CCXLVI. THE EFFECTS OF INOSITOL, CRYSTALLINE VITAMIN B₁ AND "PANTOTHENIC ACID" ON THE GROWTH OF DIFFERENT STRAINS OF YEAST.

BY ROGER JOHN WILLIAMS
AND DONALD HERBERT SAUNDERS.

From the Chemical Laboratory of Oregon State College.

(Received June 4th, 1934.)

THAT inositol is a constituent of Wildiers' "bios" and hence is involved in yeast-growth stimulation, was first suggested by Eastcott [1928], but some doubt was entertained regarding her findings by Williams, R. J., Warner and Roehm [1929] and Narayanan [1930].

The possible effect of "vitamin B" on yeast growth was suggested by Williams, R. J. [1919], and later evidence was presented [Williams, R. J. and Roehm, 1930] that the antineuritic factor is one substance involved in the growth stimulation of certain yeasts. The crystalline hydrochloride of vitamin B₁ (recrystallised from alcohol) has recently become available to us through the courtesy of R. R. Williams [Williams, R. R. *et al.*, 1934]. While conclusive evidence as to the purity of this preparation has not been published, it is effective in very small doses (5 γ are a curative dose for rats), and there is no reason at this time to doubt its essential purity, particularly from the physiological standpoint.

The term "pantothenic acid" (from Greek meaning "from everywhere") has been applied to what is believed to be a single acid substance of universal biological occurrence [Williams, R. J. *et al.*, 1933]. This acid (or possibly acids) has a striking effect on the growth of a particular strain of yeast ("Gebrüder Mayer") and its presence is tested for quantitatively by this effect. The "active principle" from whatever source is always an acid (or mixture of acids) of about the same strength. Using rice bran as a source, the ionisation constant is determined by fractional electrolysis to be about 3×10^{-5} [Williams, R. J. and Moser, 1934]. Regardless of the source, the active substance behaves in a variety of ways as essentially the same substance; it is not destroyed by catalytic hydrogenation with or without a promoter; it is not destroyed by oxidation with ammoniacal silver solution; its heat-stability at different hydrogen-ion concentrations is consistent; it is destroyed by nitrous acid; it is capable of esterification with alcohol; the esters are inactive physiologically but the activity returns on hydrolysis. The esters are more soluble in water than in ether. Diffusion studies indicate that the molecular weight (of material from different sources) is consistently of the order of 150–200.

Based upon this information we have worked out in this laboratory a method for concentrating the acid, using autolysed liver as a source, and have succeeded in obtaining it of such potency that about 2.5 parts per 10^{10} parts of culture medium can be detected by its effect on yeast growth. This is equivalent to 1 mg. per 4000 litres of culture medium. In all the numerous fractionations we have performed we have never had reason to suspect that the "active principle"

is more than one substance. Although inherently difficult to purify, it always has a tendency to become concentrated at one point in a fractionation procedure rather than to distribute itself widely as might be the case if two or more substances were involved. We constantly make use of the fact that it is an acid, and the solubilities of its salts are those that would be expected of a polyhydroxy-acid of relatively low molecular weight as previously conceived [Williams, R. J. *et al.*, 1933].

The methods of purification are undergoing active investigation and the details of procedure will be published soon. The preparation used in this study is about one-fourth as potent as the most active preparation yet made in this laboratory. While it is obviously not a pure chemical substance, we have no reason to think that it contains any substance which affects yeast growth, other than the acid which is described above.

In testing the effects of the three materials mentioned above—inositol, crystalline vitamin B₁, and "pantothenic acid"—on various yeasts we obtained results much more striking than was anticipated, and since they go so far toward clearing up the "bios question," we decided to offer them for publication without waiting for final purification of the agent which has been concentrated in this laboratory.

The strains of *Saccharomyces cerevisiae* chosen for study include those used by various workers who have attempted to determine the nature of the yeast growth stimulants. Included are Wildiers' yeast (obtained through the courtesy of Dr M. Ide), "old process" yeast (used by the senior author in 1919), W. L. Miller's yeast obtained from Toronto, Yeast #2190 obtained through the courtesy of Dr J. C. Drummond, "Gebrüder Mayer" yeast obtained from the Fleischmann Co. and used by W. H. Eddy in his studies, and yeast taken directly from a fresh Fleischmann cake. All of these with the exception of the last have been grown in stock culture for years on molasses agar, made up from the same batch of blackstrap molasses [Williams, R. J. *et al.*, 1927].

