A NEW GROWTH FACTOR REQUIRED BY CERTAIN HEMOLYTIC STREPTOCOCCI

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Studies on the factors essential for the growth of certain hemolytic streptococci have been in progress for some time. Woolley and Hutchings (1) were able to cultivate on a medium containing only chemically pure substances organisms that belonged to groups B and D of Lancefield's classification. Similar results were subsequently reported by other workers (2). Woolley and Hutchings further demonstrated that organisms of Lancefield's groups A, C, and E would not multiply on similar media. The present work will demonstrate that certain organisms of Lancefield's group A hemolytic streptococci require a factor, hitherto undescribed, for their multiplication on a chemically defined medium. The purification of this substance, as well as many of its properties, will be described below.

Materials and Methods

Organisms Used.—For purposes of routine assay, hemolytic streptococcus strain X 40, originating from the American Type Culture Collection, was employed. Stock cultures were maintained on blood broth.

Basal Medium and Assay Technique.—The basal medium contained the substances listed in Table I. Aliquots of solutions to be tested were added at the expense of the water so that the final volume in every case was 10 cc. The pH was adjusted to 7.4. All constituents except sodium thioglycollate and glutamine were mixed and sterilized in an autoclave; these two were added as a sterile solution just prior to inoculation.

Each tube was inoculated with 1 drop (0.05 cc.) of an inoculum prepared in the following manner. The cells from an 8 hour culture of the streptococcus in Todd-Hewitt broth (4) were collected by centrifugation and resuspended in an equal volume of sterile phosphate buffer. After the tubes had been incubated for 40 hours at 37°C., the turbidity of each tube was quantitatively determined as previously described (1). In order to check the results thus obtained, the acid produced in each tube was titrated with N·0.1 sodium hydroxide to the brom thymol blue end-point. In addition, plate counts of the number of organisms were occasionally made. No growth or acid production occurred in those tubes containing the basal medium. Maximal growth accompanied by maximal acid production occurred in tubes that had been supplemented with 1 mg. per cc. of the standard liver preparation (fraction A) described below. Representative data are shown in Table II. Plate counts also indicated that good growth had occurred in those tubes which became acid and appeared turbid. A "maximal unit"

(M.U.) was defined as the quantity of material that must be added per cc. of medium in order to produce maximal growth and acidity. The M.U. value of each fraction examined was determined by comparison of the response elicited by the addition of various quantities of the fractions. In general, a unit based on maximal response is not as precise as one based on half maximal response. However, in the case of the group A streptococci the maximal response has proven easier to determine than half maximum. In each series of assay tubes several dilutions of a standard liver extract were included to serve as reference points of activity.

Sources of the Growth Factor.—Several liver extracts were assayed for relative potencies. The alcohol-soluble portion of aqueous liver extract was found to be ineffective.

TABLE I

Basal Medium

Acid hydrolyzed casein	20 mg.
Tryptophane	0.5 "
Glucose	20 "
K_2HPO_4	40 "
NaCl	20 "
$MgSO_4 \cdot 7\ H_2O$	0.8 "
$FeSO_4 \cdot 7\ H_2O$	40 gamma
$MnCl_2$	12 "
Thiamin	5 "
Riboflavin	10 "
Vitamin B_6 hydrochloride	10 "
Sodium pantothenate	10 "
Adenine	50 "
Nicotinic acid	50 "
Choline chloride	50 "
Inositol	200 "
Uracil	50 "
Pimelic acid	50 "
Biotin concentrate (3)	0.5 "
Sodium thioglycollate	1 mg.
Glutamine	50 gamma
Water	to make 10 cc.

The most convenient source was that fraction of aqueous liver extract which was insoluble in 70 per cent alcohol (1 M.U. in 1 mg.). This fraction will be subsequently designated as "fraction A".[1] Yeast extract (Difco) and rice bran extract (vitab) (1 M.U. in 4 mg. and 2 mg. respectively) were also good sources of the active substance. The fact that a deficiency of a known amino acid was not involved was shown by increasing the casein hydrolysate to 1 per cent of the medium; when this was done no growth occurred, unless an active concentrate was also included.

Preliminary Concentration.—It was found that when fraction A was dissolved in a minimum of water and made strongly alkaline with barium hydroxide, the precipitate

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

after decomposition with sulfuric acid contained the active agent. Further purification could be achieved by treating the resulting solution with lead acetate. When from each fraction lead was removed with H_2S, it was found that the activity resided in the filtrate fraction. The active substance was further purified by precipitation with acetone.

