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

III. THE EFFECT OF THE ADDITION OF GROWTH-PROMOTING SUBSTANCES TO THE SYNTHETIC MEDIUM ON THE GROWTH OF *STREPTOTHRIX CORALLINUS*.

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(Received March 1st, 1928.)

In a previous paper [1927] it was shown that certain organisms could be grown in a synthetic salt-sugar medium provided that the concentration of the inorganic ions was kept within moderately narrow limits: but it was soon observed that the addition of the smallest amounts of trypsin-digested beef broth to the synthetic medium had an enhancing effect on the three organisms under experiment (*Sarcina aurantiaca*, a white *Streptothrix*, and *Streptothrix corallinus*). In fact, the growth was so much more prolific than in a purely synthetic medium that it was decided to make a detailed study of the nature of the stimulating substances in broth, and to seek for other sources.

It was reported by Mueller [1922], and by Freedman and Funk [1922] that there are present in beef and in ox-heart infusions certain substances which show a strong growth-stimulating activity on haemolytic streptococci. Mueller showed that the activating material may be divided into two fractions, active only when mixed together, by means of fractional precipitation of the first HgSO_4 precipitate by HgSO_4 , or precipitation by Ag_2SO_4 and baryta. Various workers have from time to time suggested that vitamin B or its associated substances might be responsible for the stimulation [cf. Thjotta and Avory, 1921; Kuroya and Hosoya, 1923].

Very many workers have studied the sources and properties of growth-stimulating substances for yeast, but in this paper bacterial factors only are considered; for a review of the literature on the bios question see Tanner [1925]. In order to avoid confusion in nomenclature the term *bacterial growth-promoting factor* has been used throughout, thus reserving the term *bios* for the factor augmenting the growth of yeast, as described by Wildiers [1901].

Doryland [1916], Mueller [1922] and others have discussed the possibility that unexpected relationships or dissimilarities in bacterial species may develop on the basis of food requirements, so it was decided to use one test organism only—*Streptothrix corallinus*—throughout this set of experiments. Later it is hoped to extend the studies to other species. The results recorded below, and by Orr-Ewing and Reader [1928] show that for this organism, at least, a growth-promoting factor is required. This is an organic water-soluble substance occurring in fresh beef, wheat embryo, and yeast, and closely associated though not identical with the antineuritic vitamin¹. The author takes this opportunity of modifying a statement made at the British Association Meeting in 1926 to the effect that the bacterial growth-promoting factor was inactivated by alkali. This is often true for comparatively inactive concentrates. However the more active torulin concentrates (Kinnersley and Peters) can be shown to possess a stimulant power even after the inactivation of their antineuritic constituent (vitamin B₁) by alkali. Such antineuritic concentrates therefore show a bacterial growth-promoting activity, but the reverse is not true.

EXPERIMENTAL.

The preparation of the synthetic medium and the general conditions for growth are described in Part I [Reader, 1927]. After 48 hours' incubation at 31° growth was arrested by the addition of formalin and estimated by opacity, standard flasks of a definite extract (trypsin-digested ox-heart) in varying concentrations being used for comparison.

20 cc. inorganic medium + 0.5 % glucose + 0.1 cc. broth = vigorous.

20 cc. inorganic medium + 0.5 % glucose + 0.01 cc. broth = good.

20 cc. inorganic medium + 0.5 % glucose + 0.005 cc. broth = fair.

20 cc. inorganic medium + 0.5 % glucose + nil = slight.

The standard broth was prepared by the method of Douglas [1914]. A fresh bullock's heart, after removal of fat and vessels was finely minced. To every 500 g. of mince, 1 l. of distilled water was added and the liquid made faintly alkaline to litmus. After heating slowly for 5 minutes at 75° it was cooled to 37° and 1 % liquor trypticinae co. (Allen and Hanbury) added. It was then kept at 37° for 3 hours. In order to see that the digestion is complete, the fluid should be tested for peptone by the biuret reaction. It was then made slightly acid with glacial acetic acid, brought slowly to the boil and kept boiling for 15 minutes. After standing overnight in a cool place, it should be syphoned off, made faintly alkaline to litmus, placed in a steamer for $\frac{1}{2}$ hour and then brought to the required p_H (usually 7.0) and sterilised for 20 minutes on three successive days. It is very important that at no stage in the preparation should the p_H be allowed to rise above 8.5. Reference will be made to this later.

¹ The relation of this factor to the antineuritic vitamin is fully discussed in an accompanying communication [Orr-Ewing and Reader, 1928].