The medium which has been used in these studies for several years has been modified to meet the suggestions of Elvehjem [1931] regarding the stimulating effect of iron and copper, of Richards [1932] regarding a similar effect of thallium, as well as the recognised facts that traces of boron are required by many plants and that traces of zinc, manganese and iodine are needed by at least some plant organisms. Each litre of the modified medium, therefore, contains the following solid ingredients: sucrose 20 g.; (NH₄)₂SO₄, 3.0 g.; KH₂PO₄, 2.0 g.; asparagine, 1.5 g.; CaCl₂, 2H₂O, 0.25 g.; MgSO₄, 0.25 g.; TiCl₃, 1.0 mg.; ZnSO₄, 1.0 mg.; MnCl₂, 1.0 mg.; H₃BO₃, 1.0 mg.; FeCl₃, 0.5 mg.; CuSO₄, 0.1 mg.; KI, 0.1 mg.

It should be remarked in this connection that we regard the presence of asparagine or some substitute therefor to be necessary. In unpublished experiments conducted by Truesdail it was demonstrated that asparagine (recrystallised many times) may become a definite limiting factor for the growth of "Gebrüder Mayer" yeast when plenty of nutilite (growth factor) is present. According to previous experience, this was not likely to be the case with "old process" yeast. We suspect that the effects of extracts used by other workers when added to an asparagine-free medium, may be due in part to organic nitrogen, unless the yeasts are quite different in their responses from "Gebrüder Mayer" yeast.

We have recognised for some time that traces of inorganic materials such as those added might have a stimulating effect on yeast growth. We noted in one experiment [Williams, R. J. and Truesdail, 1931] the slight improvement brought about by yeast ash. Such effects, however, are small (from the stand-

point of our usual growth responses) and in part at least due to the presence of traces of these substances as impurities in our salts. The addition of the traces of salts indicated to our medium has a detectable favourable effect on yeast growth, but the effect is too small to have had a significant effect on our previous results. We believe, however, that it is safer to introduce the traces of all of these salts knowingly, rather than to rely on chance.

The number of combinations (quantitative as well as qualitative) of the three factors we are studying might be extended almost indefinitely, hence we have had to make distinct limitations. The effect of inositol in different concentrations when used in the presence of the other factors in different amounts has not been studied because in the case of the "Gebrüder Mayer" yeast previously studied in this connection variation of the concentrations of inositol in the general range of 1–10 γ per ml. made little difference in the response obtained in our tests as ordinarily carried out. A dosage of about 20 γ per ml. is used under the conditions of the tests as carried out in the Toronto laboratories [Miller *et al.*, 1933].

Since previous publications from this laboratory, we have made an improvement in our method of seeding. The unknown materials to be tested are made up to 2 ml. with water, sterilised and then seeded with 10 ml. of a very dilute suspension of yeast in control medium. This suspension is made by dilution from a heavier suspension which is standardised by means of our thermocouple apparatus [Williams, R. J., *et al.*, 1929]. We find that this procedure allows for a more constant seeding and more uniform responses.

For all the yeasts except the last two, the seeding was 0.12 mg. moist yeast per culture. For the two others which are more rapidly growing yeasts, the seeding was 0.04 mg. The growth period was 18 hours at 30° and the crops are given in terms of moist yeast per ml. Each series of 27 cultures was run upon a separate day. The duplicate tests on the same yeasts were in most cases separated by a few weeks, and the differences between the magnitudes of the growths are no doubt due to differences in exact age and history of the particular cultures used. The relative effects of the different additions are in general concordant. The seeding from the Fleischmann cake was made directly from the centre of a cake without first isolating a pure culture. That the growth is somewhat irregular is possibly due to this cause.

Our experience agrees with that of Miller *et al.* [1933] in that, especially when growths are large, the size and shape of the vessel used may be an important factor in determining the size of the crop when the cultures are undisturbed during the growth period. We use consistently the same size (50 ml.) pyrex Erlenmeyer flasks, and in quantitative tests never rely on heavy crops or long growth periods, with the result that our ability to duplicate results using our thermocouple equipment exceeds in exactness any biological test with which we are acquainted. The use of flasks which are undisturbed during the growth period has a great practical advantage over that of rocker-tubes in a thermostat, as used in the Toronto laboratories, as we are enabled to grow 60–80 cultures simultaneously in one incubator. Handling such numbers in rocker-tubes would, we fear, entail serious expense. It may be emphasised that if the seedings and growths are much larger than those used by us, the relatively small effects produced by inositol alone, for example, may be modified owing to its becoming a limiting factor for growth. Other variations in results are to be expected on the same basis.