Dialyzability of the Active Substance.—At this point in the investigation it was found that the active substance in fraction A was not readily dialyzable, but that certain more purified concentrates contained the active compound in dialyzable form. This finding necessitated a reinvestigation of the many techniques of fractionation which had been used, for it was realized that the dialyzable and the non-dialyzable substances differed in their physical and chemical properties. For example, the non-dialyzable compound was precipitated from aqueous solution by barium hydroxide, whereas the dialyzable substance under similar conditions was not precipitated. The activity residing in fraction A was not rendered dialyzable by standing in N sodium hydroxide or N mineral acid or by heating. The best method for conversion of the non-dialyzable form to its

TABLE II

Responses of Hemolytic Streptococcus Strain X 40 to Graded Amounts of a Liver Fraction*

Addition of fraction A to basal medium	Colorimeter reading	0.1 N acid
mg. per cc.		*cc. per 10 cc.*
None	100	0
0.1	92	0.15
0.5	89	1.0
1.0	78	1.7
2.0	78	1.7

* American Type Culture Collection.

dialyzable component was the following. 100 gm. of finely powdered fraction A were stirred for 1 hour with 1 liter of alcohol and 20 cc. of concentrated HCl. The insoluble matter was filtered off and washed with alcohol, and the solvent was removed from the filtrate under reduced pressure. The filtrate contained all of the active agent (1 M.U. in 500 gamma) and in a dialyzable form.

Purification Technique.—A typical run will be described to indicate the procedure that has given the most active concentrates.

100 gm. of fraction A were dissolved in 200 cc. of water and dialyzed in a cellophane tube (Visking) for 18 hours against running water. The non-dialyzable portion was treated with saturated lead acetate until no more precipitate formed. Lead was removed from the resulting filtrate with H_2S, and the filtrate from the PbS was concentrated under reduced pressure to about 200 cc. This solution was made alkaline to phenolphthalein with a saturated methanol solution of barium hydroxide. Care was exercised not to use too large an excess of barium for, as will be shown later, the active substance was tenaciously adsorbed by barium sulfate. The precipitate of barium salts was removed by filtration and decomposed by trituration with excess sulfuric acid. The filtrate from the barium sulfate was then treated with saturated lead acetate until no more precipitate formed. The resulting filtrate was freed of lead with H_2S, the lead sulfide was removed by filtration, and the filtrate was concentrated under reduced

pressure to dryness. Various runs have yielded 300 to 800 mg. of this concentrate per 100 gm. of fraction A. Most of the preparations thus obtained contained 1 M.U. in 10 gamma. However, certain preparations have been almost inactive, and this failure has been traced to adsorption by barium sulfate. The active substance and practically all of the solids of concentrates prepared in this manner were readily dialyzable. Thus, when 180 mg. were dialyzed against 8 changes of distilled water, only 5 mg. did not dialyze, and all of the activity resided in the dialyzable portion.

Adsorption of the Growth Factor.—The active substance in the non-dialyzable portion of fraction A was adsorbed on norit only with difficulty. A fractionation procedure designed to exploit this fact has not yielded concentrates more active than those prepared by other means; but a brief description may be advantageous in that it will illustrate certain properties of the growth factor. When the non-dialyzable portion of 100 gm. of fraction A was diluted to 2 liters and adjusted to pH 1 with HCl, the activity was not invalidated by adsorption with 40 gm. of norit. The filtrate from this adsorption was then stirred with 150 gm. of norit and the resulting filtrate was found to contain only 10 per cent of the original potency. The norit was stirred with dilute aqueous ammonia and filtered. The filtrate was inactive. Activity could then be regained from the norit by 3 elutions with 70 per cent alcohol containing 5 per cent concentrated aqueous ammonia. The latter eluate contained approximately 7 gm. of solids and had about 50 per cent of the original potency. Elution could also be accomplished by stirring with alcoholic HCl.

It has not been possible to adsorb the active material from any concentrate with Fuller's earth (Lloyd's reagent), which statement holds true in respect to both the dialyzable and the non-dialyzable forms.

Barium sulfate adsorbed the active compound tenaciously. For example, when the non-dialyzable portion of 100 gm. of fraction A was treated with a solution of 50 gm. of barium hydroxide and then enough sulfuric acid was added to remove all the barium, the filtrate from the barium sulfate was inactive. Attempts to elute the growth factor with alcoholic ammonia or alcoholic HCl have not been successful.

