeck throughout his paper, has used the terms "bios" and vitamins rather loosely.

Robertson and Davis (1923) entered the investigations in this field in order to determine whether yeast could synthesize vitamin B. They used a yeast, Saccharomyces cerevisiae probably, from Fleischmann's compressed yeast. Their medium was made with dextrose and inorganic salts. They suggested that Nelson, Fulmer and Cessna's results were partially invalidated because the constituents of their media were not chemically pure. MacDonald and McCollum's work was also criticized on the basis that bios was taken over from one culture to another because 1 cc. of the agitated suspension was used for starting a new culture flask. Robertson and Davis stated that yeast could not synthesize its own "growth stimulating" substance. They worked with the following medium: asparagin (Merck) 3.4 grams; calcium chloride, 0.1 gram; dextrose, 20 gram; magnesium sulfate, 0.2 gram; potassium phosphate, (K_2HPO_4) 1 gram; sodium chloride, 5 gram; sterile distilled water, 1000 cc. This medium was adjusted to a pH of 7.0. Just what had to be added to this medium to do this was not stated. Beef heart, carrot, potato and yeast cells were said to contain water soluble substances which when added to the above medium in high diutions gave luxurious and continued growth of the yeast. In the concentrations of these substances used, the yeast would not grow. Robertson and Davis believed that the yeast cell could

take these substances over from one medium to another. This was also suggested by Eijkmann, Van Hoogenhuijze and Derks (1922). Robertson and Davis suggested that this played a rôle in the work of MacDonald and McCollum. One is led to wonder whether Robertson and Davis were justified in assuming that these water soluble substances were the so-called accessory substances. While in their summary they do not state plainly that they belong in this category, the discussion leads to this belief. It is difficult to see how Robertson and Davis could use their data as bases for criticising the work of Nelson, Fulmer and Cessna and MacDonald and McCollum. Each of these three groups of investigators used different media, different yeasts and different methods of working.

Fulmer and Nelson in reply to Robertson and Davis pointed out some possible reasons why their yeast did not grow. Robertson and Davis used another medium at 37.5°. With data secured in this manner, they could not refute Fulmer and Nelson's claim that yeast would grow indefinitely in synthetic media. To meet the criticism of Robertson and Davis that their reagents were impure, Fulmer and Nelson extracted cane sugar for seven days with 95 per cent alcohol in a continuous extractor. The only statement which Robertson and Davis made with regard to their chemicals was that all substances were chemically pure. No mention was made of the methods applied to determine this. Consequently one may wonder whether the criticism which they made of Fulmer's medium might not apply to their own. Media prepared with this cane sugar did not give any poorer growth nor did the extract give a larger growth when added to the basal medium. This statement is not in accord with the conclusions reported by Willaman and Olsen (1923). These investigators claimed that 95 per cent alcohol did not remove "bios" from commercial sugar. If this is true, Fulmer and Nelson's extraction with 95 per cent alcohol would not have much weight in meeting Robertson and Davis' criticism.

Working with Saccharomyces cerevisiae, Saccharomyces ellipsoideus and yeast XII, MacDonald reported increased growth when alcoholic or water extracts, of these yeasts, commercial

yeasts, wheat germ, malt, peptone, Liebeg's meat extract and autolyzed steak were added. Here data seemed to indicate that yeast synthesized a substance which stimulated the growth of the culture to which it was added. "Bios" differed from vitamin in that vitamin is necessary for animal growth although yeast grows, though slowly, and makes "bios." MacDonald, however, believed that the stimulating substance which she observed was "bios." She justly criticized Funk and Dubin for establishing a separate designation for this substance, thereby classing it with the vitamins. MacDonald found small seedings grew very slowly but grew more rapidly when "bios" was added. "Bios" was believed not to function as a vitamin. MacDonald believed it to be a substance which, while capable of synthesis by the yeast cell, was formed with some difficulty.

Funk and Freedman in 1922 made the whole question more complicated by stating that sucrose contained a vitamin, probably identical with their vitamin D; this favored the growth of yeasts. This substance was said to have been removed from the sugar solution with Fuller's earth or by direct crystallization from alcohol. They also stated that some "vitamin D" was carried over in transferring in sufficient amounts to allow growth of the cells. Funk and Freedman claimed that yeast could not synthesize vitamin B without vitamin D. If it is proved that cane sugar contains a growth promoting substance some of the work which has been done on this subject will have been invalidated because sucrose was used in the media. Such a state of affairs is not so improbable especially when it is recalled that plant tissues in a number of species have been found to be rich in accessory substances. This question needs further study to (*a*) confirm or disprove Funk and Freedman's contention, (*b*) demonstrate whether this substance if present is "bios" or vitamin B. The statements of Funk and Freedman concerning the use in a medium of sugar which has been extracted with alcohol, are in direct opposition to statements by Fulmer and Nelson (1923) on the same subject and Harden and Zilva (1921). It may also be further stated in this connection that there are

probably a great many grades of sucrose available. Some of these may not have been purified to such an extent as others. It would undoubtedly make a great difference whether the sucrose on which Funk and Freedman made their studies was a high grade product—chemically pure—or whether it was a product dispensed in the bulk for household purposes. With a little care in purchasing and more care in experimental work, one may quickly determine whether the sucrose which he is using is satisfactory. If it will not support the growth of a few cells (less than five perhaps) when added to a good basal medium it probably does not contain factors which hasten the growth and multiplication of yeasts. As far as can be determined there is no other test for the yeast growth promoting factor than to see whether yeasts will grow in its presence. The work might be placed on a better basis if the several investigators in this field would designate the brand and quality of sucrose which they used.

Suzuki and Suzuki (1923) separated from vitamin B by means of aluminum cream a factor which promoted bacterial growth. They called it vitamin D and said that it was a decomposition product of vitamin B. Heaton's investigations also seem to confirm Funk's findings that there is a fourth vitamin which stimulates yeast growth.

In order to further check this question of the ability of yeasts to grow in mineral salt-sugar nutrient solutions Fulmer, Nelson and White resorted to "methose" first described by Loew. This "methose" was added to Medium E in place of sucrose. Saccharomyces cerevisiae grew well. Since this methose was wholly of synthetic origin, this experiment was a little different from those reported before. The ability of yeast to grow in this medium seems to be more evidence that accessory substances are not required for yeasts. This was indeed a valuable piece of work, and if confirmed, places considerable of a burden of proof on those who claim the yeasts need "bios."

Working along the same lines as with liquid media, Fulmer and Grimes (1923) announced the preparation of a solid medium which supported typical growth of three species of yeasts, i.e.,

Saccharomyces cerevisiae, Torula spherica and a Mycoderma. The media used were prepared with pure salts and agar which had been washed several times in distilled water. Against these synthetic media malt agar was used as a control. Fulmer and Grimes stated that the colonies on the synthetic agar were from one-half to three-fourths the size of those on the wort agar but were sufficiently large to allow easy counting. Variation in the concentration of ammonium chloride did not seem to affect the size of the colonies. Bacteria were found to give poor growth thus indicating that the synthetic media were useful for purposes of separation. This work from Fulmer's laboratory would seem to be additional evidence that yeasts grow on media containing inorganic matter but that they grow better on media containing it (beer wort).

In 1923 Willaman and Olsen reported interesting data. They showed that 95 per cent alcohol was a poor solvent for "bios" and that 80 per cent alcohol was a very good solvent. They further stated that the method of MacDonald and McCollum did not remove "bios" from a commercial sugar although it was a satisfactory method for the removal of vitamin B. Willaman and Olsen explained the controversy between Eddy and Fulmer both of whom used different alcohols for extracting the accessory substances which they used. This explanation is supported by the fact that Eddy, Kerr and Williams found their crystalline "bios" to be soluble in concentrations of alcohol less than 80 per cent but insoluble in cold 95 per cent alcohol. This is borne out by growth curves secured with media made with sucrose extracted with 80 per cent alcohol in different ways. A good growth curve was secured with sucrose extracted with 95 per cent alcohol according to MacDonald and McCollum's method. The growth curves became less and less satisfactory with each extraction with 80 per cent alcohol showing that some accessory substance was removed. Willaman and Olsen were led to the conclusions that "bios" is different from water soluble B vitamin. Normal growth of yeast was said to be impossible without "bios," a conclusion which is valid only if normal growth is defined as the maximum growth. Over 60 compounds

were tested found not to be "bios" itself. Even though they believed that "bios" was different from vitamin B, Willaman and Olsen did class it with the vitamins thus supporting Funk's nomenclature. In contrast to the results with chemical compounds reported by Willaman and Oslen, we may mention the fact that Otero (1924) reported that very small amounts of puridin and nicotin stimulated the growth of beer yeast. Willaman and Olsen's work would have been a little more conclusive had they prepared media with the "bios" which they extracted from the sucrose. They showed well enough that alcohol of certain concentration removed some factor or factors from the sucrose. One is also at a loss to know why "bios" should be used to explain the different intensities of growth which resulted from the use of sugars extracted with different concentrations of alcohol. We might assume that other alcohol-soluble impurities were present in the sucrose. These might be characterized chemically if our methods were adaptable to the small quantities which might be assumed to be present. It should be pointed out, also, that Willaman and Olsen did not use Fulmer's medium and therefore did not prove that yeast would not grow in Fulmer's medium with their purified sugar. Admitting for the moment that extraction with 80 per cent alcohol did something to the sugar, is it right to explain the results solely on the basis of "bios"? The same statement may also be applied to the work of Funk and Freedman who claimed to have shown the presence of a growth-promoting substance for yeast in cane sugar.

