RELATIONSHIP OF A NEW GROWTH FACTOR REQUIRED BY CERTAIN HEMOLYTIC STREPTOCOCCI TO GROWTH PHENOMENA IN OTHER BACTERIA

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In 1941 the discovery of a new growth factor required by certain hemolytic streptococci was described in this laboratory (1). Grossowicz (2) shortly afterward was able to confirm the existence of this substance. Since that time new growth factors, distinct from the known vitamins, have been shown to stimulate growth of several types of bacteria.

Pollack and Lindner (3) showed that a substance present in peptone was necessary for growth of *Lactobacillus casei* during the early part of the incubation period, but was not essential for growth during prolonged incubation. Smith (4) showed that certain strains of *Streptococcus lactis* were made to grow better by a substance in yeast and in peptone. Kuiken *et al.* (5) observed an unknown substance in tomato juice which was needed for maximal growth of *Lactobacillus arabinosus*. Chattaway *et al.* (6) recognized a growth-promoting substance for the diphtheria bacillus, and Thompson (7) showed that certain propionic acid bacteria were unable to grow satisfactorily unless an unknown material derived from natural products was supplied. Furthermore, it was observed some years ago in the course of the work on the factor for hemolytic streptococci, that while streptococci of group A failed to grow in the absence of the factor, those of group D eventually grew well in a synthetic medium (8), but only after a lag phase of about 24 hours. When concentrates of the factor required by group A organisms were added to the medium for the group D organisms the lag phase was shortened to the usual few hours. When the growth of the group D organisms was measured after 20 hours, it was found that concentrates prepared by different procedures had the same relative potency for organisms of group A as for those of group D. The few properties of the substances described by these various authors as well as the modes of preparation of active concentrates were quite similar to those which Woolley (1) had described for the streptococcal growth factor.

These facts suggested that the same substance was responsible for the stimulatory effects noted on all of the organisms.

In order to study the relationship of these new growth factors a series of concentrates of the hemolytic streptococcus factor was prepared by a number of quite dissimilar methods. These preparations were then assayed quantitatively as growth factors for hemolytic streptococcus ×40, *Lactobacillus casei*,

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and *Streptococcus lactis* of Smith. In order to establish the identity of the growth factors for all of these bacteria it would not be enough to show that concentrates of the factor were active for all, because the concentrates might well contain more than one physiologically active compound. However, if the ratios of the activities of concentrates prepared by widely differing procedures to the activity of the original material were the same for all organisms, it would seem probable that the same, or closely related, substances were involved.

EXPERIMENTAL

The procedure for assay with hemolytic streptococcus ×40 was the same described previously (1) except that folic acid (0.0002 gamma per cc.) as a concentrate (9) and *p*-aminobenzoic acid (0.001 gamma per cc.) were added to the basal medium, and the sugar content was raised to 0.4 per cent. For the assays with *Lactobacillus casei* and with Smith's *Streptococcus lactis* the complete medium of Landy and Dicken (10) was used. This was fortified with 10 gamma of $MnCl_2 \cdot 4H_2O$ per cc. in order to avoid complicating the results of the test by manganese deficiency (11). The cells for the inoculum were grown in Todd-Hewitt broth (12), and were prepared for use by washing 3 times with phosphate buffer (pH 6.8) and diluting so that the suspension had 50 times the volume of the original culture. 1 drop of cell suspension was used to inoculate each assay tube. Each tube contained a total volume of 10 cc. A series of tubes containing graded amounts of solubilized liver extract (Wilson's fraction L)[1] ranging from 0.1 to 1.0 mg. of extract per cc. was taken as the standard, and was set up with each run. It was important to prepare fresh standard solution of solubilized liver extract from dry material at frequent intervals since the active substance lost potency when stored in solution. Graded amounts of the preparations to be assayed were added to a series of tubes as in the usual procedure in microbiological assays. Prior to assay, all concentrates were diluted in such wise that 1 cc. was derived from 10 mg. of solubilized liver extract. Similarly, the standard solution was made to a concentration of 10 mg. per cc. The potency of each fraction, as determined with all three organisms, was expressed in terms of the per cent of the activity of the same weight of the solubilized liver extract from which the fraction was derived. The tubes were incubated at 37° (30° for *Streptococcus lactis*) for 18 hours, and the determination was completed turbidimetrically in the usual fashion (13).

Under the conditions described, no growth occurred with *Lactobacillus casei* in the basal medium, while in the basal medium plus 1 mg. of solubilized liver extract per cc. maximal growth occurred. Typical responses in growth to graded amounts of the standard liver extract are shown by the data in Table I. If incubation was continued for 50 to 70 hours, good growth eventually took place in the basal medium. After 70 hours of incubation all tubes, whether supplemented or not, showed maximal growth and acid production. This state of affairs was similar to that observed by Pollack and Lindner (3) and to that noted earlier with group D hemolytic streptococci (see above). With hemolytic streptococcus ×40, a group A organism, no growth occurred in the basal medium on continued incubation. Smith's *Streptococcus lactis* behaved as did *Lactobacillus casei* in this respect except that with *Streptococcus lactis* some growth occurred in the unsupplemented basal medium during the first 18 hours. Hence *Streptococcus lactis* was less desirable as a test organism.

