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NUTRITIONAL FACTORS IN THE GROWTH
OF CERTAIN YEASTS AND BACTERIA

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By

Louis Freedman, Ph. B., M. S.

NEW YORK CITY

1922

DISSERTATION

*submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy in the Faculty of Pure Science, Columbia University, in the
City of New York.*

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AUTHOR'S NOTE.

The two papers that constitute this dissertation were reprinted from the *Journal of Metabolic Research* (1922, volume I, No. 4, pages 457-480), where they were published under the same titles in collaboration with Dr. Casimir Funk.

NUTRITIONAL FACTORS IN THE GROWTH OF CERTAIN YEASTS AND BACTERIA.†

I. VITAMINES.

By LOUIS FREEDMAN

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College of Physicians and Surgeons, New York City,
and the Research Laboratory of H. A. Metz, New York City.*

INTRODUCTION.

Since the discovery of Wildier's yeast stimulating "bios" in 1901, rapid progress has been made in determining the nutritional requirements of various yeasts and bacteria.

The early work of many investigators has shown that both yeasts and bacteria require for their growth certain unknown substances, the properties of which correspond closely to those of our present day water-soluble B vitamine. Of late, however, considerable discussion has arisen regarding the identity of the substance that promotes the growth of yeast cells with that of vitamine B, and also its relation to the substance that stimulates the growth of bacteria.

In the present work, the authors have made an attempt to remove some of the uncertainties regarding these factors, and to show that a close nutritive analogy exists between yeasts and bacteria.

EXPERIMENTAL PROCEDURE.

A strain of hemolytic streptococcus, which was kindly furnished by Dr. Mueller,** was used. The medium for the growth

† Read before the Society for Experimental Biology and Medicine, New York City, February 15, 1922.

** The authors are indebted to Dr. J. Howard Mueller of the Department of Bacteriology, College of Physicians and Surgeons, for the use of a strain of streptococcus, and for a description of the preparation of his media, and of the technique of carrying out the bacteriological tests.

of this organism was prepared as follows: A fresh beef-heart, devoid of fat, and finely chopped, was boiled for five minutes with an equal weight of distilled water; the infusion was strained through cloth and filtered, clear, through filter paper. This beef-heart infusion was boiled for 15 minutes with two per cent of its weight of norit charcoal; the decolorized liquid (to be referred to hereafter as D. I.) was filtered clear, and mixed with an equal volume of a glucose-salt solution† (to be termed G. S.). This mixture was boiled for several minutes, filtered, adjusted to a PH of 7.4 and sterilized in lots of 250 cc. at ten pounds pressure for ten minutes. This medium (referred to hereafter as D.I.-G.S.), inoculated with streptococcus, failed to give growth when used alone, whereas the original undecolorized heart infusion was favorable for the growth of this organism.

EFFECTS OF VARIOUS MEDIA ON GROWTH OF STREPTOCOCCI.

The tests on streptococci were made as follows: Duplicate tubes were prepared containing nine cc. of the D.I.-G.S. solution plus one cc. of the solution to be tested, one tube being inoculated and the other acting as a sterile control. One set of duplicate tubes of D.I.-G.S. alone, and another set containing this solution plus one per cent peptone, were used as additional sterile and inoculated controls. The inoculations were made from a 24-hour bouillon culture previously inoculated from a pure blood culture.

The original undecolorized heart infusion gave a profuse growth when inoculated with streptococci, while the decolorized medium, either alone or together with the glucose-salt solution, failed to give growth. The decolorized medium, however, gave growth when one percent peptone was added to it, while one percent peptone solution alone or together with glucose-salt solution, failed to give growth. This confirms some of the results obtained by Mueller.¹

Autolyzed brewers' yeast, diluted 20 times, when added to the D.I.-G.S. medium, supported the growth of streptococci; but in some cases the growth was retarded, due probably to the strong acid reaction of the autolyzed yeast. When the reaction

† The glucose-salt (G. S.) solution consisted of an aqueous solution of: NaCl—1.0%; CaCl₂—0.02%; MgSO₄—0.04%; K₂HPO₄—0.20%; Glucose (Difco)—0.20%.

was adjusted to an alkaline range (PH of 7.4-7.8), a more uniform growth of the microörganism was obtained. An added factor may be the possible presence in autolyzed yeast of substances which are toxic to the specific organism. The above results are given in detail in table I.

TABLE I.
Growth of streptococci on various media.

No.	MEDIA	Growth
1.	10 cc. Undecolorized beef-heart infusion.....	+++
2.	10 cc. Decolorized beef-heart infusion.....	—
3.	10 cc. Glucose-salt solution (G. S.).....	—
4.	10 cc. D. I.-G. S. solution	—
5.	10 cc. D. I.-G. S. solution, containing 1% peptone.....	++
6.	10 cc. G. S. solution containing 1% peptone.....	—
7.	10 cc. 1% peptone solution alone.....	—
8.	9 cc. D. I.-G. S. + 1 cc. 5% autolyzed yeast.....	+
9.	9 cc. D. I.-G. S. + 1 cc. 5% autolyzed yeast (PH 7.4-7.8)...	++

+++ Denotes profuse growth.
 ++ " moderate "
 + " slight "
 — " no "

EFFECTS OF THE SAME MEDIA ON THE GROWTH OF YEAST CELLS.

