XIX. THE ACTION OF YEAST-GROWTH STIMULANT.

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WILLIAMS [1919] and Bachman [1919] have confirmed the observation of Wildiers [1901] that certain yeasts are only able to grow at the expense of ammonium salts provided that a heavy inoculation is employed or a small amount of organic material (termed "bios" by Wildiers) is added to the medium. After a study of the ability of various substances to produce this effect, they suggest that the essential substance, which we will continue to call "bios" for the sake of convenience, is identical with the water-soluble *B* or anti-beri-beri vitamin. Williams [1920] has gone further, and has elaborated a method for measuring quantitatively the amount of anti-beri-beri vitamin present in any substance by observing its effect on the growth of yeast.

This suggestion has given rise to much discussion [see Eddy, 1921] and at present it is generally considered that the case for the identity of the two principles is not proven.

Lemon juice freed from citric acid by the method of Harden and Zilva [1918] when added to a mineral nutrient solution in small quantities enables a yeast to grow which could not grow in its absence. As animal feeding experiments show that the amount of water soluble *B* vitamin in lemon juice is very small relative to that in yeast extract [see Osborne and Mendel, 1920], it was thought that an investigation of its effect on the growth of yeast in mineral nutrient solutions might throw some light on the way in which the stimulation is brought about.

Preliminary experiments were performed to ascertain the smallest amount of lemon juice capable of producing growth in a nutrient solution made up as follows (Williams):

Saccharose	...	20 g.
(NH ₄) ₂ SO ₄	...	3 g.
KH ₂ PO ₄	...	2 g.
CaCl ₂	...	0.25 g.
MgSO ₄	...	0.25 g.
Distilled water	...	1000 cc.

A series of tubes was prepared containing this solution with the addition of increasing percentages of lemon juice. The yeast employed was a pure

culture of a baker's yeast isolated by Dr Harden. This was grown for 24 hours in yeast water glucose and the growth was washed three times with sterile distilled water by means of the centrifuge. A loopful of a suspension of this washed yeast was put into each tube of the series, the amount being judged so that a fair proportion of drops made, according to Lindner's method, with a mapping pen on a cover slip contained one, two, or three cells each. The coverslip was sealed over a moist chamber with vaseline and, after noting the number of cells in each drop, was incubated for 24 hours at 25° and again examined for growth.

By this method it was found that no growth took place in the mineral nutrient solution unless 5 % or more of lemon juice was added. It was also noticed however that some of the tubes containing smaller amounts of lemon juice than 5 %, which had been standing in the laboratory, contained an amount of yeast appreciably larger than the amount originally inoculated. This suggested that growth did not take place as readily in the case of one or two cells in a minute drop as in a larger amount of medium. Subsequent investigation confirmed this, and the drop method was abandoned in favour of estimating the number of cells per cc. by means of a counting chamber.

In order to avoid adding any appreciable amount of "bios" with the seed yeast, it is necessary to inoculate each tube with as small a quantity of yeast as possible. Attempts to inoculate tubes by picking up a drop of distilled water containing one, two, or three cells from a coverslip by means of sterile filter paper proved unsuccessful. The method adopted was to make a light emulsion in sterile distilled water of yeast cells prepared and washed as described above. A slide was marked with several circles with a grease pencil and a drop of the emulsion transferred to the centre of each by means of a platinum loop. The drops were dried and stained and the number of cells in each counted, and by this means an emulsion was prepared which contained somewhere between 50 and 500 cells in a loopful. One loopful was then used to inoculate each tube.

A series of ten tubes was prepared with a mineral nutrient solution containing $(\text{NH}_4)_2\text{SO}_4$ with increasing percentages of lemon juice. A similar series was prepared without the $(\text{NH}_4)_2\text{SO}_4$.

Each tube contained 7 cc. of the following solution (Williams' solution without the $(\text{NH}_4)_2\text{SO}_4$, see p. 137):

Saccharose	...	20 g.
KH_2PO_4	...	2 g.
CaCl_2	...	0.25 g.
MgSO_4	...	0.25 g.
Distilled water	...	1000 cc.

The tubes containing $(\text{NH}_4)_2\text{SO}_4$ received 1.5 cc. of a solution containing 2 g. of the salt in 100 cc. The saccharose and the $(\text{NH}_4)_2\text{SO}_4$ were purified by several recrystallisations. Repeated attempts to grow the yeast in tubes of this solution with $(\text{NH}_4)_2\text{SO}_4$ inoculated with from 50 to 500 cells in the

manner described above and without the addition of "bios" failed, no growth being observed even after a lapse of several weeks.

