X. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

IV. THE ADDITION OF MANNITOL.

By VERA READER.

From the Department of Biochemistry, Oxford.

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IN a paper by Orr-Ewing and Reader [1928], a technique was described for using the growth-promoting power of yeast concentrates upon *Streptothrix corallinus* as a test for the antineuritic vitamin. In this technique the yeast concentrate under test was put up in three dilutions, viz. 1/20, 1/400, 1/8000 in equal volumes of medium (20 cc.), and the resulting streptothrix growth compared with that in a series of standard flasks containing similar dilutions and intermediate dilutions of a concentrate of known potency. The result was expressed in terms of pigeon doses. When testing the purer antineuritic concentrates agreement with the corresponding standards was obtained in all three dilutions, e.g. 80, 70, and 75 doses in a given test sample. However, certain "anomalous results" appeared with the less pure concentrates; the tests came out thus, 500, 70, 75; i.e. there appeared to be relatively more growth-promoting activity in the higher concentrations of vitamin than in the lower. In view of this discrepancy, further work was undertaken to elucidate the phenomenon.

It has often been noted that the upper limit of concentration necessary for maximum growth is reached when approximately 1/80 of a pigeon-day-dose has been added to 20 cc. of the medium. Further addition of glucose or of vitamin concentrate at this point does not induce further growth. Now the anomalous result always occurred when the yeast concentrate was comparatively impure, and this suggested that the abnormally high results with the larger additions of vitamin were due to some added source of food for the organism. A few preliminary experiments done in 1924 had seemed to suggest that some specific carbohydrate or polyhydric alcohol might give greater growth in a given time at a given temperature than that obtained with glucose. Further, from earlier published work [Reader, 1927] it was known that "the following substances may replace glucose as a source of carbon in the medium, without loss of efficiency: glycerol, mannitol, arabinose, lactate, citrate, pyruvate, and glycogen." As these earlier experiments were carried out in a medium of pure salts + carbon compound, it was now decided to test their effect as additional substances to the seemingly complete medium.

EXPERIMENTAL.

The technique differs from that described in Part III [Reader, 1928] in one particular only, that of method of estimation. Since all the experiments in this section are concerned with the requirements for maximal growth and not with minimal dilutions for growth, it was possible to estimate the amount of growth by direct weighing. The contents of three small similar conical flasks (20 cc. fluid) were filtered through the same small, hard, dried and weighed filter-paper. The whole was then dried in a CaCl_2 desiccator to constant weight. Since the variation in twenty similar papers was not greater than $\pm 5\%$, this method was considered to be of sufficient accuracy. Growth was estimated at the end of 6 days, unless otherwise stated. Conditions of temperature are quoted at the beginning of each experiment.

Exp. 1. The amounts of synthetic medium, glucose, and torulin (vitamin) extract were kept constant throughout seven sets of three flasks, while 0.5 % of the various compounds under test was added severally to each of the sets (Table I).

Table I.

$T=1/100$ unit dose of torulin in 20 cc. fluid.
Temp. 31° for 2 days followed by 15° for 4 days.

Set	Medium	Total weight from three flasks g.
I	Salt-sugar medium + T	0.025
II	" " + T	0.025
III	" " + T + arabinose	0.024
IV	" " + T + glycogen	0.021
V	" " + T + glycerol	0.041
VI	" " + T + citrate	0.065
VII	" " + T + mannitol	0.144

It will be noted that the six-fold increase of growth with mannitol is of the order of the divergence of the "anomalous results" from the normal. Consequently Sets I and VII were repeated, dilutions being made as in the test cases described in the paper by Orr-Ewing and Reader [1928], in each case the mannitol being diluted with the torulin.

When torulin and mannitol were diluted 400 times, *i.e.* as in the third flask of the test, growth was equivalent to that with torulin without mannitol, hence a true case of "anomalous result."

Exp. 2. The growth obtained in Set VII of exp. 1 was so prolific that it was decided to extract the dried organisms with ether to find out whether the increased weight was due to increased fat production rather than to an increase in the number of organisms (see Table II).

Table II.

	Weight of organism g.	% fat-soluble material
I	Growth without mannitol 0.045	6.0
II	Growth with mannitol 0.144	6.1
III	" " 0.142	6.0

From these figures it was concluded that mannitol had not increased the fat production.

Exp. 3. The mannitol used in the above experiments was as supplied by Baird and Tatlock and was supposed to be pure. However, considering the earlier experience with traces of accessory factor present as impurity in glucose [Reader, 1928] an attempt was now made to purify the mannitol from any such factor. In the first place the mannitol was tested before and after extraction with charcoal in neutral solution, but this was found to have no effect, the respective growths being 0.135, 0.133 and 0.132 for the original mannitol and after one and two treatments with charcoal.