For the test on the growth of yeast cells, we followed the Funk-Dubin² method, which is the most convenient and which, briefly, is carried out as follows: A yeast suspension is prepared by shaking a loopful of a 48-hour pure yeast culture in 100 cc. Nägeli solution on a shaking machine for three hours. Duplicate sets of tubes are prepared containing

- (1) 4 cc. yeast suspension + 5 cc. Nägeli solution + 1 cc. water
- (2) 4 cc. yeast suspension + 5 cc. Nägeli solution + 1 cc. of the solution to be tested.

The tubes are incubated for 20 hours at 30°C., and the fermentation is then stopped by heating the tubes in a water bath to 75°C. The contents of the tubes are then transferred to special centrifuge tubes, the bottom part of which is a capillary 2.5 cm. long and is graduated in millimeters. These tubes are centrifuged for 15 minutes at about 2500 R.P.M., and the growth of the yeast cells is read directly on the tube. The reading of tube 1, which constitutes the blank, is subtracted from that of tube 2 to give the net growth of yeast cells in millimeters. In all tests, conditions were maintained which precluded the possibility of bacterial contamination.

Undecolorized beef-heart infusion gives a growth of yeast cells which compares favorably with that produced by a 5% solution of autolyzed yeast itself. The addition of glucose to autolyzed yeast has very little if any additional activating property, other than that of slightly enriching the medium. One cc. of decolorized heart infusion when added to the Nageli solution with or without the glucose-salt solution, has little or no effect on yeast cells; while the D.I.-G.S. medium containing one percent of peptone, when added to the Nageli solution, stimulates the growth of yeast cells just exactly as much as when one percent peptone solution alone is added.

TABLE II.

Effects of various media on the growth of yeast cells.

No.	Substance added to 9 cc. of standard Nageli solution	Net yeast Growth in mm.
1.	1 cc. 5% autolyzed yeast (5 cc. in 95 cc. water).....	10.0
2.	1 cc. 5% autolyzed yeast + 1 cc. 10% glucose solution (Difco) ..	11.0
3.	1 cc. D. I. solution alone	0.
4.	1 cc. D.-I.-G. S. solution	1.5
5.	1 cc. 1% peptone solution (Difco)	2.5
6.	1 cc. D. I.-G. S. solution containing 1% peptone.....	4.0
7.	1 cc. G. S. solution	0.
8.	1 cc. G. S. solution containing 1% peptone.....	2.0
9.	1 cc. Undecolorized meat infusion	7.5
10.	1 cc. Undecolorized beef-heart infusion.....	12.0

It is apparent from the results shown in tables I and II, that there is present in beef and beef-heart infusions, and in peptone and autolyzed yeast, a substance that shows comparable growth-stimulation on hemolytic streptococci and yeast cells. The question now naturally arises as to the nature of the substance that shows such a marked similarity of effect on these two organisms. From the standpoint of the favorable action on yeast cells, we are probably dealing here with one or more substances of the class of B vitamine. As for the growth-influencing action on streptococci, this either is due to a substance of an identical or similar nature; or, as suggested in the work of Davis,³ Rivers and Poole,⁴ and others, we are dealing with two unknown substances, one or both of which may belong to the class of vitamines. These authors found that certain organisms, particularly those of the hemophilic type, require two substances for

This second unknown substance present in beef-heart, thought to be in some way associated with blood, is therefore not related to the class of B vitamins, as it is not extracted by any of the adsorbing agents which have an affinity for this class of vitamins.

*Growth of yeast and streptococci on beef-heart infusions,
decolorized with 5 and 10% norit.*

† The slight decrease in activity here is due to the smaller amount of the beef-heart infusion.

SHAKING OF AUTOLYZED YEAST WITH FULLERS EARTH AND NORIT.

Funk and Dubin⁵ have shown that at least two different substances can be separated from autolyzed yeast by means of shaking with fullers earth. By this method it is now possible to separate the vitamine active for yeast growth, which has been provisionally called "vitamine D," from the anti-beriberi or B vitamine.

Several preparations of autolyzed yeast were subjected to shaking with various amounts of both fullers earth and norit, as follows: The clear filtrate of autolyzed yeast, after removal of the protein by heat coagulation, was first shaken for three hours with 50 grams of fullers earth per liter, and filtered. The filtrate showed very little loss of growth-stimulating effect on yeast cells as compared with the original material, while there was no observable loss of stimulation on bacterial growth. This filtrate was again subjected to shaking with fullers earth, this time with 100 grams per liter. The filtrate from this shaking gave a yeast growth-increase about one half as great as that given by the original 5% autolyzed yeast solution. This filtrate gave as a rule no growth of streptococci, although in one or two cases a slight growth was obtained. This second filtrate was again shaken for three hours with 100 grams of fullers earth per liter, the filtrate from this final shaking having almost no stimulating effect on yeast growth and none on bacterial growth.

We obtained practically similar results by subjecting autolyzed yeast solutions to shaking with norit. The first filtrate after shaking with 50 grams of norit per liter was still strongly active on both organisms, while the second filtrate, obtained after shaking the first with 100 grams per liter, showed a slight effect on yeast growth, but was entirely negative on the growth of streptococci, as was to be expected. The third filtrate, obtained after again shaking the previous filtrate with 100 grams of norit per liter, had lost all activity on the growth of both organisms.

