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THE VITAMINE REQUIREMENT OF YEAST
A SIMPLE BIOLOGICAL TEST FOR VITAMINE

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BY

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THE VITAMINE REQUIREMENT OF YEAST.

A SIMPLE BIOLOGICAL TEST FOR VITAMINE.*

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PLATE 6.

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INTRODUCTION.

Evidence is here presented, based upon direct microscopic observation of the growth of individual yeast cells, that the water-soluble-, beri-beri-preventing vitamine, relatively so abundant in yeast, is necessary for the nutrition of yeast cells themselves.

Pasteur in his famous researches found that yeast would grow in a solution of cane sugar, ammonium salt, and the salts of yeast ash. Since his day it has been generally believed that yeast can grow on such a synthetic medium and that an addition of broken down protein improves such a culture medium for yeast. Pasteur, however, observed the fact that a particle of yeast the size of a pin head (containing several million cells) must be used to inoculate such a solution in order to get appreciable growth and fermentation. This observation we shall see is a very important one.

Wildiers[1] made this same observation in 1901. He also observed that, by adding a little sterile water extract of yeast to a medium containing ammonium salt as its only source of nitrogen, a very small amount of yeast could be made to grow rapidly as judged by carbon dioxide production. This fact he thought to be due to a substance in the yeast extract which in addition to other known nutrients had to be supplied for the nutrition of the yeast cells. This new substance indispensable for the growth of yeast Wildiers called "bios." He found the substance to be soluble in water and 80 per cent alcohol but insoluble in ether. It was dialyzable and it was not precipitated by any of the ordinary precipitants including

* This work was carried out in connection with a fellowship for the study of Yeast Nutrition given by the Fleischmann Company.

[1] Wildiers, E., *La Cellule*, 1901, xviii, 313.

phosphotungstic acid. The substance was stable in acid solution but was destroyed by short boiling in dilute alkali. This instability was not uniform as he obtained very divergent results. Wildiers found "bios" to be absent from acid-hydrolyzed egg albumin, and from yeast ash.

Devloo[2] in 1906 claimed to have prepared "bios" in a state of purity from lecithin. Judging from his own statements, however, the material he prepared was in reality not very active in promoting yeast growth. His work will be referred to later in the discussion.

Pringsheim[3] in 1906 showed that in the synthetic solution referred to by Wildiers there was unquestionably some growth of single yeast cells, though the growth was small. He sought to refute the work of Wildiers by saying that "bios" was nothing more nor less than protein material which theoretically should be obtained in most available form for yeast, from yeast itself. Evidently this argument stood, as in more recent years no mention of Wildiers' work has been found.

In this laboratory a study of the nutrition of yeast was undertaken with the intention of first studying solutions of known composition. In working with synthetic solutions it was also thought desirable to first obtain a pure culture of yeast grown on a synthetic medium if such a thing were possible. However, using the Lindner droplet method the single cell from which the pure culture was to be grown in such a medium could never be made to produce a colony large enough to be seen with the naked eye, and consequently such a culture was out of the question. Bearing in mind Wildiers' work, other experiments along the same line were performed with the result that Wildiers' observations were largely confirmed. The possibility presented itself that the substance which Wildiers called "bios" might be the same as the vitamine known to be contained in yeast. With this possibility in mind, a preparation of "activated" fullers' earth was obtained through the courtesy of Williams & Seidell and it was found to contain a substance which in small amounts would promote remarkably the growth of single yeast cells. The other work reported below is the logical outgrowth of this initial observation.

EXPERIMENTAL.

I. General procedure.—In preliminary experiments fairly uniform results were obtained by using merely a suspension of commercial pressed yeast for seeding, but for all the experiments

[2] Devloo, R., *La Cellule*, 1906, xxiii, 361.

[3] Pringsheim, H. H., *Centr. Bakteriol., 2te Abt.*, 1906, xvi, 111.

here reported a pure culture of baker's yeast was used. All experiments except under VI were conducted with a pure culture grown in this laboratory. In these experiments a pure culture was used which was kindly furnished by Dr. R. E. Lee of the Fleischmann Company. The results obtained on this culture in that study were also confirmed with the other culture.

In order to maintain a pure culture during the necessary manipulation most of the work was carried on inside a sterile cupboard made for the purpose. This glass cupboard was sprayed inside with dilute alcohol and closed generally a day or more previous to its use. The sliding glass door was opened during the work only enough to allow free movements of the hands of the experimenter.