The actual data obtained in the tests are given in Table I and the effects of the different nutritives are summarised in Tables II, III and IV. In addition

to the results indicated in the tables we have experimented sufficiently to convince ourselves that yeast #578 (2331) of the American type culture collection behaves substantially in the same way as "old process" yeast in that when an inositol-containing medium is used, vitamin B₁ alone has a very definite effect, "pantothenic acid" alone has a smaller, but definite effect, while the effect of the two together is very striking.

Table I. *Yeast crops**.

Addition (to 12 ml. cultures)	Wildiers' yeast	Old process yeast	W. L. Miller's yeast	Narayanan's yeast # 2190	Ge- brüder Mayer	Cake Fleisch- mann
0	0-08 0-09	0-01 0-02	0-05 0-10	0-02 0-03	0-02	0-07
0	0-07 0-09	0-01 0-02	0-05 0-10	0-02 0-03	0-02	0-07
0-1γ I	0-07 0-09	0-02 0-02	0-05 0-11	0-03 0-02	0-02	0-07
1-0γ I	0-07 0-09	0-03 0-03	0-06 0-12	0-03 0-03	0-02	0-07
10γ I	0-09 0-09	0-03 0-03	0-06 0-13	0-03 0-03	0-02	0-07
100γ I	0-09 0-08	0-02 0-04	0-06 0-16	0-03 0-03	0-02	0-07
0-1γ B	0-09 0-07	0-08 0-07	0-05 0-10	0-03 0-03	0-02	0-06
1-0γ B	0-07 0-07	0-10 0-07	0-05 0-10	0-03 0-02	0-02	0-05
10γ B	0-07 0-07	0-11 0-08	0-05 0-10	0-03 0-02	0-02	0-05
0-1γ P	0-08 0-14	0-04 0-03	0-13 0-29	0-03 0-02	0-06	0-14
1-0γ P	0-31 0-44	0-05 0-03	0-61 0-72	0-09 0-03	0-22	0-44
10γ P	0-11 0-33	0-06 0-04	0-71 1-04	0-17 0-09	0-24	0-78
50γ I+0-1γ B	0-06 0-06	0-06 0-09	0-08 0-19	0-03 0-02	0-02	0-04
50γ I+1-0γ B	0-05 0-06	0-09 0-09	0-08 0-15	0-04 0-03	0-02	0-05
50γ I+10γ B	0-05 0-06	0-11 0-11	0-09 0-16	0-04 0-03	0-03	0-04
50γ I+0-1γ P	0-11 0-17	0-04 0-04	0-17 0-32	0-04 0-04	0-06	0-06
50γ I+1-0γ P	0-37 0-60	0-05 0-04	0-93 0-96	0-27 0-18	0-64	0-37
50γ I+10γ P	0-29 0-70	0-14 0-04	1-12 1-44	0-74 0-39	0-77	1-27
1γ P+0-1γ B	0-28 0-47	0-21 0-32	0-44 0-65	0-12 0-09	0-19	0-44
1γ P+1-0γ B	0-26 0-46	0-52 0-52	0-38 0-60	0-13 0-10	0-21	0-45
1γ P+10γ B	0-27 0-41	0-51 0-51	0-39 0-58	0-13 0-09	0-20	0-44
1γ B+0-1γ P	0-07 0-12	0-21 0-20	0-13 0-21	0-03 0-03	0-06	0-12
1γ B+10γ P	0-25 0-44	0-62 0-44	0-55 0-81	0-13 0-10	0-24	0-61
50γ I+0-1γ B +1γ P	0-38 0-65	0-82 1-17	0-89 0-85	0-25 0-18	0-65	0-54
50γ I+1-0γ B +1γ P	0-36 0-58	1-38 1-20	0-88 1-02	0-23 0-22	0-62	0-58
50γ I+0-1γ B +0-1γ P	0-06 0-11	0-14 0-16	0-17 0-24	0-04 0-04	0-10	0-05
50γ I+1γ B +0-1γ P	0-06 0-09	0-22 0-18	0-20 0-22	0-04 0-04	0-10	0-05

* In the table yeast crops are expressed as mg. of moist yeast per ml. I, B and P stand respectively for inositol, vitamin B₁ and "pantothenic acid" concentrate.