A few experiments were also tried with Lloyd's reagent, the results comparing favorably with those obtained with the other two adsorbing reagents, although Lloyd's reagent is less energetic an adsorber than fullers earth or norit.

EXTRACTION OF THE ADSORBED MATERIAL FROM FULLERS EARTH
AND NORIT BY MEANS OF BARYTA AND ACETIC ACID
RESPECTIVELY.

Following the method of Seidell,⁶ the fullers earth containing the adsorbed vitamine-like material was shaken for two to three minutes with one and a half volumes of a 10% solution of baryta at 60°C. The solid was then filtered off, washed with water, and the combined filtrate and washings quickly neutralized with 20% sulphuric acid. The filtrate, after removal of the barium sulphate, was concentrated in vacuum and made up to a volume equal to the original volume of the medium before treatment with the adsorbing agent. The solution was then adjusted to a PH of 7.4 and sterilized. Unless otherwise noted, one cc. of the solution was used in all tests.

The baryta extract of the fullers earth from the first shaking with autolyzed yeast (50 grams per liter) showed a comparatively strong growth stimulating action on the growth of both yeast cells and streptococci, as can be seen in table IV below. This extract contains nearly all of the vitamine B from autolyzed yeast and a small part of the newly isolated vitamine D. Its stimulating effect on yeast growth compares with that obtained by Funk and Dubin⁵ as shown in the second column of table IV. It also showed a comparatively strong stimulation of the growth of bacteria.

The baryta extract of the fullers earth from the second shaking with autolyzed yeast (100 grams per liter) had an effect on yeast growth almost equal to that of the first baryta extract; while the action on the growth of streptococci was only slightly diminished as shown by 2 (a) and 3 (a). This extract had no curative effect on polyneuritic pigeons, showing the apparent absence of vitamine B. The fullers earth, during the third shaking, failed to extract any more of the active substance, as its baryta extract showed no effect on yeast and bacterial growth.

Extraction of the norit with acetic acid: Following the method of Eddy, Stevenson, Johnson and Heft,⁷ the material adsorbed by the charcoal was extracted from it by heating the charcoal on a water bath for three hours with ten parts (by weight) of glacial acetic acid; the solution was filtered, and evaporated in vacuum to dryness; the residue was taken up in water, and the solution again evaporated. This procedure

was repeated several times to remove most of the acid. The last traces of acetic acid were neutralized by addition of normal sodium hydroxide solution. The neutralized solution was made up to a volume equal to the original volume of the autolyzed yeast, adjusted to the proper PH (7.4) and sterilized. One cc. of this extract was used in each test.

These acetic acid extracts from the charcoal gave very interesting results. As shown in table IV, Nos. 5 (a) and 6 (a), they are practically in accord with the results obtained from the baryta extracts from the fullers earth after adsorption of the vitamine-like substance. The first two extracts stimulate both yeast and bacterial growth, while the extract from the third shaking [7 (a)] shows a very slight stimulating effect on yeast growth and no effect on the growth of streptococci.

TABLE IV.

Effect of Shaking of Autolyzed Yeast with Fullers Earth and Norit.

No.	FULLERS EARTH	Net Yeast Growth* in mm. obtained by		Bac- terial growth
		Funk and Dubin	Our- selves	
1.	Autolyzed yeast (5% solution).....	14.5	12.5	++
2.	Autolyzed yeast shaken with 50 grams per liter.....	12.0	9.5	++
2 (a)	Baryta extract of fullers earth (from 2)	4.0	5.5	++
3.	Autolyzed yeast (filtrate from 2) shaken with 100 grams per liter	6.0	5.0	—
3 (a)	Baryta extract of fullers earth (from 3)	3.5	4.0	++
4.	Autolyzed yeast (filtrate from 3) shaken with 100 grams per liter	0.5	0.5	—
4 (a)	Baryta extract of fullers earth (from 4)	0.	0.	—
NORIT				
5.	Autolyzed yeast shaken with 50 grams per liter.....	13.5	10.5	+
5 (a)	Acetic acid extract of norit (from 5)	3.0	4.0	++
6.	Autolyzed yeast (filtrate from 5) shaken with 100 grams per liter	3.0	3.0	—
6 (a)	Acetic acid extract of norit (from 6)	3.0	3.0	+
7.	Autolyzed yeast (filtrate from 6) shaken with 100 grams per liter	0.5	0.	—
7 (a)	Acetic acid extract of norit (from 7)	0.	0.	—

* Slight variations between the results obtained by Funk and Dubin and by ourselves are due to the use of different preparations of autolyzed yeast.

Our results as detailed in table IV, show that we can obtain from autolyzed yeast by fractional adsorption with fullers earth and norit, and subsequent extraction of the adsorbents, a concentrated solution of vitamine D, almost free from vitamine B. This vitamine D extract is active in stimulating the growth of both yeast cells and streptococci, but is not curative for beriberi. We were, however, unable to obtain vitamine B entirely free from D as this fraction which was found to be curative for beriberi was also active in stimulating the growth of yeast cells and bacteria. We have also shown that the extraction of these active substances from the adsorbents with baryta and acetic acid is almost quantitative.