The greatest difficulty in carrying out the experiments reported below, and in obtaining uniform results, lay in obtaining yeast in the right condition so that the colonies would break apart easily when a suspension was made and at the same time contain a large proportion of live cells. If, for instance, yeast which had grown one day was suspended, the yeast cells stayed in colonies and if a suspension was shaken hard enough to break the cells apart most of the cells were killed or their vitality was so lowered that they did not grow under the conditions of the experiment. In the case of yeast which has grown for a week or more at 30°C. or a shorter time at 37°C. the cells of colonies break apart into single cells easily, but due to autolysis, a considerable number of the cells are dead, and those which do grow, grow distinctly more in a solution containing ammonium salt as the only source of nitrogen than do cells when autolyzed material is not present. The autolyzed material of such a culture contained in a speck of yeast much smaller than a pin head when added to 25 cc. of solution can be detected by the increased growth of cells in the hanging drops. For the experiments reported the yeast used was grown 3 or 4 days at 30°C. and in most cases kept in the refrigerator a day or two before being used. In such a growth the cells often had some tendency to remain in clusters but it was much more easily worked with than younger yeast. The yeast at best seemed to vary somewhat in its properties when grown apparently under the same conditions. At times during the investigation the yeast was easier to work with than at other times. All the

yeast used in the experiments reported was grown on a rich malt wort obtained from a Fleischmann factory, and solidified with agar. When yeast was grown on a wort which had been autoclaved a long time or many times the growth of cells in a solution of mineral salts and sugar was still less than that reported in the case of the more vigorous yeast.

In the preliminary experiments various solutions were used, in which asparagine and ammonium lactate were used as sources of nitrogen, but in the experiments which are reported here the simpler salt $(NH_4)_2SO_4$ was used. The solution used consisted of the following substances dissolved in a liter of distilled water.

20	gm. cane sugar.
3	gm. $(NH_4)_2SO_4$
2	gm. KH_2PO_4
0.25	gm. $CaCl_2$
0.25	gm. $MgSO_4$

Yeast cells are not sensitive to small changes in hydrogen ion concentration; nevertheless, the phosphate buffer with the very small additions made served to control the acidity of the medium.

The solutions to be tested were always sterilized, ordinarily at 10 pounds pressure for 10 minutes before being seeded with yeast. In some cases the solutions were kept in the refrigerator 2 or 3 days after sterilization before the experiment was carried on. Bacterial contamination was carefully avoided in all material. When in preliminary experiments a "mineral salt solution" became contaminated yeast cells grew in the solution better than before contamination, although the solution was sterilized before being seeded with yeast. Hence it was necessary to avoid foreign organisms and the nature of the method made it possible to be sure that this factor was controlled.

When the yeast culture and the solution were in proper condition the seeding was carried out in the sterile cupboard mentioned above. A small amount of yeast was taken out of the culture and suspended in a test-tube containing about 10 cc. of sterile water. The amount of yeast put in approximated the size of a pin head, but could be estimated only after a little practice. The suspension was then slightly cloudy. A 1 cc. sterile pipette was introduced into the suspension and attached

to a rubber tube with a pinch-cock. The rubber tube contained a good cotton plug. By blowing through the tube and pipette a uniform suspension of the yeast was insured at the moment the pipette was filled. 1 cc. of the suspension was then put for trial into a flask containing the same amount of water as the solutions to be tested. After gently shaking the very dilute suspension a sterile steel pen was dipped into it and 25 drops were made on a cover-slip and immediately inverted on a hollow ground slide prepared with vaseline for sealing the chamber air-tight.[4] The drops were then examined under the low power of the microscope to see if the seeding was about the right amount. If the seeding was much too great it was corrected by diluting the suspension with sterile water and if too small by using more of the suspension for seeding or adding more yeast to the suspension. Ordinarily there was no difficulty in obtaining the right amount of seeding as it is not necessary to have an exact and definite concentration of cells. The number of cells in the hanging drops can be varied by varying the time interval between shaking the solution and dipping the pen. If slightly too many cells are present in the drops when the pen is dipped immediately after shaking, waiting a moment before dipping the pen will give the desired results. When the correct seeding was found, each solution to be tested was seeded immediately in the same way. In this way the yeast to be seeded remained in the distilled water only a few minutes.

At least 50 hanging drops on two slides were made in the way described from each solution to be tested. By using a standard pen the drops were of fairly uniform size and by having the solution as cool as possible evaporation was avoided. The contents of each drop which contained one single cell, two cells joined together, or one single and one double cell or two of each and no more, were recorded on squared paper. In order to increase the number of observations a single cell with a small bud was counted as a single cell and a double cell with a bud was counted as a double cell. This did not change the trend of the results as all solutions were treated alike in this manner and no conclusions are drawn from differences unless they are large.