While developing a technique for the extraction of the antineuritic vitamin Kinnersley and Peters [1925] found that control of hydrogen ion concentration was an important factor in the removal of such accessory factors by charcoal adsorption. A more exhaustive attempt at removal on charcoal at various hydrogen ion concentrations was now undertaken, but again without success (Table III).

Table III.

$T = 1/100$ unit dose of torulin in 20 cc. fluid.
Temp. 31° for 2 days followed by 15° for 4 days.

Set	Medium	Weight of growth g.
I	Salt-sugar medium + T	0.033
II	" " + $T + M$ (crude, 0.1 g. in 20 cc.)	0.140
III	" " + $T + M$ (purified charcoal at $p_H=2$)	0.137
IV	" " + $T + M$ (purified charcoal at $p_H=5$)	0.138
V	" " + $T + M$ (purified charcoal at $p_H=7$)	0.135
VI	" " + $T + M$ (purified charcoal at $p_H=9$)	0.139
VII	" " + $T + M$ (hydrolysed dilute HCl for 30 mins.)	0.127
VIII	" " + $T + M$ (hydrolysed dilute NaOH for 30 mins.)	0.121

As the results show, no change was produced by these various modes of treatment. Additional proof that the growth-promoting property is due to the presence of the mannitol itself, and not to an impurity, was obtained by reducing a sample of mannose which had been previously tested and found to have no enhancing effect on the growth (Table IV). The reduction was carried out according to the directions of Fischer [1890], using Na amalgam in alkaline solution with rapid mechanical stirring for 12 hours.

Table IV.

Set	Medium	Growth g
I	Salt-sugar medium + T	0.033
II	" " + $T +$ mannose (0.5 %)	0.028
III	" " + $T +$ mannitol (0.5 %) (from mannose)	0.090

It was now decided to test other available polyhydric alcohols. Similar experiments were designed with dulcitol, sorbitol, glycerol and inositol (Table V). From exp. 5, Table V it may be seen that the effect with none of them was of the order of that obtained with mannitol, *i.e.* the enhancement with mannitol appears to be a specific one for this organism.

Table V.

Set	Medium	Weight of growth g.
I	Salt-sugar medium + T	0.033
II	" " + T + mannitol	0.140
III	" " + T + dulcitol	0.065
IV	" " + T + sorbitol	0.045
V	" " + T + inositol	0.057
VI	" " + T + glycerol	0.058

With regard to the phenomenon of the optimum concentration of the growth factor referred to in the introduction (p. 61) it now seemed possible that, in the presence of mannitol, any excess of growth-promoting factor supplied might be used up. In the following experiments the amounts of mannitol and glucose were kept constant throughout, while the torulin concentrate was varied from 1/20 to 8/20 day-dose per 20 cc. The results are shown in Table VI. From these, it may be concluded that there is a definite upper limit of torulin concentrate for maximum growth even in the presence of mannitol, but that that upper limit is much greater than with glucose alone, as is also the maximum growth obtained. It is easily seen from the curves for the four experiments cited that in each case the addition of mannitol has led to an abnormally high value for the maximum growth. Therefore, with the highest concentration of torulin each case is a true "anomalous result."

Table VI.

(All flasks contain 20 cc. inorganic salt-sugar medium.)						
$G=0.5\%$ glucose.		$M=0.5\%$ mannitol.		$T=1/20$ unit dose torulin concentrate.		
	Exp. 6.	Temperature 3 days at 33° then 6 days at $16-20^\circ$.				
	Exp. 7.	3	33°	6	20-25°	
	Exp. 8.	6	33° .			
	Exp. 9.	6	33°	3	16-20°.	
	Medium	g. growth				
		Exp. 6	Exp. 7	Exp. 8	Exp. 9	
1.	$G+T$	0.056	0.060	0.057	0.061	
2.	$G+4T$	0.056	0.061	0.050	0.065	
3.	$G+8T$	0.056	0.060	0.059	0.064	
4.	$G+M+T$	0.073	—	0.138	0.090	
5.	$G+M+2T$	0.090	0.186	0.151	0.113	
6.	$G+M+4T$	0.084	0.187	0.161	0.143	
7.	$G+M+8T$	0.111	0.180	0.154	0.171	

The variations in the results ($G+M+xT$) are due to variation in the average room temperature during the time after the cultures had been removed from the incubator. This was very puzzling at first, until it was realised that not only the torulin efficiency, but also the temperature coefficient had been appreciably altered by the addition of mannitol. Thus, in exp. 6 the temperature on the latter days varied from 16° to 20° and in exp. 7 from 20° to 25° . The difference between the results is greater than in exps. 8 and 9 where the change from the incubator temperature to that of the room occurred at a later stage in the life cycle of the organism [see Reader, 1926].