Toward the end of 1923 more publications began to appear which suggested that "bios" was not a substance of simple composition. Miller (1924) in an address before the American Association for the Advancement of Science in Cincinnati in 1923 reported experiments in support of this contention. Miller attempted to isolate the factor in beer wort which has such a stimulating effect on the growth of yeasts. He found that it was dialysable and non-volatile and that although prolonged autoclaving of the wort destroyed it, moderate heating with small amounts of acids or alkalis did not harm it. Such chemi-

cals as hydrogen sulfide, lead acetate, barium sulfate, etc., were harmless but shaking the wort with charcoal destroyed its growth-promoting powers. These things were found in some of the early work. Miller then sought a better source of "bios" than beer wort. A better source was found to be the tips of growing roots and consequently the "combings" from the malt house were used. These had about half the "bios" content of the wort but also had the advantage that only about one-sixth the solids were present that were present in the beer wort. Miller's account led on to the announcement that "bios" is not a simple substance but one made up of two components, one of which was precipitated from solution with barium sulfate; the other was left in solution. Miller proposed the provisional name "Bios I" for the one which was precipitated with barium and "Bios II" for the component which was not. "Bios I" was not absorbed by charcoal nor was it removed from solution by shaking with yeast. Sugar of lead did not precipitate it. "Bios II" was absorbed by charcoal and could be removed from solution by shaking with charcoal. It was soluble in acetone. In beer wort "Bios I" causes no increase in growth. "Bios II" is not present in beer wort in such excess since its addition resulted in an increased growth. Further work reported by Miller seemed to indicate that "Bios II" could be further fractionated.

In a footnote appended to the paper on February 18, 1924, Miller was able to report that "Bios II" had been fractionated; he stated, "Thus Wildier's 'bios' consists of at least three separable constituents, all of which must be present in the medium to ensure normal reproduction of yeast." Miller further stated that while the work of Fulmer, Nelson and White with synthetic media disproved the too wide claim that "bios" **is** "indispensable au developpement de la levure," auximones existed and the honor of their discovery rested with Wildiers.

Lucas (1923) gave a somewhat more detailed account of the chemistry of "Bios I and II" but too incomplete for any one else who might desire to confirm his results. Lucas stated that the aqueous extract of malt combings contained enough of Wildiers' "Bios" to grow 700 million yeast cells per cubic

centimeter under the conditions described by Clark (1922). After concentration and removal of proteins, pectin, etc., by alcohol, addition of baryta and alcohol throws down a precipitate from which "Bios I and II" can be prepared and purified by lead. The filtrate contains "Bios II" which can be purified by acetone. Aqueous solutions of sugar and salts to which either one of these two constituents of "Bios" has been added, will not grow yeast; but if both constituents be added, the yeast crop is nearly as large as from the original extract. Thus Wildiers' "Bios" consists of two separable constituents. Lucas published this note in May, 1923, and stated that the methods of preparation and purification would shortly be published in full. Experiments were said to be in progress which showed that neither "Bois I," "Bois II" nor both together could replace vitamin B in experiments with pigeons, rats, and guinea pigs. Eastcott (1923), working in the same laboratory, investigated the natural distribution of "Bios I" and "Bois II."

The amount of "Bios I" was determined by measuring the yeast crop obtained after twenty-four hours at 25° in a culture medium containing 1 cc. of an extract of the substance under investigation, 1 cc. of a preparation of "Bios II," and sufficient 10 per cent sugar solution salts, and yeast suspension to bring the volume to 10 cc. and the initial count to 1. "Bios II" was determined in the same way with the use of 1 cc. of a preparation of "Bios I" in place of "Bios II."

"Bios I and II" were found in approximately equivalent amounts in lemon, tomato, potato, beet, spinach, ginseng, tobacco, barley, bran, flaxseed, and egg yolk. A large excess of "Bios I" was found in chlorophyll, turnip, rhubarb, orange, strawberry, grape, pollen, corn, polished rice, cottonseed meal, alfalfa, hyacinth bulb, hyacinth roots, malt, malt extract, catnip, tea, macaroni, molasses, insulin, cinchona, buttermilk, pancreas, heart, and thymus. A large excess of "Bios II" was found in rice polishings, mushroom, coffee, malt combings, egg albumin, and liver. Very small quantities of "Bios I and II" were found in agar, rice, starch, tapioca, sago, honey, manure water, bone meal, soil from grass plat, saliva, casein, and adrenalin hydrochlorid. Horse-radish, onion, and kidney proved toxic to yeast in the presence of wort.

Lucas (1924) later reported his investigations in full. It will be impossible to do justice to his report within the scope of

a review. The apparatus used was patterned after Fraser's (1921). Lucas first of all reported that tests with some of Eddy's purified crystalline "bios" did not show the stimulation that Eddy had reported. In order to search into the discrepancies Eddy, when attending the meetings of the British Association for the Advancement of Science in Toronto during the late summer of 1924, brought some of his product with the strains of yeast which he has used in his work to Toronto where Lucas and Eddy made some observations together. Lucas reported this as follows:

> One of these cultures behaved very like the yeast used here, both with Prof. Eddy's crystals and with our "Bios" preparations; in a solution of salts and sugar alone its reproduction is very slight. The other culture, however, gave a high crop without any addition of "Bios" at all, although much more was obtained when "Bios II" or "Bios I and II" was present. This observation shews how necessary it is in work of this kind to control the strain of yeast employed, and no doubt explains repeated failures in this laboratory to duplicate the results obtained by Mr. Fulmer with his medium E.

The yeast used by Lucas was isolated in pure culture from Fleischmann's yeast. The detailed methods of preparation were listed by Lucas but these may be secured from the original publication. This more comprehensive report from Lucas and the report by Eddy published a month later now give an opportunity for others to investigate their methods of preparation.

Fulmer, Duecker and Nelson also came to the conclusion that "bios" was not a simple substance. They made four fractions with alcohol of a water extract of alfalfa according to procedures which are given in their paper and which need not be repeated here. All of these fractions stimulated the growth of yeasts to a different extent. The authors are justified in light of their own work and that of others to reach such a conclusion that "bios" is probably a very complex substance.

Miller's discussion prompted Levene and van der Hoeven (1924) to announce in a preliminary note which was followed shortly by a longer paper, the preparation of a material from

yeast which was more active than any vitamin B preparate that had been prepared. In referring to Eddy, Kerr and Williams' crystalline "bios" which is discussed later on, Levene stated that many years ago, Mandel and Dunham prepared from a commercial product, zymase, an adenine hexoside. The same substance could not be found in yeast. However, in this paper Levene reported that he had been able to prepare this substance from yeast. The composition of his product was C = 44.41, H = 5.09, N = 23.57 and the melting point was 214°. Except for the hydrogen the analytical data corresponded with those published by Eddy, Kerr and Williams. Dubin tested the Levene preparation for its stimulating effect on yeast but secured negative results. If the "bios" question is to be finally solved, it will have to be by just such attempts as are pointed toward the preparation of a pure substance.

More recently several more papers have been published by Robertson on food accessory factors (vitamins) in yeast growth. Two were published which bear on the topic being discussed. The synthetic nutritient solution used by Robertson was prepared as follows:

Asparagin	3.4	grams
Calcium Chloride	0.1	gram
Dextrose	20.0	grams
Magnesium sulfate	0.2	gram
Potassium phosphate (K_2HPO_4)	1	gram
Sodium chloride	5	grams
Sterile distilled water	1000	cc.

Robertson gave no information with regard to how this medium was arrived at and what determined the amounts of the ingredients. These substances were boiled for three minutes, brought back to original volume and the reaction adjusted to pH 7.4. All glass ware had been previously washed in $\frac{N}{1}$ NaOH, carefully rinsed and sterilized. The above medium permitted continued growth of B. coli-communis but not that of yeast. However, the addition of aqueous extracts of autolyzed yeast and grated carrot permitted the continued growth of yeast but did

not accelerate the growth of bacterium coli. The filtrate of the synthetic medium upon which Bacterium coli had grown also permitted continued growth of yeast. Robertson seems to have overlooked the fact that a filtered culture of Bacterium coli may have contained other substances than "accessory substances." Also the addition of an aqueous extract of autolyzed yeast or grated carrot probably contained a great many different food substances for the yeast. Many investigators have shown that a very slight amount of organic matter greatly stimulated growth of yeasts in synthetic media. There would probably be an appreciable amount of soluble organic matter and inorganic salts in such a product as an aqueous extract of grated carrot.