[4] Thanks are due Dr. Lee for his suggestion of the hanging droplet method of study.

The individual variations of the yeast cells are quite large as will be seen in the results below. Many observations showed that yeast cells grow practically as well whether there are one or two cells in a drop provided the growth is not carried too far. After the contents of each slide had been recorded the slide was put into an incubator at 30°C. and the time of incubation recorded. In this manner eight satisfactory slides of 25 drops each representing four solutions could be made, and the contents recorded in an hour.

After the cells had been growing 5 or 6 hours all the slides were examined. Slight growth had taken place in this time and the records of the contents of the drops were verified and the growth was recorded. In case any cells were overlooked they were noted and if more than two colonies were located in one drop, the particular drop was excluded from consideration. At the end of 20 to 24 hours the drops were examined and counts again made. If a colony could not be counted because of the presence of some debris (for instance flakes from the steel pen) it was excluded from consideration. This was also true if it could not be determined with a good degree of certainty whether a certain colony was produced by a single or double cell or both, both of which might have been in the same drop. Ordinarily, however, few drops were excluded and practically all the original cells were considered. No colonies were excluded because of irregularities of growth. In case the colonies were small the count could be accurate; larger colonies were less accurately determined as the colonies grow to some extent in three dimensions. Estimations of the larger colonies were made by counting a portion of the colony and estimating the number of times it was contained in the whole. An effort was made to make the estimates on large colonies conservative.

Often the contents of the drops were watched for several days but the results are not reported unless they have some added significance. In some experiments there were practically no single cells present, and in others few double cells were present, consequently only one kind is reported in the experiments, provided there were sufficient numbers to warrant drawing conclusions. The growth of single and double cells was always parallel.

II. Protein-Free Milk.—Osborne and Mendel have used protein-free milk extensively as a source of the water-soluble vitamine.

This experiment was performed to find out if the substance promoting the growth of yeast is also present in such material. 200 cc. of milk were made "protein-free" following the method of Osborne and Mendel.[5] The filtrate which was water-clear was evaporated to dryness on a water bath in a current of air. It was further dried by alcohol and ether treatment and kept in a vacuum desiccator. This material was extracted three times with warm ether and three times with a like quantity of warm 95 per cent alcohol. The ether extract which was very slight in amount was digested with water and made up to the original volume of

TABLE I.

	Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 1 cc. H_2O	20	12	15	4	8
II.	25 cc. control solution + 1 cc. ether extract of protein-free milk	14	4	8	4	6
III.	25 cc. control solution + 1 cc. alcohol extract of protein-free milk	18	10	180	36	108

the milk. The alcoholic extract which was much larger in amount was also digested with water and made up to the original volume of the milk.

The solutions to be tested were made up as indicated in Table I, sterilized, and seeding carried out in the manner described. Growth at the end of 22 hours was recorded. The results show plainly that the growth stimulant is present in the alcohol fraction of protein-free milk.

III. Wheat Germ.—Wheat germ has been used as a source of the water-soluble vitamine by McCollum and his coworkers as well as others.

[5] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, pts. i, ii, 1911.

In an experiment not performed specifically for this purpose and only part of which is reported (Table II), it is shown that wheat germ may be used as a source of the substance promoting the growth of yeast.

Wheat germ was extracted three times with a good quantity of hot 95 per cent alcohol, the extract was filtered, and the filtrate evaporated to dryness. The residue was digested as completely as possible in such a quantity of water that 1 cc. of the solution was equivalent to 75 mg. of the original wheat germ. After filtration 2 cc. of the solution were used for the experiment.

The results recorded in Table II were obtained at the end of 22 hours growth. There is evidence of a considerable amount of the yeast growth substance present in wheat germ.

TABLE II.

	Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 2 cc. H_2O	20	2	3	2	2.5
II.	25 cc. control solution + 2 cc. alcoholic extract of wheat germ.	18	8	135	54	91

IV. Lactose.—McCollum and Davis[6] found in feeding experiments, in which they used 20 per cent of Kahlbaum and Merck's lactose, that the lactose contained the water-soluble vitamine as an impurity. We ran tests to ascertain if the yeast growth substance was likewise present in lactose as an impurity.