*Relation of growth to amount of added growth-promoting factor
and effect of age of beef before preparing broth.*

The following experiment was designed to show at what low limit of concentration of tryptic broth the enhanced effect on growth could be observed. Inorganic medium and 0.5 % glucose were used throughout and varying amounts of broth added. There is some indication that the active substance in beef-tissue is destroyed or inactivated on keeping, since broth prepared from ox-heart fresh from the butcher on the day of killing was more potent than that prepared from ox-heart which had been allowed to stand at room temperature (18°) for 2 days. However once the broth is made and sterilised it may be kept in the laboratory for 3 months (p_H 6.5–7.0) without an apparent loss of activity.

1 cc. of broth represents 0.5 g. fresh heart muscle. In Table I, *A* = broth from ox-heart, fresh; *B* = broth from ox-heart, after standing at room temperature for 2 days; *C* = as *A*, but kept in laboratory for 3 months at p_H 6.5.

Table I.

Concentration of broth in syn- thetic medium	Growth		
	<i>A</i>	<i>B</i>	<i>C</i>
1 : 20	vigorous	vigorous	vigorous
1 : 40	"	good	"
1 : 200	"	slight	"
1 : 400	"	"	"
1 : 1000	very good	"	very good
1 : 1500	good	"	good
1 : 2000	fair	"	fair
1 : 4000	slight	"	slight

Slight growth, as will be seen from the list of standards, is equivalent to the growth obtained on a salt-sugar medium alone, *i.e.* there is no enhancement of the growth.

Physical properties of the growth-promoting factor.

(a) Water soluble; (b) insoluble in ether (see Table II); (c) dialysable (see Table II) through collodion sacs.

Table II.

Supplement to synthetic medium	Growth
(1) Control (salt-sugar)	slight
(2) Broth (1 in 200)	vigorous
(3) Ether extract of broth (equivalent 1 in 200)	slight
(4) Broth after extraction (equivalent 1 in 200)	vigorous
(5) Dialysed broth (1 in 200)	vigorous
(6) Dialysate (1 in 200)	slight

Chemical nature of the factor.

It was now decided to test the possibility of an inorganic salt or salts being responsible for the enhancing effect of broth, and also to test the inorganic portion of the bacteria themselves. (a) 20 cc. of broth *A* (Table I) and (b) 5 g. (approx.) of the organisms were ashed in a platinum dish using an Argand

burner. At three stages—(1) after heating to dryness; (2) after charring (approx. temp. = 170°); (3) after heating at a dull red heat to constant weight—an aliquot part (= 1 cc. original broth, or 0.25 g. bacteria) was taken out and tested for stimulating power.

In Table III these three parts are designated as Preparations I, II, and III.

Table III.

Medium	Growth
(1) Inorganic medium + glucose (control)	slight
(2) Control + 1 cc. broth in 20 cc.	vigorous
(3) Control + 1 cc. broth Prep. I.	vigorous
(4) Control + 1 cc. broth Prep. II.	good
(5) Control + 1 cc. broth Prep. III.	slight
(6) Control + 0.25 g. heated bacteria Prep. I.	vigorous
(7) Control + 0.25 g. heated bacteria Prep. II.	good
(8) Control + 0.25 g. heated bacteria Prep. III.	slight

The general conclusion from this table is that the substance is organic but appears only partially destroyed at the charring point (170°).

Precipitation of proteins, etc. An attempt was now made to remove from the broth all substances precipitated by mercuric chloride, lead acetate, or phosphotungstic acid, and to test the filtrate for growth-promoting power. Precipitations were carried out as follows.

(1) With HgCl_2 : the excess Hg was precipitated with H_2S and the filtrate boiled until free of gas.

(2) With lead acetate: the excess lead was removed as sulphide and the filtrate boiled.

(3) With phosphotungstic acid in 5 % H_2SO_4 and treatment with BaCl_2 .

Micro-Kjeldahl estimations before and after the precipitations showed that the N of 1 cc. of broth was lowered from 2.3 mg. to 0.3 mg. by all three methods.

Table IV.

Supplement to synthetic medium	Growth
(1) Broth (A) 1 cc. in 20 cc.	vigorous
(2) Filtrate after HgCl_2 (equivalent 1 in 200)	nil
(3) Filtrate after lead acetate (equivalent 1 in 200)	vigorous
(4) Filtrate after phosphotungstic acid (equivalent 1 in 200)	vigorous

Thus in two out of the three cases the active substance was still in the filtrate. Possibly one is not justified in assuming that a chemical separation could take place in any such precipitations, as the actual quantity of the factor present is probably so small that the conditions of adsorption on the flocculent precipitate may be the determining factor. However it seems unlikely that the inactivity of the filtrate after the HgCl_2 is due to the bactericidal action of traces of mercury in the solution as experiments have shown that concentrations of HgCl_2 less than 0.000025 % (25 γ) do not cause inhibition.