Miscellaneous Properties of the Growth Factor

It may be of use to other workers in the field to describe some of the properties of the active substance that have been observed, without, however, giving minute details of the experiments demonstrating these properties. Most of the properties have not as yet been exploited to bring about marked concentration of the active substance, but many of them have diagnostic value in differentiating this new growth factor from other growth factors that have previously been described. While the dialyzable form was soluble in alcoholic HCl, it was precipitated from alcohol solution when neutralized. Both the dialyzable and the non-dialyzable forms were not destroyed by nitrous acid or by hot acid or alkali. Even concentrated HCl at 100° did not readily destroy the substance. Both forms were precipitated from aqueous solution by phosphotungstic acid, but much loss

of activity was encountered when this reagent was used. The dialyzable form was not precipitated from alcoholic or aqueous solution by mercury salts. Butyl alcohol did not readily extract the substance from aqueous solution. The growth factor was not soluble in acetone, chloroform, or ethyl acetate. Water seemed to be the only solvent that was effective at all pH values. The non-dialyzable form was not precipitated when its aqueous solution was half saturated with ammonium sulfate, but it was precipitated from saturated ammonium sulfate solution; it was not precipitated from saturated magnesium sulfate.

Relation to Other Growth Factors

It will be observed that the basal medium used contained those substances available in pure form which have been shown to act as growth factors for microorganisms. It is not possible to establish definitely a relationship to factors not yet available in pure form. However, the properties of the growth factor differ in many respects from those described for other unidentified growth substances. Thus, the factor for yeast described by Alexander and SubbaRow (5) was soluble in many organic solvents. The factor described by Snell and Peterson (6) for their lactic acid bacteria was readily adsorbed on norit and lead sulfide. In addition, through the kindness of Dr. Snell, it has been possible to test a sample of his active picrate, which was found to be inactive for the organism used in the present experiments. The factors studied by Moller (7) have not been described in detail and hence a relationship cannot be discussed. This statement is true also of several other extracts which have been shown to increase the growth of certain microorganisms.

Pappenheimer and Hottle (8) and McIlwain (2) have reported growth of certain group A hemolytic streptococci on media of known composition. It will be noted that our basal medium contained all the substances used by Pappenheimer and Hottle, except that casein hydrolysate, rather than gelatin hydrolysate, was used. The substances described by McIlwain have proved inactive in our assay procedure.

It is possible that the active concentrates contain more than one essential growth factor. Many fractionation procedures which result in a poor yield of activity could be explained on this basis. It is more probable, however, that the poor yields are attributable to destruction of the active substance during the procedure, for in every case it has been a standard practice to assay the fractions in combination as well as singly. In no case has evidence for a multiple nature of the growth factor been found.

Effect on the Growth of Other Strains and Species

Members of Lancefield's group A hemolytic streptococci other than strain X 40 have been tested in an attempt to learn whether the new growth factor is essential for this group of microorganisms as a whole. Strains S 43, C 203, and 594 were observed not to grow in the basal medium, but to develop satisfactorily in the basal medium plus the most active concentrate. An indication that other species of bacteria require the growth factor was found when it was observed that pneumococcus D 39 R behaved in a manner similar to the streptococci. The rate of growth of yeast in the medium used by Eastcott (9) was markedly increased by additions of the active concentrates.

SUMMARY

A new factor essential for the growth of hemolytic streptococci of Lancefield's group A has been demonstrated. It has not been possible to identify this factor with any other known growth factor. The active substance occurred in liver as a water-soluble, alcohol-insoluble, non-dialyzable material. It could be rendered dialyzable by treatment in strong alcohol with HCl, but not by many less drastic procedures.

The most active concentrates prepared gave a maximal effect when approximately 10 gamma per cc. were added.

BIBLIOGRAPHY

1. Woolley, D. W., and Hutchings, B. L., *J. Bact.*, 1940, **39**, 287.
2. McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 25.
3. Woolley, D. W., McDaniel, L. E., and Peterson, W. H., *J. Biol. Chem.*, 1939, **131**, 381.
4. Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35**, 973.
5. Alexander, B., and SubbaRow, Y., *J. Biol. Chem.*, 1940, **135**, 341.
6. Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 273.
7. Moller, E. E., *Z. physiol. Chem.*, 1939, **260**, 246.
8. Pappenheimer, A. M., Jr., and Hottle, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 645.
9. Eastcott, E. V., *J. Physic. Chem.*, 1928, **32**, 1096.