10 gm. of Kahlbaum's lactose were extracted with 95 per cent alcohol continuously for 6 hours. The alcohol was cooled, filtered free from lactose crystals, and evaporated to dryness on the water bath. The residue was then dissolved in 10 cc. of water. 5 cc. of this solution were used for each of the experiments reported below. The two experiments are used together as neither one by itself was absolutely conclusive, due to the low vitality of the yeast used. The two experiments were conducted in the same way except that the yeast used in the second case had been in the refrigerator one day longer and 2 cc. of a suspension instead of 1 cc. were used for seeding.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 183.

The results of growth at the end of 22 hours of both double and single cells are reported in Table III and show definitely the presence of a small amount of the growth-promoting substance in Kahlbaum's lactose.

V. Pancreas Tissue.—Eddy[7] has found the water-soluble vitamine to be present in fairly large quantities in pancreas tissue.

This experiment was performed to determine whether the yeast growth substance is also present in pancreas tissue, pancreatin U. S. P. being used as a convenient source. 2 gm. of pancreatin were extracted directly with warm ether and filtered through a good filter. The residue was then extracted in like

TABLE III.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 5 cc. H_2O	22	3	12	7	9
II. 25 cc. control solution + 5 cc. alcoholic extract of lactose	29	12	50	18	36
	Single cells considered.				
I. 25 cc. control solution + 5 cc. H_2O	8	4	10	3	6
II. 25 cc. control solution + 5 cc. alcoholic extract of lactose	9	3	36	10	23

manner with warm 95 per cent alcohol and filtered. Each filtrate was evaporated to dryness and dissolved in 20 cc. of water. 1 cc. was used in the experiments which was equivalent to 0.1 gm. of pancreatin.

Table IV gives the results of a 22 hours growth following the usual procedure and shows the presence of a considerable amount of the yeast growth substance.

VI. Hydrolyzed Casein.—The purpose of this experiment was to test the possibility that the growth-promoting substance of yeast might be some of the amino-acids derived from an "adequate" protein like casein.

[7] Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.

5 gm. of casein Merck (Hammarsten) were digested with 25 per cent H_2SO_4 for 14 hours using a reflex condenser. The bulk of the acid was removed with $Ba(OH)_2$ and the solution brought to very slight acidity. This was made up to 500 cc. volume and sterilized on two occasions at 10 pounds pressure for 10 minutes. When ready for use, to a portion was added crystalline tryptophane up to 1.5 per cent of the original casein digested. The tryptophane had been prepared from casein by the Hopkins method. It is obvious from the previous experiment why pancreatin was not used to digest the casein. For purposes of comparison solutions were used which were known to contain the growth-promoting substance, an alcoholic extract of an alkali extract of "activated" fullers' earth being used as a source of the

TABLE IV.

	Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 1 cc. H_2O.....	14	8	10	4	5.6
II.	25 cc. control solution + 1 cc. ether extract of pancreatin.....	13	6	15	5	9.2
III.	25 cc. control solution + 1 cc. alcoholic extract of pancreatin.....	15	13	280	80	187

growth-promoting substance. Each cc. added contained 1 mg. of actual material. Because a considerable amount of the casein digest was added in this experiment additional phosphate buffer was used to control the acidity.

Table V gives the results for the first 5 hours growth and Table VI for the first 22 hours.

Noting the fact that the growth in Solution IV had slowed up it was thought desirable to watch it another day.

At the end of 48 hours the growth of the three colonies in each solution which had shown maximum growth in 22 hours were counted or estimated (Table VII).

In Solution IV the initial growth was most rapid as would be expected, but later was markedly retarded. The results clearly indicate that as the yeast grows in the solution containing both

TABLE V.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 1 cc. 10 per cent KH_2PO_4 solution + 11 cc. H_2O	17	2	3	3	3
II. 25 cc. control solution + 1 cc. 10 per cent KH_2PO_4 solution + 1 cc. fullers' earth extract	16	11	6	3	4.0
III. 25 cc. control solution + 10 cc. casein digest + tryptophane + 1 cc. H_2O	13	9	5	3	3.6
IV. 25 cc. control solution + 10 cc. casein digest + tryptophane + 1 cc. fullers' earth extract	15	12	10	3	6.1

TABLE VI.

Solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I	17	6	9	4	5.8
II	16	15	95	12	58.0
III	13	12	10	3	8.9
IV	15	14	46	17	32.0

TABLE VII.

Solution.	Average of the three maxima.	
	22 hours.	48 hours.
I	8	11
II	75	283
III	10	13
IV	43	45

casein digest and the growth-promoting substance, something harmful is formed which is not initially present. This may throw some light on the observation of Effront as well as others that too complete hydrolysis of protein injures it as a yeast food.