Robertson continued this work just discussed in which it was shown that washed yeast cells could not grow long in his synthetic medium. He used the same medium as mentioned above. In this second report data secured with Saccharomyces cerevisiae and a number of other pathogenic and non-pathogenic bacteria were published. He confirmed his former statement that yeast could not live beyond a few transplants in the synthetic medium adopted. However, when filtered cultures of the several bacteria which did grow in the synthetic medium were added, the yeast grew abundantly. These cultures were prepared by growing the organisms in 1 liter flasks at 37° for ten days. It is not at all improbable that such a preparation contained considerable organic matter. Robertson's method of determining growth was also rather indefinite. It is possible that his medium is not a good one for yeasts in general, and, in particular possibly for the species which he used. In this paper as in the former one Robertson unfortunately used the word "generation" synonymously with "transplant." Where continued growth was secured, transplants were carried through the fiftieth generation; the cultures were transferred every forty-eight hours. This transfer period may have been too frequent since Robertson may not have given his yeast time to start growing in his medium. The term generation should probably be used in the original sense—the formation of a new cell. Generation times have

been computed by a great many workers as criteria of growth and to have the word used in place of transfer or transplant might complicate the subject.

Lepeschkin (1924) reported that yeast did not grow normally in synthetic solutions containing 10 per cent of glucose, 0.1 per cent asparagin, 0.1 per cent magnesium sulfate and 0.2 per cent KH_2PO_4 were 1 or 2 cells were used as the inoculum. Larger quantities of seed allowed normal development. Addition of this vitamin B to cultures of Penicillium glaucum was effective only in the first period of growth. Lepeschkin suggested that the action of vitamin B may be catalytic or similar to that of the co-ferment in fermentation. Lepeschkin's report concerned data secured with Saccharomyces cerevisiae I Hansen. He stated that if the "Tropfchenkultur" of Lindner was made with the above named yeast in such a manner that a single cell was present in each drop of the culture solution containing ammonium sulfate as the source of nitrogen, no development of the yeast was observed. On the other hand, if the culture liquid contained peptone, the development was excellent. Lepeschkin did not tell how much medium was present in his culture flasks. He next determined to find out whether the lack of development of single cells was due to an absence of vitamin. To do this Lepeschkin prepared vitamin from yeast according to the directions of Funk. The addition of 0.001 per cent of crystallized vitamin (M.P. 233°, molecular weight 511) made possible the development of the yeast. It is interesting to note that the melting point of the material used by Lepeschkin is very close to that of Eddy's crystalline "bios." Lepeschkin also made the interesting observation that when vitamin was present in the culture fluid it not only increased the growth of the yeast but also increased its fermenting ability. He showed this by preparing a homogenous emulsion of the yeast cells so that there were 2 or 3 cells per drop when placed on the under side of a cover slip. Increasing quantities of vitamin were added to the solutions, 0.01, 0.001, and 0.0001 per cent. The results indicated that no important difference was observed between the effect produced on the growth of the yeast by 0.01

per cent vitamin and that produced by 0.001 per cent. On the contrary the 0.0001 per cent solution produced a much weaker effect. Lepeschkin concluded that the yeast cell required only a small amount of vitamin and that it was useless to increase the amount. A comparison of Lepeschkin's medium with others of similar composition, suggests that perhaps he did not have the proper amounts of inorganic salts.

Since Wildiers' work, investigators have been trying to solve the chemistry of "bios." In 1924 Eddy, Kerr and Williams made announcement of the preparation of what may be regarded as the first definite chemical substance having the properties of "bios." This product was isolated from autolyzed yeast by different absorbents and had a melting point of 223°. In amounts of 0.005 mgm. per cubic centimeter of Fulmer's Medium F it produced during a twenty-four-hour incubation at 31° a volume of yeast fifteen to twenty times that in the controls. The yield of the product was about 0.03 per cent of the autolyzed brewers' yeast. Analysis showed 43.29 per cent of carbon 8.31 per cent of hydrogen. The nitrogen content was not determined accurately but was thought to be about 25 per cent. (This was later stated to be 8.31.) The substance gave no ninhydrin or biuret reaction. It was freely soluble in cold water, in acid and alkalin solutions, and in dilute ethyl alcohol. It was only sparingly soluble in cold 95 per cent alcohol. It passed readily into solution when the alcohol was heated. It was only slightly soluble in absolute acetone but the addition of a slight amount of water brought about the solubility of the product. Eddy, Kerr and Williams (1924) later made a more exhaustive report of the methods of preparation and properties of their crystalline substance having the properties of "bios." These authors reported at this time that the action of their material seemed to have more specificity for top-growing yeasts than for bottom-growing yeasts. The crystalline form was said to be orthorhombic, the formula $C_5H_{11}NO_3$, the molecular weight approximately 133, and the index of refraction between 1.52 and 1.53. While the chemical structure was undetermined, Eddy and his colleagues thought it was a heterocyclic

nitrogen-carbon ring with a carboxyl group attached. They reported the substance[4] to lack antineuritic power showing that it was not vitamin B, but were unable to yet conclude that it was devoid of effect on mammalian growth. It is interesting to note that Eddy and his colleagues found that their "bios" preparate did produce an increase in rat growth after a period of vitamin B deficiency but the recovery was slight and temporary; furthermore, addition of "bios" to the Seidell product did not improve the growth curve. On the other hand, the

[4] The author has had helpful suggestions from Professors Fulmer and Eddy. The latter kindly sent several papers containing unpublished data to which we may briefly refer at this time. At the December 1924 meeting of the American Association for the Advancement of Science in Washington, Eddy in collaboration with Kerr and Kellogg, read a paper entitled, The Activity of Bios. At this time Eddy reported more data resulting from studies with his crystalline "bios." These experiments were stimulated by the unfavorable results reported by Lucas (1924) and Peskett (1924) with Eddy, Kerr and Williams' crystalline "bios." By using the rocking thermostat proposed by Clark and Fraser, Eddy was able to find confirmation of the activity of his pure crystalline compound. Furthermore, he found that temperature had a striking effect even over a range of two or three degrees. Thus the action of Eddy's "bios" seems to have a high temperature coefficient. Eddy reported the isolation of his crystalline "bios" from alfalfa and corn. He believed that the Toronto products of Miller and Lucas were "bioses" of a different nature from his. Eddy, on account of the similarity in constitutional formula between Mueller's new sulfur containing amino acid (Mueller, J. H., J. Biol. Chem., 56 (1923) 157) and his new crystalline bios was also led to test the "bios" properties of the former. It was found to posses as strong "bios" activity.

This paper stimulated some discussion during which it was suggested by E. C. Kendall that Eddy's "bios" was identical or closely related to thyroxin. Kendall believed this group may be attached to vitamin B. Kendall is now working toward a synthetic "bios." Perhaps we should rest the review at this point since these data are of such recent origin (January 1925). It is interesting to note, however, that the "bios" question seems to be going back toward vitamin B.

Eddy also sent the author a copy of a paper by Doctor Horacio Damianovich, entitled, Contribucion al Estudio del "Bios" O Vitamina D Extraido por el Metodo de Walter Eddy, Ralph Kerr Y R. Williams, which was read at Segundo Congreso de Quimica (Primero Sudamericano, Buenos Aires, September 18, 1924. In this report Damianovich presents data which support the claims of Eddy and his colleagues. Instead of measuring the increase of yeast cells, he measured the consumption of glucose. Dilutions of "bios" as high as one thousandth of a milligram per c.c. of medium showed great activity. Damianovich prepared Eddy, Kerr, and Williams' "bios" according to methods which were sent to him in advance of publication.