Table II. *The effect of inositol.*

"Strain" used	Added to blank medium alone	Supplement to vitamin B ₁	Supplement to "pantothenic acid"	Supplement to vitamin B ₁ plus "pantothenic acid"
Wildiers'	No effect	No effect	Definite though small increase	Same as without B
Old process	Slight increase	Very slight increase	Very slight increase	Striking increase
W. L. Miller's	Slight increase	About same as without B	Definite increase	Same as without B
Narayanan's # 2190	Very slight increase	Same as without B	Striking increase	Same as without B
Gebrüder Mayer	Not detectable here	No effect	Marked increase in larger growths	Same as without B
Fleischmann Cake	No effect	No effect	Marked increase in larger growths	About same as without B

Table III. *The effect of vitamin B₁.*

"Strain" used	Added to blank medium alone	Supplement to inositol	Supplement to "pantothenic acid"	Supplement to inositol <i>plus</i> "pantothenic acid"
Wildiers'	No effect	Slightly inhibitory	Variable, slight	Slightly inhibitory
Old process	Definite increase	Definite increase	Most striking increase	Most striking increase
W. L. Miller's	No effect	Slight increase	Slightly inhibitory	No effect
Narayanan's # 2190	No effect	Increase questionable	Slight increase	Practically no effect
Gebrüder Mayer	No effect	No effect	Inhibitory questionable	Inhibitory questionable
Fleischmann Cake	Slightly inhibitory	Slightly inhibitory	No effect	No effect

Table IV. *The effect of "pantothenic acid."*

"Strain" used	Added to blank medium alone	Supplement to inositol	Supplement to vitamin B ₁	Supplement to inositol <i>plus</i> vitamin B ₁
Wildiers'	Striking increase	Striking increase	Same as without B	Same as without B
Old process	Definite slight increase	Slight increase	Striking	Most striking
W. L. Miller's	Striking increase	Striking increase	Good growth, less than without B	Same as without B
Narayanan's # 2190	Definite increase	Striking increase	Definite increase	Practically same as without B
Gebrüder Mayer	Striking increase	Striking increase	Same as without B	Same as without B
Fleischmann Cake	Striking increase	Striking increase	About same as without B	About same as without B

It is to be noted that in the summaries (Tables II, III and IV) the results as tabulated were, in the case of the first four yeasts, obtained twice in independent experiments each involving two to three cultures. Since there is no disagreement between the different experiments (except possibly in the case of a single culture), we feel that the differences are significant, and that the five pure cultures listed represent different species of organisms. As they have been grown on the same medium for years, the differences can hardly be accounted for on any other basis. The individual behaviour of "old process" (or # 578) yeast is very striking and cannot be questioned. The difference in behaviour between the yeast of Miller and that of Wildiers, particularly with respect to the response toward inositol and crystalline vitamin B₁ together, is, in our opinion, significant. Wildiers' yeast has also throughout a period of years been peculiarly susceptible to the effect of toxic substances which appear in extracts. This was particularly apparent in the experiments of Williams, R. J. and Truesdail [1931]. The fact that these two yeasts react similarly towards the crude preparations of Miller *et al.* [1933] does not constitute proof of the identity of the active ingredients of the extracts, though from our experiments there remains no doubt that both "pantothenic acid" and inositol are common factors for the two yeasts.

There is no doubt in our minds that there is an unknown basic factor effective for Wildiers' yeast which is not vitamin B₁. We have repeated some of the

fractional electrolysis experiments of Williams and Truesdail on rice bran extracts and confirm their findings regarding the supplementary action of acidic and basic components of the extract. We find also that the basic component and our concentrated "pantothenic acid" supplement each other. However, there is a significant difference in our results in that the acid factor alone (containing "pantothenic acid") now causes very distinct stimulation, whereas in the earlier experiments, it did not. We attribute the difference to the probability that in the earlier experiments toxic material (which was constantly encountered in the more acid cells) was not removed completely, and hence masked what stimulating effect the acidic component ("pantothenic acid") would have had.

A careful analysis of the results given in Table I reveals the fact that sometimes the nutrilites which, under one set of conditions promote increased growth, under a different set of conditions may inhibit growth. We believe there is some evidence that to promote most rapid growth some yeasts require a "balance" between the different stimulating factors. This matter requires further study.