Aside from this question the experiment shows that the growth-promoting substance is not one of the commoner amino-acids known to be contained in an acid digest of casein.

VII. Adsorption from Malt Wort by Fullers' Earth.—Seidell[8] has found that the water-soluble vitamine is adsorbed nearly quantitatively from autolyzed yeast filtrate by certain varieties of fullers' earth and is thus removed from the bulk of the original material.

As has been mentioned it has been found that such a preparation contains the substance which stimulates yeast growth in relatively large quantity. The experiment reported below was performed to confirm this fact and to determine if there is anything unadsorbed by the fullers' earth which may take the place of the adsorbed material.

Fresh malt wort obtained from one of the Fleischmann factories was evaporated on the steam bath to about one-half its original volume. It was filtered and a portion diluted 1:20, while a 25 cc. portion was shaken continuously with 1 gm. of fullers' earth (Eimer and Amend) for 1 hour. The fullers' earth was filtered off, the filtrate diluted 1:20, and the fullers' earth washed once with water. The fullers' earth was then extracted by shaking 10 minutes with saturated $Ba(OH)_2$, filtered, and the barium removed with H_2SO_4. The resulting solution was diluted so that 2 cc. were equivalent to 3 cc. of the diluted wort. This experiment was carried on with the pure culture furnished by the Fleischmann Company using the general method previously described.

Table VIII gives the results of 6 hours of growth.

At the end of 24 hours, due to the large number and size of the colonies, only the three in each solution which had shown maximum growth in 6 hours were considered (Table IX).

One effect is observed here but not recorded in any other experiments. The yeast used for seeding had remained in the

[8] Seidell, A., *Bull. Hyg. Lab., U. S. P. H.*, 1916, xxxi, 364.

refrigerator a week, during which time autolysis had evidently taken place. A large percentage of cells was not able to grow even in the good medium. Furthermore, the growth of those which did grow in the control solution was greater than in other

TABLE VIII.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 5 cc. H_2O	25	10	4	2	2.4
II. 25 cc. control solution + 3 cc. diluted fullers' earth-treated wort + 2 cc. H_2O	22	10	8	2	3.2
III. 25 cc. control solution + 3 cc. diluted fullers' earth-treated wort + 2 cc. alkaline extract of fullers' earth	19	11	10	2	5.5
IV. 25 cc. control solution + 3 cc. original untreated diluted wort + 2 cc. H_2O	23	15	13	2	5.6

TABLE IX.

Solution.	Number considered.	Number growing.	Growth of the three maxima.			Average growth of the three maxima.
I	25	13	2	16	10	15
II	22	11	51	40	42	44
III	19	12	180	300	180	220
IV	23	12	200	375	300	292

experiments, due evidently to the autolyzed material added with the live cells. That the effect is not due to the vigor of the yeast was shown by the fact that the same yeast was used in a previous unreported experiment, where, before the slight autolysis had taken place, the cells had grown much less than in this experiment.

The foregoing experiment shows that the growth-promoting substance is taken out by shaking a solution with fullers' earth, just as vitamine is known to be. Probably it can be removed even more quantitatively by observing the right conditions of acidity, quantity of earth used and time of shaking; conditions which as yet have not been definitely determined.

VIII. Phosphotungstic Acid Precipitation.—Since the earliest work of Funk, precipitation by phosphotungstic acid has been used as a means of purification of the vitamine which prevents beri-beri. Wildiers found the so called "bios" not to be precipitated by phosphotungstic acid. The conditions of precipitation which he used were not definitely stated. Consequently, the experiment was repeated to determine if under proper conditions the yeast growth substance can be precipitated with phosphotungstic acid.

20 gm. of "activated" fullers' earth were shaken with 200 cc. of saturated $Ba(OH)_2$ for 5 minutes and filtered. The filtrate was brought with H_2SO_4 to very slight acidity, filtered, and the filtrate concentrated *in vacuo* to about 40 cc. and filtered from organic residues. The filtrate was divided into two equal portions. To one portion was added 50 per cent H_2SO_4 until it contained 5 per cent acid, and to it was added as a precipitant altogether 10 cc. of a 20 per cent solution of phosphotungstic acid dissolved in 5 per cent H_2SO_4. This was a slight excess of phosphotungstic acid. It was allowed to stand over night. The precipitate of phosphotungstates was filtered off and washed thoroughly with 5 per cent H_2SO_4 containing 0.5 per cent of phosphotungstic acid. The filtrate was made slightly alkaline with $Ba(OH)_2$ and filtered, the precipitate being washed thoroughly with half saturated $Ba(OH)_2$. The filtrate was then made very slightly acid, filtered, and after dilution to 200 cc. was used in the experiment as the "phosphotungstic acid filtrate." The phosphotungstates were decomposed with five 20 cc. portions of saturated $Ba(OH)_2$ grinding each time in a mortar. The material was in contact with the alkali a total of over an hour and the last two portions of alkali were warmed gently with the precipitate. The material was filtered and the precipitate washed on the filter with saturated $Ba(OH)_2$. The filtrate was made very slightly acid with H_2SO_4 and again filtered.