Seidell product is admittedly a mixture and it gave a "bios" test. These tests, therefore, leave unsettled the question of whether vitamin B can produce "bios" effects or whether "bios" can affect mammalian growth if polyneuritis is prevented. In reference to the several "bioses" which have been reported by Miller (1924) and Fulmer and his colleagues (1924) Eddy, Kerr and Williams stated that their results did not negate the possibility of more than one "bios." The relative stimulation secured with addition of their pure product and the yeast autolysate suggested that the latter contained more than one growth stimulant. Those desiring the procedure for the preparation of this "bios" may consult the original publication. We may soon hope for confirmation of Eddy's conclusions for if they are confirmed, we will have made, perhaps, a long step toward the solution of the chemistry of vitamins. There will also be better bases for testing the "bios" properties of some of the chemically definite substances which have similar chemical properties. The statement of Eddy, Kerr, and Williams concerning the solubility of their crystalline "bios" is of some special interest. As stated it was soluble in cold alcohol as long as the concentration is less than 80 per cent, becoming almost insoluble in cold 95 per cent alcohol. It was readily soluble in 95 per cent hot alcohol. Eddy and his colleagues used these solubilities to explain in part the controversy between Fulmer and themselves and to justify Willaman and Olsen's use of 80 per cent alcohol instead of 95 per cent to free the sugar used in their media from contaminating "bios." When Fulmer replied to the criticism of Robertson and Davis, he stated that he extracted "with 95 per cent alcohol in a continuous extractor." This means of course that he used hot 95 per cent alcohol which Eddy himself states is a good solvent for "bios." Does not this invalidate Eddy's statement about Fulmer's work? Seidell's (1924) review of the chemistry of vitamins is interesting in this connection. Lucas (1924) had difficulty in securing the stimulation with Eddy's pure "bios" which Eddy had reported. Deas (1924) reported that malt rootlets and combination infusion of the same and fractions known and "Bios I" and "Bios

II" of the yeast growth promoting vitamin either separately or in combination are insufficient to produce the growth of rats. Consequently Wildiers' "bios" and Funk's Vitamin D are not identical with the rat growth promoting vitamin B. It was also said not to be identical with the antiscorbutic vitamin C.

It would be wrong not to attempt to secure help in the analysis of this problem from experiments in adjacent fields. Lack of space prevents a broad application of this idea but we may mention briefly the work of T. B. Robertson with the protozoa. He was concerned in studying the rate of development of isolated cells of protozoa. He called attention in an interesting discussion that a protozoan culture may exhibit youth, age and the phenomenon senescence that is, not merely the slowing down of multiplication which is age, but that diminution of the capacity to multiply, even under suitable conditions and in the presence of abundance of food-stuffs, which is senescence.

It will be impossible for us to give much space to Robertson's work. We may, however, point out some of the salient parts of his conclusions which seem to bear on the topic being discussed in this review. He reported that the rate of multiplication of a single cell of an infusorian Enchelys farcinem Ehr. fell off at first very rapidly and later more slowly with increasing age of the culture. He was unable to detect any accumulation of a retarding substance in old cultures. Bacterisation of the hay infusions greatly stimulated the reproductive capacity of the isolated organisms and delayed the loss of reproductive capacity which occurs with advancing age. Heating of the bacterized infusion did not upset the multiplication rate of the organisms. The stimulation in reproduction was also found to be due to a filterable, thermo-stable non-volatile factor to which Robertson gave the name X-substance and not to the presence of the bacterial cells. Robertson also stated that when large numbers of cells were present in a culture the organism could withstand adverse effects such as high temperature. In the next paper he reported that when two individuals from the same parent culture were placed in a drop of culture medium not twice as many but from four to six or eight times as many

cells were produced as were produced in cultures started with one cell. He further showed that some substance to which he had given the name X-substance was responsible for the accelerated growth of the protozoan in contact with other cells. The possibility that the X-substance might be related to the vitamins was suggested; however, it was said not to be vitamin B because it resisted boiling. This hypothetical substance was also believed not to be an accelerator in itself of multiplication but was converted by the animal cells into an accelerator. Cutler and Crump working with Colpidium colpoda could not confirm Robertson's conclusions with regard to allelocatalysis. Sherman and Albus (1924) were also unable to demonstrate allelocatalysis with cells of Bacterium coli.

In their work on the need of vitamins by certain of the bacteria Hosoya and Kuroya (1923) have also studied some yeasts. They used "Berlin" and "Yebisu" beer yeasts (Saccharomyces cerevisiae), "Johannesburg" grape vine yeast (Saccharomyces ellipsoideus), "Saké" yeast (Saccharomyces saké) in Nägeli's medium. The yeasts were grown on "koji" and from this 2 mgm. of a two-day growth were transferred to 10 cc. of Nägeli's medium. One cubic centimeter of this was transferred to 9 cc. of the same medium and 0.5 cc. of the last emulsion was used for inoculation of the synthetic media. It is interesting to note that they adjusted the reaction of Nägeli's medium by some means probably those usually used by bacteriologists. Where Nägeli's medium was used without any accessory substance the yeasts did not grow and ferment; however, when 0.0625 mgm. of Tsukie's (spelled Tsukiye by the original author) vitamin B was put in a large amount of gas was formed in from two to three days. These authors also tested the effect of the addition of various amino acids on the growth and fermentation of yeasts These amino acids phenylalanine, alanine, tyrosine, glycocoll, mixed crystals of dl-leucine and dl-isoleucine had no effect while Tsukie's vitamin B had a profound effect on the growth of yeast.

The characteristics of the yeast vitamin caused these investigators to conclude that the vitamin essential for hemolytic

streptococci is different from the vitamin required by yeast. Thus the subject is becoming increasingly more complex. The chemical and physical properties of the yeast vitamin were listed as follows:

"*a.* This factor is most stable to heat. It remained potent by heating in neutral solution in the autoclave at 182° to 185° for two hours.

b. It is very stable to alkalis, and even when heated in the autoclave at 140° and 3.5 atmospheric pressure for two hours in degree of alkalinity of normal sodium hydroxide.

c. It is adsorbable with animal charcoal from neutral solutions but not by Fuller's earth."

Consequently, the vitamin required by hemolytic streptococci was said to be different from the vitamin required by yeasts (vitamin D). At first thought it might appear that the experiments and conclusions of Hosoya and Kuroya confirmed the work of R. C. Robertson and Davis. It is well to keep in mind, however, that these groups of investigators worked with different media and yeasts. The former used a medium of their own with one culture of yeast, the origin of which was Fleischmann's compressed yeast, while Hosoya and Kuroya used Nägeli's medium with several species of yeasts. It is very doubtful whether these should be looked upon as confirmatory reports.

The work by T. B. Robertson stimulated Peskett to test Robertson's conclusions on Infusoria with yeast. Peskett grew yeast in bacteria-free solutions—in hanging drops with one, two, three, and in a few cases, five cells of yeast. He used a basal medium composed of primary potassium phosphate (KH_2PO_4) 5 grams; ammonium chloride, 2.5 grams; magnesium sulfate, 0.35 gram; calcium chloride, 0.25 gram; distilled water, 1000 cc. From this three other media were prepared as follows: I, Basal medium plus 60 grams per liter commercial cane sugar. II, Basal medium plus 50 grams, per liter of recrystallized cane sugar. III, Basal medium plus 50 grams per liter recrystallized cane sugar plus one-fourth of its volume of a solution containing 12 mgm. per 100 cc. of a solid "bios complex." This "bios"

complex was prepared according to the method of Eddy, Kerr and Williams. The cane sugar was recrystallized from hot 80 per cent alcohol according to method of Willaman and Olsen. The culture used was Saccharomyces cerevisiae secured from the Lister Institute. A modification of Williams' technic was used for following multiplication. While Peskett could not secure evidence that growth was allelocatalytic, it is interesting to see that multiplication took place even when one cell was present in the hanging drop. Peskett stated that within the limits of experimental error, the growth of two cells was approximately double that of one cell. The growth of living yeast was not therefore accelerated by the presence of other living yeast cells. Dead cells, also, did not seem to acclerate growth which conflicts with the opinions of Henneberg, Kossowicz, and others. Peskett found that Eddy's crystalline "bios" did not stimulate the growth of yeast. In fact in the mount to which it was added, there was less growth than in the mounts which did not contain it.

Henneberg remarked, that a single yeast cell in a hanging drop may be benefited by other cells from which it may secure utilizable materials. Such studies were carried out by Lindner (1905), Lindner and Stockhausen (1906) and Stockhausen (1908). Going to the other extreme, it is well to point out that Clark (1920) reported that "crowding" of cells had little effect.

Tanner, Devereux and Higgins (1925) reported the use of a great number of different species of yeast. They stated that most of the work of other investigators had been carried out with but one or few species of budding fungi in one medium. In some cases generalizations had been made which were not warranted. Fifty pure species of budding fungi and twenty-two cultures which had been isolated from sore throats by Tanner and Dack (1922) were grown in Nägeli's, Fermi's, and Fulmer and Nelson's Medium F. Fulmer and Nelson's Medium F was found to be more satisfactory than either Nägeli's or Fermi's. Eddy had also reported that Medium F was a better medium than Nägeli's medium. These authors also pointed out that the solid matter in a yeast cell 5 microns in diameter,

assuming that the specific gravity was 1, would be about 0.000,000,009,817,5 mgm. indicating that a "bios"-containing extract would have to be diluted far more than any of them have been, to avoid an acceleration in growth that might result from the addition of chemically definite substances in the extract. Under such a condition it was wrong to attribute the increase in growth to "bios" when other factors which are chemically known might be responsible. Tanner and his colleagues also stated that each species of yeast probably had its own dietary requirements making one medium better than another. Consequently some preliminary attention should be given the medium and the requirements of the species of yeast which the investigator intended to use, in order to be certain that the yeast was being given a fair opportunity to develop. They finally stated that it was unnecessary to assume the requirement of "bios" or any other hypothetical substance to explain the absence of multiplication of a yeast in a synthetic nutrient solution. Lack of growth might be due to other things; conversely, the stimulation of growth and multiplication following the addition of a supposedly "bios" containing substance did not indicate that "bios" is necessary since the stimulation might be due to other factors in the "bios" preparate. It was admitted, however, and some statements were made in the paper to support it, that yeasts developed better in a medium to which yeast water had been added. Whether the stimulation in growth was due to "bios" or to the other materials in the extract was not known. The statement of these investigators that each yeast may have its own most favorable medium confirms a statement made by Henneberg in 1907. Henneberg in this investigation studied very carefully the effect of minute changes in the composition of the synthetic medium.