SHAKING OF BEEF-HEART INFUSIONS WITH FULLERS EARTH AND NORIT.

A beef-heart infusion, prepared as described in the early part of this paper, when shaken for three hours with 50 grams of fullers earth per liter, lost most of its stimulating effect on yeast growth, while it still retained enough of the active substance to promote the growth of streptococci. The filtrate, after a second shaking with fullers earth, (100 grams per liter) showed no activity on the growth of either yeast or bacteria.

The baryta extract of the fullers earth from the first shaking, peculiarly showed only a very slight growth-stimulating activity on yeast; even the addition of 2 cc. of the extract to the medium increased the growth of yeast cells only slightly. The baryta extract of the fullers earth from the second shaking was correspondingly less active on yeast growth, showing a net growth of only 1 mm.; while it was only slightly active on bacterial growth. The effects on the two organisms, however, were comparable, as shown in table V.

Norit removed from beef-heart infusions practically all of the substance which stimulates the growth of yeast when the infusion was shaken with 2% of its weight (approximately 20 grams per liter) for three hours. The infusion was, however, still effective for the growth of streptococci. A second shaking, this time with 50 grams per liter, removed all activity on yeast and bacterial growth.

The acetic acid extract of the norit from the first shaking showed great growth stimulation on both organisms, this extract giving almost as much stimulation as the original medium. The net growth stimulating activity of the extract on

yeast varied from 5.5 to 9 mm. with an average of about 7.5 mm. This activity compared favorably with that of 1% peptone solutions. Extraction of the norit from the second shaking failed to give any action on yeast or bacteria. Table V gives in detail the results of these extraction experiments with beef-heart infusions.

An interpretation of these results shows that the growth-stimulating substances are present in smaller amounts in beef-heart infusions than in autolyzed yeast; and that they are more easily adsorbed from the former than from the latter. This adsorption is more complete, at least from beef-heart infusions, with norit than with fullers earth. Baryta fails to extract any active substance from the norit after adsorption, while glacial acetic acid is ineffective on fullers earth.

TABLE V.

Effect of Shaking of Beef-Heart Infusions with Fullers Earth and Norit.

No.	FULLERS EARTH	Net Yeast growth in mm.	Bacterial growth
1.	Beef-heart infusion — 1 cc. (equiv. to 2/5 gm. beef-heart)	12.0	++
2.	Beef-heart infusion shaken with 50 gms. per liter, 1 cc.	3.0*	++
2 (a)	Beef-heart infusion shaken with 50 gms. per liter, 2 cc.	3.5*	++
2 (b)	Baryta extract of fullers earth (from 2) 1 cc.	1.0	+
2 (c)	Baryta extract of fullers earth (from 2) 2 cc.	1.0	+
3.	Beef-heart infusion (filtrate from 2) shaken with 100 grams per liter, 1 cc.	0.	—
3 (a)	Beef-heart infusion (filtrate from 2) shaken with 100 grams per liter, 2 cc.	0.	—
3 (b)	Baryta extract of fullers earth (from 3) 1 cc.	1.0	+
3 (c)	Baryta extract of fullers earth (from 3) 2 cc.	1.0	+
NORIT			
4.	Beef-heart infusion shaken with 20 grams (2%) per liter, 1 cc.	0.	+
4 (a)	Acetic acid extract from norit (from 4) 1 cc.	7.5*	++
5.	Beef-heart infusion (filtrate from 4) shaken with 50 grams per liter, 1 cc.	0.	—
5 (a)	Acetic acid extract of norit (from 5) 1 cc.	0.	—

* Average result of several extractions.

It is quite possible that the nature and reaction of the media play an important rôle in these adsorption experiments. Inhibiting substances may also be present, so that a solution may be active after adsorption, although inactive before that procedure. How important this hypothesis is, we cannot at present state, as we did not fully investigate these solutions before extraction with the adsorbents.

A summary of the results obtained shows that the substances which promote the growth of bacteria and yeast (as extracted from beef-heart, peptone, autolyzed yeast, etc.) belong to the class of vitamins of the water soluble B type, but are not identical with B vitamin; that they are comparable in activity and show similar properties in that they are easily extracted from their natural sources by the same adsorbents, and are again readily recovered from these adsorbents without appreciable loss in activity. These results point strongly to the conclusion that they are either identical with vitamin D, or so similar to it in their physiological behavior, that only by actual isolation, purification, and complete chemical analysis will it be possible to differentiate them.

The separation of these vitamin-like substances from the bulk of impurities with which they are associated in nature, by adsorbing them in fuller's earth and norit and their subsequent extraction by chemical treatment of the adsorbents, gives us these unknown substances in a highly concentrated and comparatively pure form. This procedure is a distinct advantage over working with these substances in their natural media.

SUMMARY OF CONCLUSIONS.

1. There are present in beef and beef-heart infusions, peptone and autolyzed brewer's yeast, certain substances which show a strong growth-stimulating activity on both hemolytic streptococci and yeast cells.

2. These active substances can be extracted from their natural sources by shaking with certain adsorbents, such as fuller's earth and norit charcoal, and can be recovered by extracting the adsorbents with baryta and acetic acid respectively.