This was also diluted to 200 cc. and used in the experiment as "decomposed phosphotungstates." The original untreated solution was likewise diluted and equivalent amounts (2 cc.) of each solution were used. Each portion added was equivalent to 0.1 gm. of the "activated" fullers' earth. Each of the solutions had been heated in the autoclave a total of 10 minutes at 10 pounds pressure.

Hanging drops were made in the usual way. Table X shows the results at the end of 24 hours.

To get the best quantitative interpretation of the results the number growing in the solution should be considered as well as

TABLE X.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 2 cc. H_2O	16	3	5	2	3.3
II. 25 cc. control solution + 2 cc. original untreated extract	15	13	70	14	35.
III. 25 cc. control solution + 2 cc. "phosphotungstic acid filtrate"	18	4	33	2	25.
IV. 25 cc. control solution + 2 cc. "decomposed phosphotungstates"	14	10	70	11	51.

the average growth. Solution IV was most active, Solution II next in activity, and III much weaker as shown by the small percentage which grew. The "decomposed phosphotungstates" show greater activity than the original extract. Later the explanation for this result was found in the effect of acid and alkali on the material and will be discussed later. The activity of all the solutions except Solution I was considerably less than would be expected from the amount of earth used, judging by other experiments. Possibly some activity was lost when the original material was filtered after concentration *in vacuo*. That the substance is precipitated by phosphotungstic acid cannot be doubted. A similar result to that reported was obtained in other

experiments, the precipitation being more incomplete with more dilute solutions.

IX. Heat Stability.—Considerable divergence of opinion exists as to the degree of heat stability of the water-soluble vitamine. The more recent work indicates that in foods and in ordinary preparations it is quite stable to heat, not being destroyed at boiling temperature and withstanding considerable heating under pressure. No doubt preparations vary depending on various factors.

An alcoholic extract of dried yeast was tested for the heat stability of the substance promoting the growth of yeast. To each of two flasks were added 6 cc. of a solution of yeast extract, which had been prepared by extracting dry yeast with 95 per cent

TABLE XI.

	Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 6 cc. H_2O	12	3	4	3	3.3
II.	25 cc. control solution + 6 cc. autoclaved extract	20	12	6	3	4.3
III.	25 cc. control solution + 6 cc. untreated extract	11	7	10	3	5.6

alcohol, and to a third flask 6 cc. of distilled water. Each of the samples of yeast extract was equivalent to 0.3 gm. of dry yeast. One of the portions of extract was heated in the autoclave for 30 minutes at 15 pounds pressure and brought to its original weight with distilled water. To each of the three flasks were then added 25 cc. of the control solution and they were sterilized as usual for 10 minutes at 10 pounds pressure. After 6 hours growth in hanging drops results were obtained as recorded in Table XI.

At the end of 23 hours estimations were made on the three colonies in each solution which in 6 hours had shown the maximum growth (Table XII).

The substance promoting the growth of yeast in the condition in which it was used was partially destroyed by heating under 15 pounds pressure for 30 minutes.

X. Treatment with Acid or Alkali.—The water-soluble vitamine has been found to be quite stable in acid solution even at the boiling temperature. Vedder and Williams[9] found it to be markedly changed in its physiological properties by acid treatment and suggested a hydrolysis as a possible explanation. On the other hand it has been found to be unstable in alkaline solution. On this point there has been a wide divergence of results. At least one worker has reported its destruction by as weak a base as ammonia. McCollum and Simmonds[10] found that as prepared from wheat germ it was destroyed by short boiling with very dilute alkali. Williams and Seidell[11] have found the preparation extracted from "activated" fullers' earth to be quite stable to alkali, though the alkali alters it in some way without destroying its property of preventing polyneuritis in pigeons. Again a hydrolysis was suggested to explain the change. Recent

TABLE XII.