Before leaving the subject it is well to point out that even wort may be "improved" as a culture fluid for yeasts. This review has already become too long and only a paper by Baetslé (1924) will be mentioned because this is of recent date and quite pertinent. This author found that added vitamins, amino-acids and certain inorganic salts stimulated the development

of yeast. A brewery wort of 12° gravity was used as the medium. The vitamins were added in the form of a cold water extract of green malt or of undecorticated rice; a six hours' extract at 113°F. of green malt, afterwards kept at 284°F. for an hour to destroy vitamins, furnished the amino-acid addition, while the source of mineral matters was a malt steep water, which was rich in potassium phosphate. The untreated and treated worts were seeded with the same amounts of the same yeast, one series with a small and a second series with a large quantity. Fermentations took place either at 77°F. without aeration or at room temperature with aeration. On completion of the fermentations, determinations were made of the weight of yeast produced, the free acidity, the "formol" nitrogen, and the gravity of the wort. Fulmer, Sherwood and Nelson (1924) also showed that the addition of the optimum concentration of ammonium chloride to beer wort incubated at 42° increased the yeast crop six fold. These authors were led to state as part of their conclusions that the main rôle of "bios" in yeast growth cannot be the same as that of ammonium chloride—i.e., the maintenance of the proper state of hydration of protein. The generalization was stated that if the addition of ammonium salt to a medium increases its ability to dehydrate gluten the addition will likewise improve the medium for the growth of yeast, the effect being maximum in any combination in which the gluten is least swollen.

The presence of vitamins was not indispensable, since, even after their destruction almost as heavy a yeast crop was obtained. Vitamins and amino-acids stimulated the fermenting power as well as the growth of the yeast, large seedings resulting in an increase of fermenting power, while, with small seedings the increase in growth was more marked. Aeration produced a great increase of yield and in the presence, both together, of vitamins and amino-acids an excessive attenuation was produced. To obtain commercially the maximum of yeast, a mixed infusion of vitamins and amino-acids should be added to the wort. As a preliminary, it is advisable to neutralize with sulphuric acid two-thirds of the malt alkalinity and to make a peptonization to increase the amount of amino-acids. No one in-

terested in the general subject of yeast growth should overlook Clark's (1922) and Fraser's (1921) paper in which the "rocker" thermostat or tube was used for studying yeast growth.

SUMMARY

It would be almost impossible to summarize the various data and opinions that have been published on this question. Most of the investigators since 1901 have been concerned with establishing the correctness or disproving Wildiers' statement that an hypothetical substance to which he gave the name "bios" was necessary for the normal growth of yeasts. It is interesting to note, however, that very few, especially those in America, have tried to use Wildiers' technic. Perhaps a better starting point would have been the use of these various factors such as medium, species of yeast, and other details of technic.

One who has special training in the methods of microbiology also wonders whether the technic used in some of the experiments which have been reported, has been in accord with that used by trained bacteriologists and microbiologists. For instance, some authors relied on pasteurization, another on boiling for sterilizing their media. It is well known that numerous bacteria exist which form very resistant spores. One bacteriologist has reported an organism, the spore of which resisted boiling for 17 hours. Other illustrations could be mentioned to support this. It is obvious that the methods of sterilization should be rigorous and sufficient to insure sterility. A perusal of the descriptions of technic also causes one to question the sterility of the preparates even though sterile media may have been used. In some cases no attempt seems to have been made to determine whether bacteria were present. It is known that the mutual relationships of microorganisms are important. Some bacteria may inhibit the development of yeasts, others may favor it. Several statements appear in the publications on "bios" to indicate that the growth of the yeast was influenced by contaminating bacteria.

Fulmer and Nelson (1922) also pointed out another fact

that the terms vitamin, "bios" and auximones should not be confused. They defined them in 1922 somewhat as follows:

Vitamin: materials of unknown constitution, necessary in the diet other than fats, proteins or carbohydrates, or mineral salts, for animals.

"Bios": substances of unknown composition supposed to be necessary for best growth of yeasts.

Auximones: substances of unknown composition supposed to be necessary for the growth of plants. Their existence is very doubtful.

This suggestion was borne out by reports later on from several different laboratories that vitamin B and "bios" were not only different but that "bios" itself was composed of several different components. Drummond (1924) has also pointed out complications which arise from different definitions. He has stated that from the present state of our knowledge we may reasonably regard a substance as a vitamin if it is essential to the life and well-being of an organism which does not possess the power to synthesize that substance, and also if it is organic in nature, and does not belong to any one of the three great classes of foodstuffs, proteins, fats and carbohydrates. Drummond pointed out a fact which should be kept in mind by those who are working on the "bios" question—that we must differentiate between substances essential for life and growth and those which appear to act more as stimulants of growth.

Is "bios" a vitamin? Keeping in mind Drummond's definition of a vitamin which has been quoted above, and admitting that yeasts may grow and multiply in pure mineral salt-sugar solutions as shown by Fulmer and Nelson (1922), MacDonald (1923) and Tanner, Devereux and Higgins (1925), we must conclude that in the light of present experimental evidence, that "bios" is not a vitamin as suggested by Williams (1919) and by Funk and Dubin. In this relation the recent report by Eddy, Kerr and Williams (1924) that their crystalline "bios" produced an increase in rat growth after a period of vitamin B-free diet, although the recovery was slight and temporary, causes one to wonder whether Eddy, Kerr, and Williams have "bios," a vitamin as a mixture of the two.

It seems to have been fairly well established that the methods of experimentation have had much influence on the data which have been secured. Lindner used this explanation to explain the original controversy between Pasteur and Liebig. Willaman and Olsen also explained the controversy between Eddy and Fulmer on the basis of different methods of extracting materials for accessory substances. Tanner, Devereux and Higgins (1925) called attention to the possible confusion that results when different criteria of growth are taken. In this connection they mentioned the statement of Slator, who after discussing different methods that could be used for determining growth, stated that the results obtained by these various methods were sometimes different. Slator reported that yeast cells grew in size after multiplication had stopped.

One group of investigators denies the existence or need on the part of the yeast plant, of a substance like "bios." They feel that yeasts will grow without this accessory substance.

Another group believes that "bios" is necessary for the growth of yeasts. They are unable to secure growth of yeasts in pure solutions without it. Certain of these investigators have reported fractionation of "bios" into components which are necessary to one another.

A third group of investigators believe that yeast will grow in pure nutrient solutions without "bios" but that the addition of a bios containing substance may cause increased growth. Whether this acceleration in growth following the addition of a "bios" containing substance is due to "bios" or to some other factor in the preparate has not been satisfactorily established. In this connection it is well to point out that even a medium as beer-wort which is rich in "bios" may be improved by the addition of other "bios" containing substances.

A fourth group may also be recognized including those who have isolated "bios" or substances having "bios" properties.

BIBLIOGRAPHY

AMAND, A. 1902 Le "Bios" de Wildiers ne joue pas le role d'un Contrepoison, *La Cellule*, **20**, 225.

AMAND, A. 1904 La Disparition du Bios de Wildiers dans les Cultures de Levure, *La Cellule*, **21**, 329–346.

BACHMANN, F. 1919 Vitamine Requirements of Certain Yeasts, *Jour. Biol. Chem.*, **39**, 235–257.

BAETSLÉ, R. 1924 Substances Stimulating the Growth of Yeast, (Trans. Title) *Bull. Ass. Anc. Eléves de l'Inst. Superieur des Fermentations de Gand.*, **7**, *Jour. Inst. Brewing*, **30**, 932.

BEHRENS, J. 1902 *Berichte d. Grossherz. landw. Versuchsanstalt Augusteberg.* (Quoted from Kossowicz, 1911.)

BEIJERINCK, M. W. 1894 Schizosaccharomyces octosporus eine achtsporige Alkoholhefe, *Cent. Bakt.*, **16**, 49.

BERDNIKOW, A. 1924 Limite du Developpement des Microbes dans les Milieux Artificiels, *Comp. Rend. Soc. Biol.*, **90**, 1305.