The concentrates to be assayed were prepared in the following manner. They are listed in Table II along with their activities as determined with various organisms.

1. Dialysis.—Solubilized liver extract was dissolved in water and dialyzed in a cellophane tube against running water for 24 hours. The non-dialyzable portion was tested.

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[1] We wish to thank Dr. David Klein of the Wilson Laboratories, Chicago, for gifts of this material.

2. Lead Acetate-Norit Separation.—Solubilized liver extract was dissolved in water and treated with excess lead acetate. The soluble portion was freed of lead with H_2S, and the filtrate from PbS was acidified to pH 3, stirred with an amount of norit equal in weight to that of the liver extract used, and filtered. The filtrate was neutralized and assayed.

3. Silver Precipitation.—Preparation 2 above was treated with excess silver sulfate, followed by excess barium hydroxide. The filtrate was freed of reagents and tested.

TABLE I

Response of Lactobacillus casei to Graded Amounts of Solubilized Liver Extract

Amount added	Colorimeter reading*
mg. per cc.	
0	100
0.1	77
0.2	66
0.3	56
0.6	47
1.0	40

* Colorimeter readings represent the per cent of incident light transmitted by the cultures. Transmission is decreased as the growth becomes more dense, and is reported in relation to the transmission through an uninoculated tube taken as 100 per cent.

TABLE II

Relative Potencies of Concentrates As Growth Factors for Bacteria

Fraction prepared by	Recovered activity for		
	×40	*L. casei*	*S. lactis*
	per cent	*per cent*	*per cent*
1. Dialysis	54	54	50
2. Lead acetate-norit separation	40	39	—
3. Silver precipitation	27	—	26
4. Separation with solvents	50	52	—
5. Alcoholic butylamine extraction	25	27	21
6. Neuberg precipitation	13	17	—
7. Partial hydrolysis of casein	17	19	18

4. Separation with Solvents.—A lead acetate filtrate (see preparation 2) of solubilized liver extract was made to pH 1 with HCl and extracted 6 times with butanol. The aqueous residue was concentrated under reduced pressure to a 10 per cent solution and treated with 4 volumes of acetone. The precipitate was collected and tested.

5. Alcoholic Butylamine Extraction.—Finely powdered solubilized liver extract was extracted with 20 parts of a mixture of alcohol and *n*-butylamine (4:1). The extract was freed of solvents by evaporation under reduced pressure, and the resulting residue was extracted with butanol. The butanol residue was freed of butanol by evaporation and assayed. The active substance contained in this fraction was not soluble in alcohol, but was soluble in alcohol plus butylamine or ammonia.

6. Neuberg Precipitation.—Powdered solubilized liver extract was extracted with alcohol

plus sufficient HCl to give a final pH of 2. The extract was treated with acetone and the precipitate which resulted was dried and dissolved in alcohol. The alcoholic solution was treated with phosphotungstic acid and the phosphotungstic acid filtrate was decomposed by extraction with butanol-ether-water (1:1:2). The aqueous solution was then treated with sodium carbonate and mercuric acetate (Neuberg's reagent). The precipitate was decomposed with H_2S and the resulting filtrate was assayed.

7. *Partial Hydrolysis of Casein.*—Vitamin-free casein (Labco) suspended in 50 parts of N HCl was autoclaved (15 pounds pressure) for 1 hour. The solution was neutralized, filtered and assayed. Untreated casein was inactive as was also fully hydrolyzed casein.

DISCUSSION

From the data in Table II it can be seen that the active substance for all three organisms was recovered approximately to the same extent after the various concentration procedures. These facts can be taken to indicate that the same substance or closely allied substances were responsible for the effects. However, the identity of the growth factors cannot be established until the pure material has been isolated. As an aid to isolation the *Lactobacillus casei* test appeared to be preferable to the hemolytic streptococcus ×40 test previously used. The assay with the former organism required less time. Furthermore, this organism grew more luxuriantly and had the advantage of being non-pathogenic.

Several properties of the growth factor in addition to those previously described (1) may be deduced from the fractionations described above. Thus it can be seen that the growth factor was insoluble in alcohol, but soluble in either alcohol and HCl or alcohol and *n*-butylamine. It is probable, therefore, that the factor is amphoteric and was soluble in the acid or alkaline alcohol because its acidic or basic salts were soluble. The occurrence of the growth factor in partially hydrolyzed casein was puzzling in view of the fact that unhydrolyzed or fully hydrolyzed casein was inactive.

It is convenient to have a descriptive name for a new substance which is not as cumbersome as "a new growth factor for certain hemolytic streptococci," and not as lacking in specificity as, let us say, "norit filtrate factor." The name "strepogenin" is suggested for this new growth factor since its presence is necessary for streptococci of group A to generate. This name may suffice until more systematic nomenclature is possible.

SUMMARY

By a series of quantitative assays of concentrates prepared in a variety of ways, it has been shown that the previously described growth factor now called "strepogenin," required by certain hemolytic streptococci, is very similar to and possibly identical with the recently described growth factors for *Lactobacillus casei* and Smith's *Streptococcus lactis*. An improved procedure for assay of strepogenin with the aid of *Lactobacillus casei* has been described, and additional properties of the growth factor have been presented.

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