Solution.	Growth of three maxima.			Average of three maxima.
I.....	9	8	10	9
II.....	160	180	150	163
III.....	400	320	200	307

work by Daniels and McClurg[12] has shown that as the vitamine exists in foods it is not readily destroyed by cooking under pressure with very dilute alkali.

The study of the behavior of the growth-promoting substance of yeast toward acid and alkali has proven to be a complicated problem, due apparently to the interference of toxic substances formed. This study is therefore not complete although a large number of experiments has been performed. An indication of the trend of the results will be given.

The fullers' earth preparation has been found to be quite stable in alkaline solution, confirming the observations of Williams and

[9] Vedder, E. B., and Williams, R. R., *Philippine J. Sc.*, 1913, viii, 180.
[10] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 88.
[11] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 432.
[12] Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, xxxvii, 201.

Seidell[11] for the vitamine. There seems no doubt from our experiments that this material when acted on by acid or alkali is changed (presumably by hydrolysis) to a form which is more immediately available for the yeast. The treatment does not change the total amount of yeast which is produced in 2 or 3 days but makes the initial growth for 24 hours as much as three times as fast. These results are in accordance with the observations mentioned above on the supposed hydrolysis. This, it is thought, adequately explains the result obtained in the experiment on phosphotungstic acid precipitation as both the "filtrate"

TABLE XIII.

	Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 25 cc. H_2O	25	3	6	4	4.7
II.	25 cc. control solution + 1 cc. H_2O + 1 cc. ether extract of egg yolk	17	5	14	7	9
III.	25 cc. control solution + 1 cc. H_2O + 1 cc. alcoholic extract of egg yolk	29	15	120	29	68
IV.	25 cc. control solution + 1 cc. ether extract of egg yolk + 1 cc. alcoholic extract of egg yolk	26	20	200	23	59

and "decomposed phosphotungstates" had stood in 5 per cent H_2SO_4 for some time.

The results obtained on various other preparations would suggest that the substance promoting the growth of yeast, as it exists in yeast and wheat germ (alcoholic extracts), is not very unstable in the presence of alkali. However, a previous exhaustive extraction with dry ether seems to render the alcoholic extract obtained afterward more labile to alkali. We have some evidence that this is true also of the fullers' earth preparation. It seems to be unstable to alkali when the material is extracted with ether before alcoholic extraction. This will be referred to later.

XI. Fat-Soluble Vitamine.—It was thought desirable to find out whether the fat-soluble vitamine has any effect on yeast growth.

The yolk of hard boiled egg was selected as a source as it contains both water-soluble and fat-soluble vitamines in good quantity. The yolk was removed from a hard boiled egg, partially dried in a desiccator, then ground with plaster of Paris, and thoroughly dried over calcium chloride in a vacuum desiccator. The material was continuously extracted for 10 hours with sodium-dried ether, taking extra precaution to avoid the presence of moisture. It was then extracted for 3 hours with 95 per cent alcohol. Each extract was evaporated to dryness, digested as completely as possible in water, and filtered. The solutions were made so that 1 cc. was equivalent to 0.1 gm. of dry egg yolk.

The results obtained after 22 hours growth are given in Table XIII. The effect of the fat-soluble vitamine, if there is any effect, is not marked either as a substitute for the alcohol-soluble substance, or as complementary to it. If the yeast has a requirement for the fat-soluble vitamine it is exceedingly minute.

DISCUSSION.

In the preceding experiments it has been shown that the substance promoting growth of yeast occurs in the same materials as those in which vitamine has been found; namely, protein-free milk, wheat germ, lactose, yeast, egg yolk, and pancreatin. The substance is none of the commoner amino-acids contained in an acid digest of casein with tryptophane added. It has the same properties of solubility, precipitation by phosphotungstic acid, adsorption in fullers' earth, heat stability, and behavior toward acid and alkali, as nearly as we know those properties, as the water-soluble vitamine, and in addition the two substances so far as known have no divergent properties. A warning should be given that the results reported here in different experiments are not comparable one with another and were *not* designed to show quantitative comparisons of different materials. As far as we are able to judge, however, taking into consideration the apparent hydrolysis factor which probably is important, as well

as the method of extraction, those substances which are richest in vitamine are richest in the yeast growth substance. The fullers' earth preparation is richest of any of the sources tested. Wheat germ and yeast are poorer than the fullers' earth preparation and are of the same order as has been found to be true of the vitamine content in animal experiments. Milk is still poorer in the yeast growth substance as is also true of its vitamine content.

From the cumulative evidence offered we believe we are justified in concluding that as far as present knowledge is concerned the substance or substances which stimulate the growth of yeast is or are identical with the substance or substances which in animal nutrition prevent beri-beri or polyneuritis.