BOKORNY, T. 1907 Die "Bios" -Frage und die mineralische Ernährung der Hefe, *Allg. Brauer- u. Hopfen-Ztg.* 1907; *Cent. Bakt. Abt.*, **19**, 331–332.

CHRZASZCZ, T. 1904 Zur Kenntniss des Hefewachstums in mineralischer Nährlösung, *Cent. Bakt.*, II, Orig., **13**, 144–149.

CLARK, N. A. 1922 The Rate of Formation and the Yield of Yeast in Wort, *Jour. Physical Chem.*, **26**, 42–60.

CUTLER, D. W., AND CRUMP, L. M. 1923 The Rate of Reproduction in Artificial Culture of Colpidium Colpoda, Part II., *Biochem. Jour.*, **17**, 878–886. (See also pp. 174–186; **18** (1924), 905–912.)

DEAS, J. 1924 Yeast Growth-Promoting Vitamin Tested for Its Effects on Animals, *Jour. Biol. Chem.*, **61**, 5–8.

DELBRÜCK, M. 1903 Some Facts About the Reproduction of Yeast, (Trans. Title). *Wochenschr. Brau.*, **20**, 25.

DELBRÜCK, M. 1908 Uber Giftwirkungen von Getriede auf Hefe. *Jahresb. d. Versuchs- und Lehranst. f. Brauerei in Berlin*, **11**, 31; *Kochs Jahresb.*, **19**, 235.

DELVO, R. 1906 Purification du Bios de Wildiers, *Chem. Absts.*, **1** (1907) 1311; *La Cellule*, **23**, 361–424.

DRUMMOND, J. C. 1924. Some Modern Tendencies of Vitamin Research. *Chem. and Ind.*, **43**, 908–911.

DUCLAUX, M. 1864 Sur la fermentation alcoolique, *Comp. Rend. Acad. Sci.*, **58**, 1114–1116.

DZIERZBICKI, A. 1909 Einige Beobachtungen über den Einfluss der Humusstoffe auf die Entwicklung der Hefe und auf Alkoholgärung, *Anzieger der Akad. d. Wissensch. in Krakau*, 651.

EASTCOTT, E. V. 1924 The Natural Distribution of Bios I and II, *Proc. and Trans. Roy. Soc. of Canada*, Section 3, 3rd Series, **17**, 157–158.

EDDY, W. H., HEFT, H. L., STEVENSON, H. C., AND JOHNSON, R. 1921 Studies in the Vitamin Content. II., The Yeast Test as a Measure of Vitamin B, *Jour. Biol. Chem.*, **47**, 249–275.

EDDY, W. H., HEFT, H. L., STEVENSON, H. C., AND JOHNSON, R. 1921 The Yeast Test as a Quantitative Measure of Vitamine. *Proc. Soc. Exp. Biol. and Med.*, **18**, 138–140.

EDDY, W. H., HEFT, H. L., AND STEVENSON, H. C. 1922 A Reply to Fulmer, Nelson, and Sherwood Concerning Medium F, *Jour. Biol. Chem.*, **51**, 83–85.

EDDY, W. H., KERR, R. W., AND WILLIAMS, R. R. 1924 The Isolation From Autolyzed Yeast of a Crystalline Substance Melting at 223°C. Having the Properties of Bios, *Jour. Amer. Chem. Soc.*, **46**, 2846–2855.

EDDY, W. H., KERR, R. W., AND WILLIAMS, R. R. 1924 The Isolation of a Crystalline Substance (M.P. 223°C.) Having the Properties of "Bios," *Proc. Soc. Exp. Biol. and Med.*, **21**, 307–308.

EDDY, W. H., AND STEVENSON, H. C. 1919 The Suitability of the Bachmann Test for Water Soluble B Vitamin, *Proc. Soc. Exp. Biol. and Med.*, **17**, 52–56.

EDDY, WALTER H., AND STEVENSON, HELEN C. 1920 Further Studies in the Measurement of Vitamine Content. *Proc. Soc. Exp. Biol. and Med.*, **17**, 122–128.

EDDY, W. H., AND STEVENSON, H. C. 1920 Some Results with a New Technique in Vitamine Measurement, *Proc. Soc. Exp. Biol. and Med.*, **17**, 218–220.

EDDY, W. H., AND STEVENSON, H. C. 1920 Studies in the Vitamin Content, *Jour. Biol. Chem.*, **43**, 295–309.

EHRLICH, F. 1911 Über die Bildung des Plasmaeiweiszes bei Hefen und Schimmelpilzen, *Biochem. Zeit.*, **36**, 477–497.

EIJKMANN, C., HOOGENHUIJZE, C. J. C., AND DERKS, T. J. G. 1922 The Vitamin Content of Microorganisms in Relation to the Composition of the Culture Medium, *Jour. Biol. Chem.*, **50**, 311–314.

EMMETT, A. D., AND LUROS, G. O. 1920 Water-Soluble Vitamines. I. Are the Antineuritic and the Growth-Promoting Water-Soluble B Vitamines the Same? *Jour. Biol. Chem.*, **43**, 265–286.

EMMETT, A. D., AND STOCKHOLM, M. 1920 Water Soluble Vitamines. II. The Relation of the Antineuritic and Water Soluble B Vitamins to the Yeast Growth Promoting Stimulus, *Jour. Biol. Chem.*, **43**, 287–297.

EULER, H. VON AND PETTERSON, A. 1921 Vitamin B (Biokatalysatoren) und Co-Enzyme, *Zeit. physiol. Chem.*, **114**, 4–16; *Exp. Sta. Rec.*, **46**, 866, (1922).

EULER, H. VON AND MYRBÄCK, K. 1921 Vitamin (Biokatalysatoren) B und Co-Enzyme II, *Zeit. physiol. Chem.*, **115**, 155–169; *Exp. Sta. Rec.*, **46**, 866, 1922.

FERNBACH, A. 1901 Die Entwicklung der Hefe in einem mineralischen Medium, *Ann. de la Brasserie et de la Distillerie*, 510.

FLEMING, W. D. 1921 Vitamin Content of Rice by the Yeast Method Organic Nitrogen as a Possible Factor in Stimulation of Yeast, *Jour. Biol. Chem.*, **49**, 119–122.

FRANKEL, S., AND SCHWARZ, E. 1920 Ueber wasserlösliche Vitamine und garungsbeschleunigende Verbindungen. *Biochem. Zeit.*, **112**, 203–235; See also Vol. **126**, 189–226, 227–264, 265–268, 269–280.

FRASER, C. G. 1921. Methylene-Blue As Indicator in Determining the Toxicity of Phenol and Phenol-Salt Solutions Towards Yeast. *Jour. Physical Chem.*, **25**, 1–9.

FULMER, E. I., DUECKER, W. W., AND NELSON, V. E. 1924 The Multiple Nature of Bios, *Jour. Amer. Chem. Soc.*, **46**, 723–726.

FULMER, E. I., AND GRIMES, M. 1923 The Growth of Yeasts on Synthetic Agar Media, *Jour. Bact.*, **8**, 585–588.

FULMER, E. I., AND NELSON, V. E. 1922 Is Bios a Single Substance? *Proc. Iowa Acad. Sci.*, **29**, 371.

FULMER, E. I., AND NELSON, V. E. 1922 Water Soluble B and Bios in Yeast Growth, *Jour. Biol. Chem.*, **51**, 77–81.

FULMER, E. I., AND NELSON, V. E. 1923 Studies on Yeast. VI. On the Continuous Growth of Saccharomyces cerevisiae in Synthetic Mediums, *Jour. Inf. Diseases*, **33**, 130–133.

FULMER, E. I., NELSON, V. E., AND SHERWOOD, F. F. 1921 The Nutritional Requirements of Yeast II. The Effect of the Composition of the Medium on Growth of Yeast, *Jour. Amer. Chem. Soc.*, **43**, 191–199.

FULMER, E. I., NELSON, V. E., AND SHERWOOD, F. F. 1921 The Nutritional Requirements of Yeast. I. The Rôle of Vitamines in the Growth of Yeast, *Jour. Amer. Chem. Soc.*, **43**, 186–191.

FULMER, E. I., NELSON, V. E., AND WHITE, A. 1923 The Growth of Yeast on a Medium of Wholly Synthetic Origin, *Jour. Biol. Chem.*, **57**, 397.

FULMER, E. I., SHERWOOD, F. F., AND NELSON, V. E. 1924 Effect of Ammonium Chloride upon the Growth of Yeast and the Hydration of Gluten in Beer Wort. *Ind. and Eng. Chem.*, **16**, 921.

FUNK, C. 1921 The Antiberiberi Vitamin, *Jour. Ind. Eng. Chem.*, **13**, 1110–1111.

FUNK, C., AND DUBIN, H. E. 1920 A Test for Antiberi-beri Vitamin and Its Practical Application, *Jour. Biol. Chem.*, **44**, 487–498.