That inositol is a factor in the growth stimulation of all the yeasts studied is a striking fact, and Miss Eastcott working in Dr Miller's laboratory deserves full credit for the discovery. That several other workers have not noted a positive effect of inositol, has been due in part to the reasons outlined by Miller *et al.* [1933], and also to the lack of proper supplements. In our laboratory an important factor has been the fact that we have consistently dealt with small growths and under such conditions the effect of inositol alone or in combination is very small, except in the case of the yeast from the Toronto laboratories which we found to be affected [Williams, R. J. and Bradway, 1931]. To be effective inositol evidently must be present in much larger amounts than the other nutrilites. We are basing this statement partly on unpublished experiments on "Gebrüder Mayer" yeast and partly on the results obtained in the Toronto laboratories.

The striking effect of crystalline vitamin B₁ as a supplement to "pantothenic acid" (also alone) in stimulating "old process" yeast shows that the senior author's original suggestion as to the probable identity of "bios" (for "old process" yeast) and the water-soluble B vitamin [Williams, R. J., 1919] was not wholly without substantial basis. There are, however, other complications which have previously been noted [Williams, R. J. and Roehm, 1930].

The results with "pantothenic acid" on the different yeasts are much more striking than we anticipated, especially the marked effects which it has when used without any supplement. It is apparent to us that this material stands alone in so far as its ability in minute doses to stimulate different strains of yeast is concerned.

Since our results and claims in this respect are striking and must be confirmed by other workers, our laboratory will be glad to furnish interested investigators in this field with samples of our concentrates in order that they may carry out whatever confirmatory tests on yeast they may desire to make.

SUMMARY.

1. The different strains of so-called *Saccharomyces cerevisiae* used in the studies of Wildiers, R. J. Williams, W. L. Miller, Eddy and Narayanan have been studied.

2. Inositol is found to be at least a detectable factor in the growth-stimulation of all the yeasts investigated. Alone, it in general has very little effect, but in conjunction with the other substances mentioned, it may cause a striking

increase. The amounts necessary to produce effects are relatively large (1 γ to 10 γ per ml. of medium).

3. Crystalline vitamin B₁ has very little effect on any of the yeasts except "old process" yeast on which the original work of the senior author was done and on yeast 578, also investigated in his laboratory. As little as 0.008 γ per ml. has a distinct effect alone on these yeasts, and in conjunction with pantothenic acid, the effect of doses of this size and larger is very striking.

4. A highly concentrated "pantothenic acid" preparation was found to be effective alone in doses of 0.008 γ per ml. upwards, on all the yeasts investigated. This effect was in general enhanced more or less either by inositol or vitamin B₁ or both. In some cases both the individual and supplementary effects are most striking. The different yeasts, in most cases, show characteristic responses to the different materials.

5. The results are so conclusive and striking that we feel that the "bios question" is to a considerable extent answered. No single (unknown) substance is wholly responsible for yeast growth stimulation. The three substances studied play important rôles, of which that of "pantothenic acid" is outstanding.

We acknowledge with thanks the generous support of Standard Brands Inc., successors to the Fleischmann Company, which has made this work possible.

REFERENCES.

- Eastcott (1928). *J. Physical Chem.* **32**, 1094.
Elvehjem (1931). *J. Biol. Chem.* **90**, 111.
Miller, Eastcott and Maconachie (1933). *J. Amer. Chem. Soc.* **55**, 1502.
Narayanan (1930). *Biochem. J.* **24**, 6.
Richards (1932). *J. Biol. Chem.* **96**, 405.
Williams, R. J. (1919). *J. Biol. Chem.* **38**, 465.
— and Bradway (1931). *J. Amer. Chem. Soc.* **53**, 783.
— Lyman, Goodyear, Truesdail and Holaday (1933). *J. Amer. Chem. Soc.* **55**, 2912.
— McAlister and Roehm (1929). *J. Biol. Chem.* **83**, 315.
— and Moser (1934). *J. Amer. Chem. Soc.* **56**, 169.
— and Roehm (1930). *J. Biol. Chem.* **87**, 581.
— and Truesdail (1931). *J. Amer. Chem. Soc.* **53**, 4171.
— Warner and Roehm (1929). *J. Amer. Chem. Soc.* **51**, 2764.
— Wilson and Von der Ahe (1927). *J. Amer. Chem. Soc.* **49**, 227.
Williams, R. R., Waterman and Keresztesy (1934). *J. Amer. Chem. Soc.* **56**, 1187.