The lemon juice was prepared by the method of Harden and Zilva [1918], namely as follows: calcium carbonate was stirred into fresh lemon juice in excess of the amount required to neutralise all free acid. Absolute alcohol to twice the volume of the original lemon juice was added, and the whole filtered. The filtrate was evaporated down almost to dryness *in vacuo* at 35°. and the residue made up to the original volume of the lemon juice with distilled water. The sample used contained 0.0868 mg. of nitrogen per cc. of which 0.0112 mg. was ammoniacal nitrogen. The desired amount of this lemon juice was added to each tube of the series which was then made up to 10 cc. with distilled water. The tubes were sterilised by steaming for 30 minutes on each of three successive days. Each was then inoculated with from 50 to 500 cells as described above, the tubes were aerated by blowing air into them through a sterile glass tube plugged with wool and having its end drawn out to a fine point, and were incubated at 25°.

The tubes were examined each day and the number of cells counted by means of a Thoma haemocytometer. At least four drops were counted from each tube and if any considerable discrepancy was found between them the counting was continued until the margin of error was reduced below 5%. Owing to the number of tubes it was not possible to count all of them every day, but the different rates at which the yeast grew in the various tubes rendered this unnecessary. The results are set out in Table I.

Table I.

Number of tube	Percentage of lemon juice	(NH ₄) ₂ SO ₄	Days: growth in millions of cells per cc.						
			1	2	3	4	5	6	9
1	0.1	0	s.g.	s.g.	s.g.	s.g.	—	s.g.	1.6
11	0.1	+	"	"	"	"	—	1.5*	29.0†
2	0.25	0	"	"	"	"	—	1.8	1.0
12	0.25	+	"	"	"	"	—	4.3*	32.6†
3	0.5	0	"	"	"	1.3	—	3.5	1.0
13	0.5	+	"	"	"	1.7	—	2.1*	34.2†
4	0.75	0	"	"	"	0.9	—	2.5	1.8
14	0.75	+	"	"	"	1.0	—	4.9*	26.7†
5	1.0	0	"	"	2.8	3.1	—	2.7	6.1
15	1.0	+	"	"	1.1	1.4	—	4.6*	22.6†
6	2.5	0	"	2.8	4.3	—	6.3	—	—
16	2.5	+	"	3.1	5.5	—	21.2	—	—
7	5.0	0	"	4.9	7.1	—	—	—	—
17	5.0	+	"	4.8	27.4	—	—	—	—
8	7.5	0	"	6.7	9.2	—	—	—	—
18	7.5	+	"	21.1	19.4	—	—	—	—
9	10.0	0	"	12.0	12.1	—	—	—	—
19	10.0	+	"	26.5	29.4	—	—	—	—
10	15.0	0	"	15.9	19.6	—	—	—	—
20	15.0	+	"	24.3	40.6	—	—	—	—

S.g. means some growth but too slight to count.

* Involution forms present.

† Films formed. Accurate counting impossible owing to clumping of cells. Many involution forms.

It is well known that the growth of yeast is susceptible to slight variations of conditions which cannot be controlled absolutely, such as the degree of aeration and the formation of products of metabolism and fermentation. Further, the accuracy of the counting is affected by the smallness of the drop examined in the low counts and by the tendency of the cells to form clumps in the higher counts or in the later stages of growth, so that below 3,000,000 and above 15,000,000 cells per cc. the counts of different drops of the same sample are found to be noticeably less uniform than between those figures, and the counts made after more than six days of growth can only be regarded as approximate. In considering the results obtained regard must be paid to these facts, and account taken only of large differences in the numbers. The table shows, however, that, until the yeast reaches a concentration of somewhere in the neighbourhood of five or six million cells per cc., the rate of growth is independent of the presence or absence of $(\text{NH}_4)_2\text{SO}_4$ and depends on the concentration of the lemon juice.

It does not appear unreasonable that it should take longer for an individual cell to collect sufficient nutriment to enable it to multiply when the nutriment is dilute than when it is concentrated, and possibly the fact that cells were unable to multiply in a minute drop in dilutions that enabled growth to take place in larger quantities of medium is explained by the fact that the whole drop did not contain sufficient nutriment.

After the yeast reaches a concentration of five or six million cells per cc. it is able to continue growing freely in the $(\text{NH}_4)_2\text{SO}_4$ tubes. Further it was observed that after six days involution forms, as described by Will [1895] in his investigation of film formation, begin to appear in all tubes containing $(\text{NH}_4)_2\text{SO}_4$ where the growth had not reached the critical point of five or six million cells per cc., and by the ninth day there was a heavy growth of film yeast on all these tubes. All the remaining tubes after a lapse of three weeks showed only very rare involution forms and no film formation, but most of the cells were highly refractile with many large vacuoles and granules and a thick membrane, similar in appearance to the permanent cells described by Will. It would appear, then, that after an interval of six or seven days the cells in smaller concentrations than five or six million per cc. are able to adapt themselves to the use of $(\text{NH}_4)_2\text{SO}_4$ and that film formation results.