FUNK, C., AND DUBIN, H. E. 1920 Experiments on a Quantitative and Qualitative Test For Antiberiberi Vitamin, *Proc. Soc. Exp. Biol. and Med.*, **17**, 175–177.

FUNK, C., AND DUBIN, H. E. 1921 Vitamin Requirements of Certain Yeasts and Bacteria, *Jour. Biol. Chem.*, **48**, 437–443.

FUNK, C., AND FREEDMAN, L. 1922 Nutritional Factors in the Growth of Yeasts, II. Protein Hydrolysates, *Jour. Metabol. Res.*, **1**, 469–480.

FUNK, C., AND FREEDMAN, L. 1922 Nutritional Requirements in the Growth of Yeasts and Bacteria. I. Vitamins, *Jour. Metabol. Res.*, **1**, 457–468.

FUNK, C., AND FREEDMAN, L., 1923. Can Yeast Grow in a Chemically Pure Medium? *Proc. Soc. Exp. Biol. and Med.*, **20**, 311–313.

FUNK, C., AND FREEDMAN, L. 1923 The Presence of An Yeast Growth-Promoting Vitamin in Cane Sugar, *Jour. Biol. Chem.*, **56**, 851–860.

FUNK, C., AND PATON, J. B. 1922 Studies on Vitamine B and D, *Jour. Metabol. Res.* **1**, 737–775.

GOY, P. 1921 Les Vegetaux Inferieurs et les Facteurs Accessoires de Croissance, *Comp. Rend. Acad. Sci.*, **172**, 243–244.

HARDEN, A., AND ZILVA, S. S. 1918 The Antiscorbutic Factor in Lemon Juice, *Biochem. Jour.*, **12**, 259–269.

HARDEN, A. A., AND ZILVA, S. S. 1921 The Synthesis of Vitamin by Yeasts, *Biochem. Jour.*, **15**, 438–439.

HEATON, T. B. 1923 On the Vitamin D, *Biochem. Jour.*, **16**, 800–810.

HENNEBERG, W. 1904 Zeit. Spiritusind, 11. (Quoted from Kossiwicz, 1911.)

HENNEBERG, W. 1910 Zum Verhalten der Kulturheferassen in zusammengesetzten Nährlösungen, *Beiträge zur Physiologie der Hefe*, Berlin Inst. f. Gärungsgewerbe; *Wochenschr. Brauerei*, **24**, 542–546, 575–579, 581–586, 596–601, 609–620.

HENNEBERG, W. Zum Verhalten der Kulturheferassen in zusammengesetzten Nährlösungen, *Beitrage zur Physiologie der Hefe*, Berlin.

HENRY, J. 1902 Uber das Bios des Wildiers, *Ann. Brasserie et Distillierie*, 129.

HOSOYA, S., AND KUROYA, M. 1923 Water-Soluble Vitamin and Bacterial Growth. Second Report, With Special Reference to Chemical and Physical Properties of Vitamin Essential for the Growth of Hemolytic Streptococci, Scientific Reports Gov., *Inst. for Inf. Dis.*, **2**, 265–285.

IDE, M. 1907 Über Wildiers' Bios, *Cent. Bakt.*, II, 18, 193–199.

IDE, M. 1921 The "Bios" of Wildiers and the Cultivation of Yeast, *Jour. Biol. Chem.*, **46**, 521–524.

KITA, G. 1914 Zur Frage der Assimilierbarkeit der Maltose durch Hefen, *Zeit. Tech. Biol.*, **4**, 321–322.

KOSSOWICZ, A. 1903 Untersuchungen uber das Verhalten der Hefen in Mineralischen Nährlösungen, *Z. Landw. Versuchswesen, Osterreichs*, **6**, 27.

KOSSOWICZ, A. 1906 Uber den Einfluss von Mycoderma auf die Vermehrung und Gärung der Hefen, *Zeit. f. Landw. u. Versuchsw. Oesterr.*, **17**, 688.

KOSSOWICZ, A. 1911 Die alkoholische Gärung und die Biosfrage, *Einfuhrung in die Mykologie der Genussmittel und in die* Gärungsphysiologie, Berlin.

KRIEGER. 1901 Bericht über die Mitteilungen von Wildier's Betrachtungen, *Amerikanischer Bierbrauer*, 712.

KURONO, K. 1905 Significance of Oryzanin for the Nutrition of Fermentative Organisms, *J. Coll. Ag., Imp. Univ. Japan*, **5**, 305.

LEPESCHKIN, V. 1924 The Influence of Vitamins Upon the Development of Yeasts and Molds, *Amer. Jour. Bot.*, **11**, 164–167; *Chem. Absts.*, **18**, 1683–1684.

LEVENE, P. A., AND VAN DER HOEVEN, B. J. C. 1924 On Concentration of Vitamin B, *Science* (Short Letter), **59**, 276.

LEVENE, P. A., AND VAN DER HOEVEN, B. J. C. 1924 The Concentration of Vitamin B, *Jour. Biol. Chem.*, **61**, 429–443.

LIEBIG, J. V. 1871 Sur la Fermentation et la Source de la Force Musculaire, *Annal de Chim. et Phys.* (4th Series), **23**, 5–49.

LINDET. 1917 Le Dechet de la Fermentation Alcoolique, *C. R. Acad. Sci.*, **164**, 58–61.

LINDNER. 1905 Die Assimilierbarkeit der Selbsverdauungs-produkte der Bierhefe durch verschiedene Heferassen und Pilze, *Wochenschrift für Brauerei*, 22, 528.

LINDNER, P. 1919 Zur Verflüchtigung des Biosbegriffe, *Zeit. Tech. Biol.*, **7**, 79–87; *C. B.* Abt. II, **59**, 143. 1920.

LINDNER, P. 1920 Die Biosproblem in der Hefeforschung, *Deut. Essigsind*, **24**, 103–105.

LINDNER, P. 1921 Mikrobenfettung, die Biosfrage und die Bekämpfung des Tuberkelbazillus in seiner Eigenschaft als Fettpilz, *Zeit. tech. Biol.*, **9**, 100–107. (See also p. 115.)

LINDNER, P., AND STOCKHAUSEN, F. 1906 Die Assimilierbarkeit der Selbstverdaunngsprodukte der Bierhefe durch verschiedene Heferassen und Pilze II, *Wochenschr. Brauerei*, 519.

LINOSSIER, G. 1920 Les Vitamines et les Champignons, *C. R. Soc. Biol.*, **83**, 346–349.

LINOSSIER, G. 1919 Les Vitamines et les Champignons, *C. R. Soc. Biol.*, **82**, 381–384.

LOBECK, A. 1922 Contribution a l'Etude des Facteurs Accessoires du Développement (Auximones), Dissert., Univ. of Geneva.

LUCAS, G. H. W. 1924 Chemical Study of Bios, *Proc. and Trans. Roy. Soc. of Canada*, Section 3, 3rd Series, **17**, 157.

LUCAS, G. H. W. 1924. The Fractionation of Bios and Comparison of Bios with Vitamins B and C, *Jour. Physical Chem.*, **28**, 1180–1200.

LUMIERE, A. 1921 Influence des Vitamins et des Auximones sur la Croissance des Vegetaux, *Ann. Inst. Past.*, **35**, 102–123.

MCCOLLUM, E. V., AND SIMMONDS, N. 1918 A Study of the Dietary Essential Water Soluble B. In Relation to its Solubility and Stability Towards Reagents, *Jour. Biol. Chem.*, **33**, 55–89.

MACDONALD, M. B. 1922 The Synthesis of Water Soluble B by Yeast Grown in Solutions of Purified Nutrients, *Jour. Biol. Chem.*, **54**, 243–248.

MACDONALD, M. B. 1923 The Synthesis of Bios by Yeast Grown in a Solution of Purified Nutrients, *Jour. Biol. Chem.*, **56**, 489.

MACDONALD, M. B., AND MCCOLLUM, E. V. 1921 The Cultivation of Yeast in Solutions of Purified Nutrients, *Jour. Biol. Chem.*, **45**, 307–311.

MACDONALD, M. B., AND MCCOLLUM, E. V. 1921 The Bios of Wildiers and the Cultivation of Yeast, *Jour. Biol. Chem.*, **46**, 525–527.

MARTINAND, V. 1908 *Revue de Viticulture*, **29**, 29. (Quoted from Kossowicz, 1911.)

MATTHEWS, C. G., AND LOTT, F. E. 1902 Fermentation from the Point of Yeast Crop, *Jour. Fed. Inst. Brewing*, **8**, 313-340.

MAYER, A. 1869 Untersuchungen über Alkoholgärung.

MICKO, K. 1904 Untersuchung von Fleisch-, Hefen-, und anderen Extrakten auf Xanthinkörper, *Zeit. Unters. d. Nährungs Genussm.*, **8**, 225–237.