Much further work is needed upon this point, more especially as the issue

is complicated by the fact that the organism under observation is a *Streptothrix*, that is, it is continually segmenting, and the rate of the segmentation is also varied by slight changes in temperature.

Fig. 1. Weight of organisms in 14 days, i.e. after growth is complete.
($M=0.5\%$ mannitol; $I=0.5\%$ inositol; $G=0.5\%$ glucose; $T=1/20$ unit dose of torulin.)

Table VII.

Temperature 7 days at 33° , then 7 days at 20°

$I=0.5\%$ inositol.

$M=0.5\%$ mannitol.

$T=1/20$ unit dose torulin concentrate.

	Medium	Weight of growth in 14 days g.
1.	$G+T$	0.050
2.	$G+2T$	0.051
3.	$G+M+1/10T$	0.070
4.	$G+10M+\frac{1}{2}T$	0.160
5.	$G+M+\frac{1}{2}T$	0.155
6.	$G+1/10M+\frac{1}{2}T$	0.154
7.	$G+10M+T$	0.170
8.	$G+M+T$	0.179
9.	$G+1/10M+T$	0.175
10.	$G+10M+2T$	0.169
11.	$G+M+2T$	0.175
12.	$G+1/10M+2T$	0.178
13.	$G+10M+4T$	0.174
14.	$G+M+4T$	0.165
15.	$G+1/10M+4T$	0.170
16.	$G+I+\frac{1}{2}T$	0.064
17.	$G+I+T$	0.062
18.	$G+I+2T$	0.067
19.	$G+\frac{1}{2}I+T$	0.057
20.	$G+I+4T$	0.063

A final, and perhaps more conclusive experiment was carried out (see Fig. 1). In this the weighings were taken at the end of 14 days. The curve for inositol is added for comparison. The results are given in Table VII. As the growth was independent of concentration of mannitol, at least within these limits, only one curve is drawn ($G + xT + M$) (Fig. 1).

Thus it may be seen that even with 1/200 of a unit dose (1/10 T) the growth with mannitol is in excess of the maximum growth with glucose alone, and also that the maximum growth with mannitol is not reached until 1/20 of a unit dose is supplied in 20 cc. of medium. From this table it is obvious that in the presence of mannitol a true "anomalous result" is artificially reproduced.

DISCUSSION.

Whether the results obtained by Orr-Ewing and Reader with crude torulin extracts were due to the presence of mannitol in the watery extracts of yeast has not yet been proved. Indeed it would appear that the prolonged extractions and purification of yeast extract necessary to show the presence of mannitol would be unlikely to meet with success as the work is complicated by the presence of inositol [see Eastcott, 1928] and possibly other polyhydric alcohols of very similar properties.

At this point it is interesting to consider the parallel case reported by Eastcott [1928]. She claims to have isolated "bios I" [see Lucas, 1924] for yeast, and to have identified this substance with inactive inositol, the "phaseo-mannite" of Vohl [1856]. However the degree of stimulation reported in her experiments is not greater than that recorded in this paper for the action of mannitol on the growth of *S. corallinus*. Thus, from her experiments, it may be seen that in 48 hours, in solutions containing salts, sugar and "bios II," 113 cells of yeast were produced, while with salts, sugar, crude "bios II" and inositol, some 365 cells resulted from the same size of inoculation. This increase is of the same order as that shown in Tables VI and VII of this paper. Certainly I do not consider that the mannitol effect may be explained as due to a true "growth-promoting factor," but rather as a specific source of food-supply and would suggest a similar interpretation of the results of Eastcott.

SUMMARY.

Further work on the cultural requirements of *S. corallinus* is reported.

1. The "anomalous results" obtained by Orr-Ewing and Reader when crude watery extracts of yeast were added to the culture medium, can be artificially reproduced by adding mannitol to the purer extracts.
2. The increased weight of bacteria grown in the presence of mannitol was not due to increased fat production.
3. Within the limits of these experiments, the amount of growth was independent of the concentration of the mannitol.

4. Exhaustive treatment with charcoal at various hydrogen ion concentrations and subsequent recrystallisation failed to remove any growth-promoting factor from the mannitol.

5. A sample of mannitol was prepared from mannose which had previously been shown to have no growth-promoting effect. This mannitol gave an enhancement of growth equal to that obtained with mannitol from natural sources as usually supplied for laboratory purposes.

6. It is not possible to substitute dulcitol, sorbitol, glycerol, or inositol for the mannitol.

It is concluded that the effects obtained are due to either the whole or part of the mannitol molecule acting as a specific source of food-supply rather than as an additive "growth-promoting factor."

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