If this conclusion is true the water-soluble vitamine must be a most fundamental nutritional requirement playing an indispensable rôle for a great variety of organisms. It has not before been shown conclusively to be necessary for the nutrition of any members of the plant kingdom. Some suggestions have been made as to its possible importance in the nutrition of bacteria. We have accidentally observed that some species of molds are apparently able to produce it. Pacini and Russell[13] have shown that typhoid bacilli must be able to produce it.

As it is apparently possible to cause a single yeast cell to produce from 20 to several thousand cells in 24 hours by varying the vitamine content of the culture medium, we hope the method may be valuable both as a qualitative and ultimately as a quantitative test for vitamine. Work is now being done to make the test more applicable quantitatively and will be reported upon later. As a qualitative test extremely small amounts may be detected. The most serious obstacle in the purification and study of vitamines has been the difficulty of obtaining sufficient material to work with and test. Yeast can be used to test for the water-soluble vitamine and only infinitesimally small amounts need be used for testing. The accompanying photomicrographs (Figs. 1 and 2) show the effect of the addition of 0.5 mg. of actual crude material to about 30 cc. of control solution and subsequent incubation for 24 hours. The drops in which the cells were growing weighed approximately 0.03 mg. each and contained therefore

[13] Pacini, A. J. P., and Russell, D. W., *J. Biol. Chem.*, 1918, xxxiv, 43.

about one millionth of the 0.5 mg. of total material added. The material added was crude, being obtained simply by adsorption on fullers' earth from a malt wort and subsequent extraction with alkali. The test can be easily applied after a little practice. The vitamine if present in sufficient but very small quantity can be detected in the course of 4 or 5 hours.

Already the method has contributed something in the way of additional information in connection with the apparent hydrolysis for which the evidence is now more certain.

It seems that we have at least a partial explanation for the divergence of results in stability of the vitamine toward alkali, in the effect of previous ether extraction. In recent work McCollum and Simmonds[10] found the vitamine to be very unstable toward alkali. The material they used was previously extracted for 18 hours with ether. Voegtlin and Lake[14] observed that meat which had been freed from fat lost its antineuritic properties under the influence of heat and alkali much more easily than meat with the fat present.

The difficulty of obtaining pure materials and the likelihood of wrong inferences drawn from work with impure material is emphasized by our work. In earlier work on yeast, yeast no doubt grew better in "synthetic" media than it would have if the asparagine, ammonium tartrate, sugar, etc. used had been purer. Devloo's results on the preparation of "bios" from lecithin parallel the findings of an early investigator who cured polyneuritis on "pure" lecithin.

At this point I wish to thank Professor F. C. Koch for helpful suggestions and interest throughout the work and for his generosity in providing equipment.

CONCLUSIONS.

1. A substance of unknown nature, which is a constituent of yeast, is necessary in addition to the ordinary nutrients for the nutrition of yeast cells.

2. This substance (or substances) based upon identical occurrence and various properties is concluded to be identical with the beri-beri-preventing vitamine.

[14] Voegtlin, C., and Lake, G. C., *Am. J. Physiol.*, 1918–19, xlvii, 558.

3. The fat-soluble vitamine apparently has no effect on yeast growth.

4. The growth of single cells of yeast may be used as a simple biological test for vitamine, and it is hoped may be used to advantage in quantitative studies.

EXPLANATION OF PLATE.

FIG. 1. Growth produced by a single cell in 24 hours in "synthetic" solution.

FIG. 2. Growth produced in 24 hours under identical conditions except for the addition of one part in 60,000 of crude vitamine-containing material (cf. p. 484).

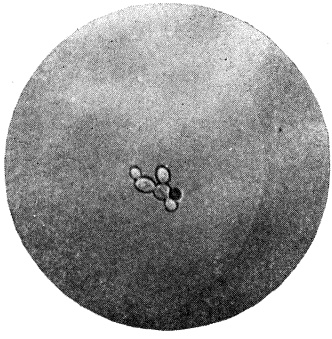

FIG. 1.

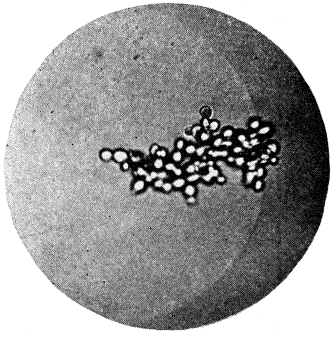

FIG. 2.

(Williams: Vitamine requirement of yeast.)