SYNTHETIC MEDIA FOR CULTURE OF CERTAIN HEMOLYTIC STREPTOCOCCI[1]

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Hemolytic streptococci are usually believed to be organisms of rather exacting requirements, and are invariably grown in media which contain tissue extracts. For some time attempts have been made in this laboratory to grow these organisms in media of known composition. It was hoped that this study would not only add to the general knowledge on growth requirements of living cells, but also would be of aid to those who investigate the immunological and metabolic reactions of hemolytic streptococci. It has recently been shown (Woolley and Hutchings, 1939) that a wide variety of these bacteria require riboflavin, pantothenic acid, and a suitable reducing compound. The present work describes the cultivation of some hemolytic streptococci in media in which every constituent was supplied as a pure compound.

EXPERIMENTAL PART

Cultures and assay technique

The organism used in this study, unless otherwise stated, was one of the Lancefield group D, namely *Streptococcus zymogenes* strain H-6905. This was used in preference to other members of the group because it had been noted (Woolley and Hutchings, 1939) that this strain grew more luxuriantly and appeared to require fewer growth factors than most other hemolytic streptococci tested. The assay method was the same as that previously

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described by us (1939). Hence, the colorimeter readings in the tables indicate the per cent of incident light transmitted by the cultures; a large reading denotes poor growth.

The assay tubes were inoculated by dipping a fine wire needle into a 12-hour culture of the organism in veal infusion, and then dipping this needle into the medium under examination. This method proved reliable and much more rapid than inoculation from colonies picked from plates and suspended in sterile water. The growth period allowed was 72 hours, since turbidity did not increase beyond this time. The tubes were also examined quantitatively at 38 hours.

It should be pointed out that because of unknown variation in the inoculum used at different times, the maximum turbidity reading obtained with the same medium at different times was not the same. This maximum varied between the values of 10 and 30. For this reason, all readings recorded in any one table, except when separated by a horizontal line, represent values obtained with the same inoculum.

Growth factors required

The basal medium was composed of acid-hydrolyzed, vitamin-free casein 0.2 per cent, tryptophane 0.01 per cent, glucose 0.1 per cent, salts,[2] riboflavin 1 microgram per ml., "reduced" iron (Merck's finely-divided "iron by reduction") 400 micrograms per ml., and a highly-purified pantothenic acid concentrate which supplied 0.1 microgram of pantothenic acid per ml. (compare Woolley and Hutchings, 1939). The medium was adjusted to pH 7.4 and autoclaved for 15 minutes at 15 pounds pressure. In this mixture negligible growth occurred. Luxuriant growth invariably resulted when the alcohol-insoluble portion of aqueous liver extract[3] was added. Yeast extract was also effective, but not as good as the liver fraction.

[2] The salt mixture supplied the following materials and amounts per ml. of medium. NaCl 5 mgm., $MgSO_4 \cdot 7H_2O$ 0.1 mgm., Na_2SO_4 0.2 mgm., KH_2PO_4 1 mgm.

[3] This material was the portion of an aqueous liver extract which was precipitated when the extract was treated with enough alcohol to produce a concentration of 70 per cent. The precipitate was then rendered completely water soluble by a mild enzyme treatment. We wish to thank Dr. David Klein of the Wilson Laboratories for generous gifts of this substance.

Fractionation of the liver concentrate was undertaken in an attempt to isolate the substance required for growth. It was found that the active material was precipitated by lead acetate, but that it was adsorbed by the lead sulfide when the precipitate was decomposed with hydrogen sulfide. It could be eluted from the lead sufide by refluxing with a mixture of alcohol and and dilute ammonia. The factor could then be adsorbed from the acidified eluate by repeated treatment with fuller's earth, and it could be eluted from the latter with a mixture of pyridine (1 volume) and 80 per cent alcohol (25 volumes). The resulting eluate promoted good growth when 1 microgram was added per ml. of medium.

Effect of vitamin B_6

These properties of the factor, especially the behavior with fuller's earth, suggested trials of various nitrogenous substances which have been shown to stimulate the growth of other organisms. Nicotinic acid, uracil, thiamin, and vitamin B_6[4] in all possible combinations were tried, and it was found that vitamin B_6 was effective. A detailed description of the fractionation procedures has been omitted, since their chief value was to suggest the possible relation of the factor to the basic vitamins.