3. The properties of these substances show them to be of a vitamin-like nature; and they are either identical with the vita-

mine D described by Funk and Dubin⁵ or of a similar nature.

4. There is also present in beef and beef-heart infusions another substance which is necessary for the growth of hemolytic bacteria, and this substance is thought to be associated with hemoglobin.

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NUTRITIONAL FACTORS IN THE GROWTH OF CERTAIN YEASTS AND BACTERIA.¹

II. PROTEIN HYDROLYSATES.

By LOUIS FREEDMAN

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and the Research Laboratory of H. A. Metz, New York City.*

INTRODUCTION.

The use of proteins and protein hydrolysates for enriching culture media for the growing of bacteria is well known; and we find proteins to be a definite constituent of many culture media. Bainbridge¹ showed that certain bacteria required proteins for growth, while Robinson and Rettger² used a casein hydrolysate to enrich bacterial media. However, the cases in which it has been definitely shown that the growth of the bacterial organisms was due to the presence of the protein alone or to any of its hydrolytic cleavage products, are extremely rare. In most cases it has been shown that vitamins were present as an added constituent or as an adhering impurity.

The possible presence in proteins of a hitherto unknown substance which is now thought to be of biological importance in nutrition, has of late been suggested by several investigators. In this connection, Mueller³ thought that the growth-stimulating substances for hemolytic streptococci, which he obtained from certain proteins, were definite constituents of the protein molecule, and he described the isolation of two such substances from a preparation of enzyme-digested milk proteins called "aminoids". After his final precipitation of these substances, however, he found them to be inactive. Goy⁴ also described the isolation, from certain fungi, of a nitrogen-free acid which he

¹ Read before the Society of Experimental Biology and Medicine, New York City, February 15, 1922.

claimed stimulated the growth of bacteria and yeast, but he also found the finally purified substance to be inactive.

In a previous communication⁵ we have shown that we can obtain from beef and beef-heart infusions, peptone and autolyzed brewer's yeast, by adsorption with fullers earth and norit charcoal, certain substances which show a strong growth-stimulating activity on yeast cells and hemolytic streptococci. We have also shown that these substances can be extracted from their combination with fullers earth and charcoal by treating these adsorbents with baryta and glacial acetic acid respectively. The properties of these substances were found to be similar to those of the vitamine D described by Funk and Dubin⁶.

In the following work, the authors have tried to show the relationship between the substances found in proteins, and vitamine D; and that these substances are not present in purified proteins in general but are found associated only with certain proteins.

EXPERIMENTAL PROCEDURE.

For this work we have prepared a series of well known proteins, consisting of twelve animal and ten vegetable proteins. The proteins of animal origin varied to a great extent, and included the milk proteins, casein and lactalbumin, three ox-blood proteins, three egg proteins, muscle protein, liver albumin and globulin, and gelatin.

For the proteins of vegetable origin, we have prepared edestin (from hemp seed), the wheat and corn proteins, the three proteins from the pea, oryzenin (from rice), hordein (from barley), and yeast protein. These proteins were prepared according to the standard published methods, particular care being taken to have the proteins, in so far as it was possible, free from vitamins.

HYDROLYSIS OF THE PROTEINS.

All of the above proteins were hydrolysed by boiling them with seven parts of concentrated hydrochloric acid (specific gravity 1.19) for eight to ten hours. After cooling, the humin

substance was filtered off and the filtrate evaporated to dryness in vacuum. The residue was taken up in water and the solution again evaporated to dryness. This operation was repeated twice, making four evaporations, to remove the bulk of hydrochloric acid. The remaining acid was then neutralized by addition of normal sodium hydroxide; the solution was made up to a volume corresponding to a 10% solution of the protein, adjusted to a PH of about 7.3 and sterilized at ten pounds pressure for ten minutes.

EFFECT OF THE PROTEIN HYDROLYSATES ON THE GROWTH OF STREPTOCOCCI.

The procedure for testing the effect of the hydrolysates on bacterial growth was the same as described in our previous paper⁵. One c.c. of the hydrolysate was added to the sterile D.I.-G.S. medium and the whole, after suitable inoculation, incubated at 37°C. for 24 hours. Of the animal proteins tested, only two, casein and commercial gelatin, gave a definite positive stimulation to the growth of streptococci, while fibrin and lactalbumin gave only a slight activity. Sulphuric acid hydrolysates of casein were also strongly active. Of the hydrolysates of the proteins of vegetable origin, we found only two to support the growth of streptococci,—namely, edestin and yeast protein.

TABLE I.

Effect of Protein Hydrolysates on Growth of Streptococci and Yeast.