A similar series of tubes was prepared with increasing percentages of aqueous yeast extract instead of lemon juice. This was prepared by Osborne and Wakeman's method [1919]; about 250 g. of wet brewer's yeast was washed and boiled for two minutes with 1 litre of distilled water containing 0.01 % of acetic acid and the liquid was separated off in the centrifuge. The solid residue was again heated with 500 cc. of 0.01 % acetic acid and the extracts united and concentrated to 500 cc. This extract contained 2.856 mg. of nitrogen per cc., of which 0.2273 was ammoniacal nitrogen. As this is rather more than 30 times the amount contained in the lemon juice, the amounts added to the various tubes of the yeast extract series were much smaller than in the lemon juice series. The results are set out in Table II.

Table II.

Number of tube	Percentage of yeast extract	$(\text{NH}_4)_2\text{SO}_4$	Days: growth in millions of cells per cc.						
			1	2	3	4	5	6	11
21	0.01	0	s.g.	s.g.	s.g.	s.g.	—	—	3.1
31	0.01	+	"	"	"	"	—	—	10.6*
22	0.02	0	"	"	"	"	—	—	3.0
32	0.02	+	"	"	"	"	—	—	10.5*
23	0.05	0	"	"	"	"	—	—	4.0
33	0.05	+	"	"	"	"	—	—	15.0*
24	0.1	0	"	"	"	"	—	1.6	10.0*
34	0.1	+	"	"	"	"	—	15.1*	20.0*
25	0.15	0	"	"	"	6.1*	—	10.0*	—
35	0.15	+	"	"	"	3.6	—	11.5*	—
26	0.2	0	"	"	0.5	3.6	5.1	—	—
36	0.2	+	"	"	0.5	3.7	15.5	—	—
27	0.5	0	"	4.9	7.6	—	—	—	—
37	0.5	+	"	4.4	23.7	—	—	—	—
28	1.0	0	"	5.3	14.5	—	—	—	—
38	1.0	+	"	11.8	39.5	—	—	—	—
29	2.0	0	"	13.6	27.2	—	—	—	—
39	2.0	+	"	19.9	43.0	—	—	—	—
30	3.0	0	"	} Very heavy growth. Not counted.					
40	3.0	+	"						

S.g. means some growth but too slight to count.

* Involution forms present. Very slight film formation.

On comparing the results obtained with the yeast extract with those obtained with the lemon juice it will be seen that their general trend is similar, and that the yeast extract is approximately ten times as effective as the lemon juice although its nitrogen content is more than 30 times as great. The results obtained are somewhat less uniform than those obtained with the lemon juice, and tubes Nos. 24 and 25 fall out of line on the 11th and 4th days respectively. Film formation does not occur so readily, only very slight traces being present in tubes Nos. 31, 32, 33, 34 and 35 by the 11th day.

These differences may be due to differences in the nature of the nitrogen in the two substances under examination or to the presence of toxic substances in the yeast extract which are absent from the lemon juice. The yeast experiment, however, affords confirmation of the fact that the rate of growth is at first independent of the presence or absence of $(\text{NH}_4)_2\text{SO}_4$ and depends on the concentration of the "bios" until the yeast has reached a concentration of somewhere in the neighbourhood of five or six million cells per cc., after which it proceeds further in the presence of the $(\text{NH}_4)_2\text{SO}_4$.

GENERAL CONCLUSIONS.

The suggestion that "bios" may be of the same nature as the vitamins, which are widely held to be necessary for the nutrition of higher animals, and the further suggestion that it may be identical with one of the recognised vitamins, open up possibilities for investigating the subject of vitamins which are particularly attractive, owing to the ease with which a biological process

can be studied in a yeast as compared with a similar study in the case of a more complex organism. Before we can proceed to investigate the general question of vitamins by such methods it is necessary, firstly, to be certain that "bios" is actually a vitamin, *i.e.* a substance whose presence in the food in relatively small quantities is necessary to enable the remainder of the foodstuffs to be properly assimilated and utilised by the organism, and secondly that the substance in question is identical with one of the recognised vitamins. The present investigation points to the fact that "bios" does not enable the yeast to assimilate $(\text{NH}_4)_2\text{SO}_4$ simply by its presence or by being consumed at the same time, but that the yeast grows solely at the expense of the "bios" until it reaches a certain degree of concentration, and after that it is able to use the $(\text{NH}_4)_2\text{SO}_4$. No explanation is offered of this phenomenon which requires further investigation.

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