Amino acid requirements

Shortly before the effect of vitamin B_6 was discovered, it was found that the casein hydrolysate could be replaced by a mixture of purified amino acids. This mixture was compounded to simulate the composition of casein hydrolysate, and supplied the following amounts of amino acids per 10 ml. of medium: glycine 0.1 mgm., dl-alanine 0.4 mgm., dl-valine 1.6 mgm., dl-leucine 1.0 mgm., dl-isoleucine 1.0 mgm., dl-serine 0.2 mgm., l-proline 1.0 mgm., l-hydroxyproline 0.1 mgm., dl-phenylalanine 1.0 mgm., l-tyrosine 0.7 mgm., l-cystine 0.1 mgm., d-arginine hydrochloride 0.8 mgm., l-histidine hydrochloride 0.4 mgm., dl-lysine dihydrochloride 2.0 mgm., l-tryptophane 0.3 mgm., dl-methionine 0.8 mgm., l-aspartic acid 0.5 mgm., d-glutamic acid 2.5 mgm., and

[4] We wish to thank Dr. S. Lepkovsky for generous gifts of crystalline vitamin B_6.

dl-threonine 0.6 mgm. (The dl acids were added in twice their concentration in casein hydrolysate.) The amino acids were purified materials obtained from the following sources. The dl acids were all synthetic, recrystallized products purchased or made in this laboratory. The optically active ones were C.P. grade, purchased from the Eastman Kodak Company, or isolated in analytically pure condition in this laboratory.

It can be seen from table 1 that the mixture of amino acids (plus the other constituents of the basal medium except casein hydrolysate) supported negligible growth, while the amino acids

TABLE 1

Effect of vitamin B_6 in purified media

MEDIUM	COLORIMETER READING	
	After 38 hours	After 72 hours
(1) Basal	100	100
(2) Basal + 19 amino acids	94	74
(3) Basal + amino acids + B_6 (0.5 micrograms per ml.)	82	18
(4) Basal + amino acids as in (11), table 3	98	94
(5) (4) + B_6, 2 micrograms per ml	86	30
(6) (4) + B_6, 0.5 micrograms per ml	90	30
(7) (4) + B_6, 0.05 micrograms per ml	92	39
(8) (4) + B_6, 0.0001 micrograms per ml	94	80

Basal composed of glucose, inorganic salts, pantothenic acid, riboflavin, and reduced iron as described in text. Concentration of amino acids as described in text.

plus vitamin B_6 enabled the organisms to grow luxuriantly. Two consecutive transfers in this synthetic medium showed that the organisms would still grow well.

With the above medium which contained only highly purified materials, it was possible to study the essential nature of various amino acids. A series of experiments were performed in which each amino acid in turn was withheld from the mixture of nineteen. In this way, the essentiality of each amino acid was tested in the presence of the other eighteen. By this method it was demonstrated that no growth would occur in the absence of glu-

tamic acid or of tryptophane (table 2). The essential nature of glutamic acid was interesting in view of the finding that glutamine is a growth factor for certain hemolytic streptococci (McIlwain, *et al.*, 1939).

When only glutamic acid and tryptophane were supplied, no growth occurred. Addition of ammonium sulfate or of asparagine to correct a possible nitrogen deficiency of the medium did

TABLE 2

Effect of omitting one amino acid

AMINO ACID OMITTED	COLORIMETER READING AFTER 72 HOURS
Glycine	10
Alanine	10
Valine	10
Leucine	10
Isoleucine	10
Serine	10
Threonine	10
Proline	10
Hydroxy proline	10
Phenylalanine	10
Tyrosine	12
Cystine	9
Methionine	8
Aspartic acid	12
Glutamic acid	100
Tryptophane	88
Arginine	12
Histidine	10
Lysine	10

Basal same as in table 1, except vitamin B_6 (0.5 microgram/ml.) and eighteen amino acids added. Concentrations of amino acids as in table 1.

not make growth possible. A large number of experiments were then performed in an effort to discover the additional necessary amino acids. These experiments will not be enumerated, but only the mixture of acids which proved effective will be listed. The addition of any one or any pair of the seventeen other acids to tryptophane and glutamic acid, in the presence or absence of asparagine, gave negative results. A mixture of isoleucine,

lysine, arginine, cystine and tyrosine in connection with glutamic acid and tryptophane proved to be entirely sufficient. A few representative data are shown in table 3. No additional nitrogen in the form of ammonium sulfate or asparagine was required.