No.	Hydrolysates of Animal Proteins. 1 cc. used.	Net yeast growth in mm.	Bacterial growth
1.	Casein (Purified) HCl hydrolysate.....	1.0	+
2.	“ “ H ₂ SO ₄ “	0	+
3.	“ (Technical) HCl “	1.0	+
4.	Lactalbumin	0.5	±
5.	Fibrin	—	—
6.	Serum globulin	—	—
7.	Serum albumin	—	—
8.	Beef-heart (muscle) protein	—	—
9.	Egg globulin	0	—
10.	Egg albumin	—	—

No.	Hydrolysates of Animal Proteins. 1 cc. used.	Net yeast growth in mm.	Bacterial growth
11.	Vitellin (egg yolk)	0	±
12.	Liver globulin (beef)	—	—
13.	Liver albumin "	—	—
14.	Gelatin (commercial—"Silver Label" brand).....	—	+
15.	Gelatin (prepared from bones and marrow).....	—	—
16.	Gelatin (prepared from bones without marrow)..<	—	—
Hydrolysates of Vegetable Proteins. 1 cc. used.			
17.	Edestin (hemp seed)	—	+
18.	Gliadin (wheat)	—	—
19.	Zein (corn)	—	—
20.	Glutelin (corn)	0	—
21.	Legumin (peas)	0	—
22.	Vicilin (peas)	0	—
23.	Legumelin (peas)	0	—
24.	Oryzenin (rice)	0	—
25.	Hordein (barley)	0	—
26.	Yeast protein	1.0	+

+ denotes growth.

± denotes slight growth.

— (under Bacterial column) denotes no growth.

0 (under Yeast column) denotes that growth was same as control.

— (" " ") " inhibition.

Quantitative Results: We attempted to devise a method for the determination of the amount of stimulation of the growth of streptococci by centrifuging the bacteria, as is done in the method for yeast described by Funk and Dubin.⁷ This procedure, however, was found to be impractical, due to the difficulty of stopping the growth of bacteria, and also because of the occasional precipitation of substances which were not due to bacterial formation.

A better method for comparative results was devised, based on the change in hydrogen ion concentration due to the fermentation of the glucose in the D.I.-G.S. medium by the streptococci. This change in hydrogen ion concentration was determined by means of the Sörensen⁸ indicator method, using the Clark and Lubs⁹ indicator set. Cole and Jordan¹⁰ used a similar method for diagnosis, by fermentation tests, of the gonococcus, meningococcus and other pathogenic organisms.

The variation in hydrogen ion concentration, in all of our tests, covered a range from a PH of 7.4 to a PH of 4.0. For this test, we prepared standard solutions consisting of mixtures of

Na_2HPO_4 and NaH_2PO_4 for the alkaline range, and solutions consisting of mixtures of acetic acid and sodium acetate, according to Walpole¹¹, for the acid range. With these standard solutions, we used the following indicators:

Phenol red, for pH 8.0—6.8
 Brom thymol blue, for pH 7.6—6.0
 Brom cresol purple, for pH 6.9—5.2
 Methyl red, for pH 6.0—4.4

Three-tenths cc. of the required indicator solution was added to 10 cc. of the standard solution. The colored solutions were then put into uniform test tubes and the tubes sealed. The tests were made as follows: Duplicate tubes were prepared, each containing 9 cc. of the D.I.-G.S. medium and 1 cc. of the protein hydrolysate. One tube was inoculated with the streptococci and the other kept sterile. Both tubes were incubated together. After twenty-four hours, 0.3 cc. of the indicator necessary, which was determined roughly by the amount of bacterial growth formed, was added to each tube and the resulting color compared with the standard tubes.

As we used a control check for each test, we found this method to be very convenient and fairly accurate for comparative results. We have for convenience tabulated the results obtained with our protein hydrolysates in their stimulating action on the growth of streptococci, in tables IIa and b.

TABLE II (a)

Quantitative Effect of Protein Hydrolysates on the Growth of Streptococci.

pH of standard D.I.-G.S. solution = 7.3			
No.	Hydrolysates of Animal Proteins. (1 cc. used in each test)	Growth	pH after incubation
1.	Casein (purified) HCl hydrolysate.....	+	5.8
1 (a).	“ “ (sterile control).....	—	7.3
2.	Casein (purified) H_2SO_4 hydrolysate.....	+	5.3
2 (a).	“ “ (sterile control).....	—	7.0
3.	Casein (technical) HCl hydrolysate.....	+	5.8
3 (a).	“ “ (sterile control).....	—	7.3
4.	Lactalbumin	$\pm$	7.0
4 (a).	“ (sterile control)	—	7.3
5.	Filbrin	—	7.3
5 (a).	“ (sterile control)	—	7.3

TABLE II. (a) (*continued*)

No.	Hydrolysates of Animal Proteins. (1 cc. used in each test)	Growth	pH after incubation
6.	Serum globulin	—	7.3
6 (a).	" " (sterile control)	—	7.3
7.	Serum albumin	—	7.3
7 (a).	" " (sterile control)	—	7.3
8.	Beef-heart (muscle) protein.....	—	7.2
8 (a).	" " " (sterile control) ..	—	7.3
9.	Egg globulin	±	7.0
9 (a).	" " (sterile control)	—	7.3
10.	Egg albumin	—	7.3
10 (a).	" " (sterile control)	—	7.3
11.	Vitellin (egg yolk)	—	7.3
11 (a).	" " " (sterile control).....	—	7.3
12.	Liver globulin	—	7.2
12 (a).	" " (sterile control)	—	7.3
13.	Liver albumin	—	7.3
13 (a).	" " (sterile control)	—	7.3
14.	Gelatin (commercial)	+	6.0
14 (a).	" " (sterile control).....	—	7.2
15.	Gelatin (prepared from bone and marrow)....	—	7.2
15 (a).	" " " " " (sterile control)	—	7.3
16.	Gelatin (prepared from bone without marrow)	—	7.2
16 (a).	Gelatin (prepared from bone without marrow) (sterile control)	—	7.3