MILLER, ELIZABETH W. 1921 The Effect of Certain Stimulating Substances on the Invertase Activity of Yeast, *Jour. Biol. Chem.*, **48**, 329–346.

MILLER, W. L. 1924 Wildiers' Bios, *Science*, **59**, 197–199.

MÜLLER-THURGAU, H. 1892–1893 Weitere-untersuchungen über die Physiologie der Hefe und die Bedentung ausgewälter und rimgezüchteter Heferassen für die Weingährung, *Jahresb. d. Schw. Versuchstat. in Wädensweil*, **11.** (Quoted from Kossowicz, 1911.)

NÄGELI, C. 1879 Ehrnährung der niederen Pilze durch Kohlenstoffund Stickstoffvergindungen, *Nachtrag zur Stiz. d. Mathem. Physi. Klasse*, **5.**

NÄGELI, C. 1903–1905 Ueber Oligodynamische Erscheinungen in liebenden Zellen, *Neve Denkschriften naturfarschenden Gescleschaft*, **33** and **34**, 1-51.

NAUMANN, H. 1919 Die Lebenstätigkeit von Sprosspilzen in mineralischen Nährlösungen, *Zeit. tech. Biol.*, **7**, 1.

NELSON, V. E., FULMER, E. I., AND CESSNA, R. 1921 The Nutritional Requirements of Yeast III Synthesis of Water Soluble B by Yeast, *Jour. Biol. Chem.*, **46**, 77–81.

NELSON, V. E., HELLER, V. G., AND FULMER, E. I. 1923 Studies on Yeast. VII. The Dietary Properties of Yeast. *Jour. Biol. Chem.*, **57**, 415–424.

OSBORNE, T. B., AND WAKEMAN, A. J. 1919 Extraction and Concentration of Water Soluble Vitamin from Brewer's Yeast, *Jour. Biol. Chem.*, **46**, 383–394.

OTERO, M. J. 1924. Role de quelques Stimulants sur l'Activite Fermentative de la Levure. *Comp. Rend. Soc. Biol.*, **88**, 375–376.

PASTEUR, L. 1860 Memoires sur la Fermentation Alcoolique, *Annales de Chim. et Phys.*, 3rd Series, **58**, 323–426.

PASTEUR, L 1871 Note sur un Memoire de M. Liebig Relatif aux Fermentations, *Comp. Rend. Acad. Sci.*, **73**, 1419–1424.

PASTEUR, L. 1872 Note sur un Memoire de M. Liebig, Relatif aux Fermentations, *Annals de Chim. et Phys.*, 4th Series, **25**, 145–151.

PASTEUR, L. 1874 Production de la Levure dans un Milieu Mineral Sucre, *Comp. Rend. Acad. Sci.*, **78**, 213–217.

PESKETT, G. L. 1924 Allelocatalysis and the Growth of Yeast, *Biochem. Jour.*, **18**, 866–871.

PRINGSHEIM, H. 1906 Ueber die Sogenannte "Bios-Frage" und die Gewohnung der Hefe an gezuckerte Mineralsalznährlösungen. *Cent. Bakt.*, **16**, 111–119.

PRINGSHEIM, H. 1907 Uber die Stickstoffernährung der Hefe, *Biochem. Zeit.*, **3**, 121–286.

ROBERTSON, R. C. 1924 Food Accessory Factors (Vitamins) in Bacterial Growth VIII. Relation of Substances Formed by B. coli to the Growth of Yeast, *Jour. Inf. Dis.*, **34**, 395–399.

ROBERTSON, R. C. 1924 Food Accessory Factors (Vitamins) in Bacterial Growth IX. Growth of Several Common Bacteria in a Synthetic Medium and Relation of Substances Formed by Them to Growth of Yeast, *Jour. Inf. Dis.*, **35**, 311–314.

ROBERTSON, R. C., AND DAVIS, D. J. 1923 Food Accessory Factors (Vitamins) in Bacterial Growth. VII. Observations on the Ultimate Source of Accessory Growth of Substances for Yeast, *Jour. Inf. Dis.*, **32**, 153–158.

ROBERTSON, T. B. 1921 The Influence of Mutual Contiguity Upon the Productive Rate and the Part Played Therein by the "X Substance" in Bacterised Infusions which Stimulates the Multiplication of Infusoria, *Biochem. Jour.*, **15**, 612–619.

ROBERTSON, T. B. 1921 Experimental studies on cellular multiplication I. The Multiplication of Isolated Infusora, *Biochem. Jour.*, **15**, 595–611.

SAMMARTINO, V. 1921 Vitamins, *Biochem. Zeit.*, **125**, 25; *Internat. Med. and Surg. Survey*, 1a–140.

SEIDELL, A. 1921 The Chemistry of Vitamins, *Jour. Ind. Eng. Chem.*, **13**, 72–75.

SEIDELL, A. 1924 The Chemistry of Vitamins, *Science*, **60**, 439–447.

SHERMAN, J. R., AND ALBUS, W. R. 1924 Is There An Allelocatalytic Effect in the Growth of Bacterial Cultures, *Absts. Bact.*, **8**, 6.

SOUZA, G. DE P., AND MCCOLLUM, E. V. 1920 A Study of the Factors Which Interfere With the Use of Yeast as a Test Organism For Antineuritic Substances, *Jour. Biol. Chem.*, **44**, 113–129.

STERN, A. L. 1902 Some Final Considerations on the Nutrition of Yeast. The Nutrition of Yeast, Part IV., *Jour. Federated Institutes of Brewing*, **8**, 690–707.

STOCKHAUSEN, F. 1908 Uber die Assimilierbarkeit der Selbstverdauungsprodukte der Bierhefe durch verschiedene Heferassen, *Jahrb. d. Versuchsanst. f. Brauerei*, **11**, 673.

SUZUKI, V., AND SUZUKI, B. 1923 The Chemical Nature of Vitamin D, *Jour. Chem. Soc. Japan*, **44**, 228; *Chem. Absts.*, **17**, 2907.

SWOBODA, F. 1920 A Quantitative Method For the Determination of Vitamin in Connection With Determinations of Vitamin in Glandular and Other Tissues, *Jour. Biol. Chem.*, **44**, 531–551.

TANNER, F. W., AND DACK, G. M. 1924 A Study of Yeasts from Sore Throats, *Cent. Bakt.*, Pt. 1, **91**, 282–299.

TANNER, F. W., DEVEREUX, E. D., AND HIGGINS, F. M. The Multiplication of Yeasts and Yeast-Like Fungi in Synthetic Nutrient Solutions. In press.

TAYLOR, E. M. 1924 The Toxicity of Acids Towards Yeast, *Proc. and Trans. Roy. Soc. of Canada*, 3rd Series, Section 3, **17**, 158.

THOLIN, T. 1921 Über die Thermostabilität des Co-Enzyms und Seine Abscheidung von Hefevitamin B., *Zeit. Physiol. Chem.*, 115, 235-256; *Exp. Sta. Rec.*, **46**, 309 (1922).

TSUKIYE, S. 1922 Beiträge zur Kenntniss des Vitamins (B) nebst Darstellungsmethode, *Biochem. Zeit.*, **131**, 124–139.

VILLAROEL, M. J. La Vitamine B Comme Activateur des Fermentes. *Comp. Rend. Soc. Biol.*, **88**, 376–377.

VOLK, W. 1915 The Utilization by the Animal Organism of Yeast Cultivated in Solutions of Sugar and Inorganic Salt. (Trans. Title), *Z. Spiritusind.*, **38**, 235.

WILDIERS, E. 1901 Une Nouvelle Substance Indispensible au Developpement de la Levure, *La Cellule*, **18**, 313.

WILLAMAN, J. J., AND OLSEN, A. G. 1923 The Bios Requirements of Baker's Yeast, *Jour. Biol. Chem.*, **55**, 815–836.

WILLIAMS, R. J. 1919 The Vitamine Requirement of Yeast, A Simple Biological Test for Vitamine, *Jour. Biol. Chem.*, **38**, 465–486.

WILLIAMS, R. J. 1920 A Quantitative Method for Determination of Vitamine, *Jour. Biol. Chem.*, **42**, 259–265.

WINDISCH, W. 1902 Uber "Das Bios von Wildier spielt nicht die Rolle eines Gegengiftes," *Wochenschr. Brauerei*, **19**, 527–532.

WINDISCH, W. 1902 Kritische Bemerkungen zu der Abhandlung von Wildier's "Bios" eine neue, zur Entwicklung der Hefe unumganglich notwendige Substanz., *Wochenschr. f. Brauerî*, **19**, 2.

WINDISCH, W. 1902 Poisonous Influence of Infinitesimal Traces of Substances on Living Matter, *Wochenschr. Brau.*, **19**, 27–30.

WRIGHT, O. K. 1922 The Action of Yeast Growth Stimulant, *Biochem. Jour.*, **16**, 137–142.