The growth-promoter may have been precipitated by the HgCl_2 , but tests of the precipitate failed to demonstrate this (see also the effect of HgCl_2 on torulin, Kinnersley and Peters [1925]). Possibly the factor was adsorbed on the mercuric sulphide precipitate, but so far it has never been recovered from this.

Other sources of this bacterial growth-promoting factor.

Aqueous extracts of the following animal and vegetable substances were prepared and tested to find what weight of raw material was necessary to give growth equal to 1 g. beef tissue (1 in 200 dilution being used as control).

	g.
Ox heart	1.0
Rabbit muscle	1.5
Serum	5.0
Yeast	0.2
Apple	No effect
Wheat germ	0.25
Crude thyroid gland	10.0
Purified thyroid	No effect
Impure glucose	10.0
Glucose	No effect

To ascertain whether a concentrated extract of yeast would have a greater growth-promoting effect than marmite, a sample of freshly prepared torulin concentrate [Kinnersley and Peters, 1925] was tested in dilutions of 1 pigeon day dose in 200 cc. to 1 in 20,000,000 cc. of inorganic-glucose medium. Even in concentrations of 1 in 2,000,000 a fair growth-promoting effect was obtained for *S. corallinus*. Since this preparation had an activity of 1/10 mg. per day dose the actual concentration of solid matter added was 5×10^{-7} mg. per 100 cc. To get the same degree of growth it is necessary to add 0.7 mg. weight of broth solids per 100 cc.

Effect of p_H on the activity of the factor.

In Table I it was shown that broth could be kept at p_H 6.5 for 3 months without loss of activity. Experiments were now arranged in which sufficient NaOH was added to two portions of broth to make them 1/10 *N*. One was kept at room temperature (18° approx.) for 3 months and the other was boiled for 1 hour. In both cases the activity was decreased to 10 % of its former value. A similar experiment with marmite revealed the same result. However with a much purer product the activity remained practically constant even when boiled with *N*/4 NaOH for 60 minutes (see Table V). The extract used in this experiment had an activity for the pigeon of 0.2 mg. *per diem*.

Table V.

	Volume of solution necessary to give growth equal to 0.5 cc. broth
1. Control (xxx)	0.050
2. Control boiled <i>N</i> /200 NaOH for 5 mins.	0.050
3. Control boiled <i>N</i> /40 NaOH for 5 mins.	0.045
4. Control boiled <i>N</i> /4 NaOH for 5 mins.	0.045
5. Control boiled <i>N</i> /200 NaOH for 60 mins.	0.045
6. Control boiled <i>N</i> /40 NaOH for 60 mins.	0.045
7. Control boiled <i>N</i> /4 NaOH for 60 mins.	0.045
8. Control autoclaved $p_H=10$ for 30 mins. at two atmospheres	0.050
9. Control autoclaved $p_H=7$ for 30 mins. at two atmospheres	0.050
10. Control autoclaved $p_H=2$ for 30 mins. at two atmospheres	0.050

Thus it would appear that the bacterial growth-promoting property of the torulin concentrate is stable to alkali. The only other possibility is that some inhibitory substance was formed by the action of NaOH on the more crude broth and yeast, thereby masking the growth-promoting activity. That in this property of stability to alkali the bacterial growth-promoting factor for *S. corallinus* differs from the antineuritic vitamin will be considered again in an accompanying paper [Peters, Kinnersley, Orr-Ewing and Reader, 1928].

SUMMARY.

From these results it may be concluded that the substance in tryptic beef broth that exerts a stimulating action on the growth of *S. corallinus* is organic, water-soluble, and dialysable and is not precipitated by either neutral or basic lead acetate. It is also present in yeast preparations, rabbit muscle, serum, and wheat embryo. In the fractionation of the antineuritic vitamin from yeast this bacterial growth-promoting factor is associated with the vitamin up to the 1/10 mg. per pigeon day dose stage, *i.e.* the addition of 1×10^{-7} mg. of this preparation in 20 cc. of medium increases the growth 200 times. To what extent Funk and Dubin [1920] were justified in claiming to estimate quantitatively the presence of vitamin B by growth of micro-organisms other than yeasts will be considered in the next paper [Orr-Ewing and Reader, 1928].

In conclusion the author takes this opportunity of thanking Profs. J. C. Drummond and R. A. Peters for their advice and criticism and the Medical Research Council for a grant.

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