TABLE II (b)

No.	Hydrolysates of Vegetable Proteins.	Growth	pH after incubation
17.	Edestin	+	6.5
17 (a).	" (sterile control)	—	7.3
18.	Gliadin	—	7.0
18 (a).	" (sterile control)	—	7.1
19.	Zein	—	7.3
19 (a).	" (sterile control)	—	7.3
20.	Glutelin (corn)	—	7.0
20 (a).	" (sterile control).....	—	7.2
21.	Legumin	—	7.0
21 (a).	" (sterile control)	—	7.2
22.	Vicilin	—	7.0
22 (a).	" (sterile control)	—	7.2

TABLE II. (b) (*continued*)

No.	Hydrolysates of Vegetable Proteins.	Growth	pH after incubation
23.	Legumelin	—	7.3
23 (a).	" (sterile control)	—	7.3
24.	Oryzenin	—	7.0
24 (a).	" (sterile control)	—	7.2
25.	Hordein	±	7.0
25 (a).	" (sterile control)	—	7.3
26.	Yeast protein	+	4.9
26 (a).	" " (sterile control).....	—	7.3

These quantitative results confirm our first results, namely, that only two of our animal and two of our vegetable proteins contain the growth stimulating vitamine for streptococci in appreciable amounts. Of the proteins whose hydrolysates contain the active substances, yeast protein is by far the richest, with casein second, commercial gelatin closely following, and edestin containing the smallest amount of the active substance.

EFFECT OF PROTEIN HYDROLYSATES ON GROWTH OF YEAST.

The protein hydrolysates as described above were tested on yeast; and, with the exception of casein and yeast protein, all the hydrolysates either failed to stimulate, or actually inhibited, the growth of yeast cells. Even the action of casein and yeast protein hydrolysates were, however, practically negative, the net increase in growth of yeast cells due to the vitamine activity of the hydrolysates amounting to one mm. for each of these proteins.

These results are not at all surprising, as Vansteenberge¹² found that leucine, tyrosine and asparagine inhibited the growth of yeast cells but not of the lactic bacteria. All of our hydrolysed proteins contain large amounts of leucine, while most of them contain varying amounts of tyrosine and aspartic acid.

A close analysis of the results obtained with these proteins together with their method of purification and their physical structure will, if anything, tend to confirm our view that the growth-stimulating action is due, not to any constituent part of the protein molecule, but to the tendency of the protein to adsorb vitamine and hold on to it in spite of all attempts at purification. Thus, yeast protein, the hydrolysate of which gave considerable stimulation to the growth of streptococci, was ob-

tained from a medium which we know to be very rich in vitamine, particularly the newly isolated vitamine D. This protein was prepared by heating the filtered autolyzed yeast until the protein had coagulated. As this protein separates in a form similar to milk curd, we see the possibility of great adsorption of vitamins. Although this protein underwent considerable washing, and was suspended in a dialyzing bag for several days, it still retained most of the vitamine which it had originally adsorbed.

The same is true of casein. This protein, which is precipitated by means of dilute acid and is purified by repeatedly redissolving and reprecipitating, separates at the start in the form of a coagulum, and, as other investigators have shown, has great adsorbing powers.* Consequently, no amount of redissolving and reprecipitating will entirely free it from vitamins, as its physical structure and adsorbing properties remain the same.

With gelatin, we have a somewhat analogous situation. Gelatin, which we prepared in the laboratory, from bones with and without the marrow, was entirely freed from vitamins by the process of purification described by Van Name.¹⁴ This consists, essentially, in precipitating the gelatin in absolute alcohol, extracting with ether, redissolving the protein in water, concentrating the solution to a jelly, reprecipitating in absolute alcohol and again extracting with ether. Commercial gelatin, ("Silver Label" brand) however, contains enough of the active substance to stimulate the growth of streptococci, as we have shown in tables I and II. It seems apparent therefore that the present commercial process for purifying gelatin is not adequate for freeing this protein entirely from the vitamins contained in the bones and parts of the hide that are used. In this connection it might be mentioned that Boyer¹⁵ used an hydrochloric acid extract of finely divided bones for the cultivation of streptococci.

With edestin, we have a protein which was prepared in a somewhat different manner. We prepared this protein by extracting the ground hempseed with five percent sodium

* The following far-reaching statement was made by Prof. F. G. Hopkins¹³ in his Chandler Lecture given at Columbia University, 1921. "It is remarkable what a considerable portion of the vitamins present in milk is adsorbed by precipitated casein. A failure to recognize this has often obscured the results of feeding trials."

chloride solution at 60°C. The solution was allowed to cool gradually when the edestin separated as a crystalline precipitate. It was purified by redissolving in warm saline solution and again cooling the solution. Apparently, edestin also has great adsorbing properties. Osborne, Wakeman and Ferry¹⁶ were unable to free edestin entirely from vitamines.

Regarding the proteins, the hydrolysates of which showed no growth stimulating activity, a review of their methods of preparation and purification may throw some light on this point. Thus the prolamines, e.g., wheat gliadin, zein and hordein from cereals, were all extracted by means of hot alcohol, the alcoholic solutions evaporated to small volumes and the protein precipitated either in water or very dilute salt solutions. As the water soluble vitamines are also soluble in alcohol, we can readily see how all or nearly all of the vitamines would remain in solution. Those proteins, such as the albumins and globulins, which were salted out either by half saturation or complete saturation with ammonium sulphate were all purified by dialysis, and we know that vitamines are dialysable. Thus it is possible that these albumins and globulins have very little adsorbing properties, and therefore can be freed from vitamines by thorough purification. The same apparently holds true for the vegetable globulins with the exception of edestin.

The glutelins probably were free from vitamines even before extraction with dilute alkali, as they were obtained from the cereal residues after the extraction of the gliadins by means of alcohol. At any rate, the vitamines, if present would probably be destroyed by the continued action of the alkali.

The active proteins, from published analyses, show nothing in common that cannot be shown to apply to the other, inactive, proteins, so that we can safely rule out the question of amino acids or nitrogen content.*

* McLeod and Wyon¹⁷, in a recently published report on the "Supposed importance of vitamines in promoting bacterial growth," used among other extracts, alcoholic extracts of the kidneys of the guinea pig, and they believed that the growth was due to vitamine B, although they stated that the amino acid content of the extracts was also a factor. They also obtained better results with hydrolysed casein than with casein. They also found that "marmite," which is supposedly rich in vitamines, was inactive for the pneumococcus and meningococcus, and that charcoal, devoid of vitamines, when suspended in their bouillon cultures, stimulated the growth of these two organisms. The part of the work of these authors dealing with charcoal and "marmite" will no doubt need confirmation.

In summarizing the above results, three points stand out in explanation of the growth stimulating power of the hydrolysates of proteins.

1) The source of the protein; whether it is obtained from a medium rich in water-soluble vitamins of the B type such as Vitamin D.

2) The physical structure of the protein and its ability to adsorb vitamins.

3) The method of purification.

EXPERIMENTS WITH NEUTRAL SODIUM CASEINATE.

If these active substances present in casein and three of the other proteins, as described, are nothing more than adsorbed vitamins, and if the protein could be brought into solution without subjecting it to hydrolysis, then the vitamins would also go into solution and could be removed by adsorbing it with fullers earth or some other adsorbing agent.

To test this theory, we prepared a solution of neutral sodium caseinate by suspending our purified casein in water, and adding five percent sodium hydroxide solution until the casein had practically all dissolved. A small amount of insoluble denatured casein, formed by the action of the alkali, was filtered off, and the solution was then made neutral to litmus. This neutral sodium caseinate solution (containing about 5% casein) was shaken for three hours with ten grams (50 grams per liter) of fullers earth.

After shaking, the fullers earth was separated from the solution by centrifugation, as filtration was very slow, due to the formation of an emulsion, and the supernatant liquid was then filtered. The fullers earth was repeatedly washed with water and centrifuged to remove small amounts of sodium caseinate adhering to it, pressed out on a suction filter, washed with alcohol and dried. This fullers earth was then extracted with baryta and worked up as described in paper I.⁵ The resulting solution was made up to a volume corresponding to a ten percent solution of the original casein, adjusted to a PH of 7.4 and sterilized. This solution, when tested, was found to have a strong stimulating effect on the growth of streptococci, but had no action on the growth of yeast cells.

The neutral sodium caseinate solution, after shaking with

fullers earth, was subjected to hydrolysis by adding concentrated sulphuric acid until a 30% solution was obtained and boiling for 36 hours. This hydrolysate failed to give any growth stimulating activity on either streptococci or yeast.

It is therefore apparent that most or all of the vitamine was removed from the casein as described. This shows that the active substance in the protein is not necessarily a constituent part of the protein molecule, and does not have to be isolated by breaking up the protein.*

SUMMARY OF CONCLUSIONS.

1. We can obtain from purified casein, commercial gelatin, yeast protein and edestin, by hydrolysis of these proteins, certain substances which show marked growth stimulating activity on hemolytic streptococci. Hydrolysates of purified egg globulin, lactalbumin and hordein, show traces, whereas hydrolysates of the other proteins examined are devoid of these activating substances.

2. We have found that these active substances are not a constituent part of the protein molecule, and that the amount of the substances present in the protein depends on the physical and adsorptive properties of the protein and the method and degree of purification.

3. The properties of these active substances, as obtained from the proteins described above, show that they are probably related to if not identical with the water-soluble vitamins obtained from brewers' yeast, particularly vitamine D.

4. We have described a method for determining the amount of these active substances by the use of Sørensen's indicator method, which is based on the change in hydrogen ion concentration due to the fermentative action of the bacteria. This method is advocated for comparative results only, and not as a direct quantitative method.

* The experiments with neutral sodium caseinate, just described, may be of practical importance in that they may lead to a method of preparing a vitamine-free casein. The casein thus obtained gives positive tests for all the color reactions known for the various amino acids, while its nitrogen content is the same as that of the original casein from which it was prepared. Further work is now in progress on this problem.

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VITA.

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