With the aid of this simplified amino acid mixture, an attempt was made to learn the response of the organisms to different levels of vitamin B_6. The simplified mixture was used in preference to all nineteen acids because the slight growth obtained in the absence of B_6 with the latter mixture was practically eliminated with the former. This fact suggested that some of the amino acids probably contained traces of the growth factor (Hutchings and Woolley, 1939). The data in table 1 show that maximum effect was obtained with approximately 0.05 microgram of vitamin B_6 per ml. The vitamin could be detected even at 0.0001 microgram per ml. When this quantity is compared with the amounts of amino acids added, it can be realized that only slight contamina-

TABLE 3

Effect of amino acids in addition to tryptophane and glutamic acid

ADDITION TO BASAL	COLORIMETER READING	
	After 38 hours	After 72 hours
(1) None	98	98
(2) Asparagine	98	98
(3) Isoleucine + cystine	93	82
(4) Lysine + cystine	100	94
(5) Arginine + cystine	96	94
(6) Lysine + arginine + cystine	96	94
(7) Isoleucine + lysine + cystine	100	98
(8) Isoleucine + lysine + arginine	96	96
(9) Isoleucine + arginine + cystine	100	96
(10) Isoleucine + arginine + cystine + lysine	92	58
(11) Isoleucine + arginine + cystine + lysine + tyrosine	84	20
(12) 17 Amino acids	86	31
(13) 11 + aspartic acid + histidine + glycine + proline + hydroxy proline	48	24

Basal same as in table 1 except vitamin B_6 (0.5 micrograms per ml.), tryptophane and glutamic acid added. Concentrations of amino acids as in table 1.

tion of any one with the vitamin would be sufficient to cause growth in the basal medium. Apparently, the acids in the simplified mixture were free of vitamin B_6.

The inhibitory as well as the stimulatory effect of some amino acids was noted. This effect applied particularly to the lag phase of growth. As a routine matter, all tubes were read at 38 hours as well as at 72. It was noted that with the mixture of nineteen amino acids, growth did not begin until after 38 hours incubation. However, with only tryptophane, glutamic acid, aspartic acid, lysine, histidine, arginine, isoleucine, proline, hydroxy proline, tyrosine, and glycine, good growth was obtained at 38 hours. This difference applied only to the rate, and not to the extent of growth, for after 72 hours, the turbidity in all tubes was the same (see table 3). Since practically all the amino acids in the mixture which permitted rapid growth were optically active, it may be that the unnatural isomers present in the mixture of 19 acids retarded growth. This hypothesis has not yet been tested experimentally.

Sulfur requirements

In connection with the experiments on amino acid requirements it was of interest to determine if a sulfur-containing amino acid was required, or if inorganic sulfate or sulfide could meet the needs of the organisms. To settle this question, a basal medium containing inorganic salts, glucose, riboflavin, pantothenic acid, reduced iron, vitamin B_6, tryptophane, glutamic acid, arginine, lysine, isoleucine, and tyrosine, as described above was used. Some indication had been found that the tyrosine, even though recrystallized, contained cystine. This was not surprising when it was recalled that these two amino acids are the water-insoluble ones, and are usually made by no more elaborate fractionation than direct crystallization from a suitable protein hydrolysate, followed by recrystallization from water. In order to remove any cystine from the tyrosine the latter was dissolved in a large excess of normal NaOH and heated at 100° for an hour. The solution was acidified with HCl, concentrated under reduced pressure to half its original volume, and adjusted to pH 7. The

tyrosine was crystallized by cooling the solution. By this procedure any cystine was decomposed, and the sulfur was eliminated as H_2S. In the basal medium poor growth occurred. It can be seen from table 4 that cystine, methionine, or sodium sulfide was effective in supporting growth. However, cystine appeared to meet the requirements of the organisms better than did methionine. These findings are similar to those reported for *Staphylococcus aureus* by Fildes and Richardson (1937). *Streptococcus zymogenes* does not seem to be quite as fastidious in sulfur requirements as *S. aureus*.

TABLE 4

Sulfur requirements

ADDITION TO BASAL	COLORIMETER READING	
	After 38 hours	After 72 hours
(1) None	86	72
(2) Cystine	82	36
(3) Methionine	84	54
(4) Sodium sulfide (100 micrograms per ml.)	82	32

Basal as described in text.

Replacement of the pantothenic acid concentrate with a synthetic product

Pure pantothenic acid has as yet not been obtained. The material used in this work was a concentrate made as previously described (Woolley and Hutchings, 1939). This concentrate was free of sulfur and of amino acids, and hence was adequate for the experiments on amino acid and sulfur requirements. It was possible, however, that it contained pimelic acid or nicotinic acid. In order to determine if pantothenic acid was the only compound in the concentrate which was required by the organisms, a synthetic substance was tried. This was N- (α, δ-dihydroxy valeryl)-β-alanine synthesized by Woolley, *et al.* (1939) and found by them to have some pantothenic acid activity. This substance completely replaced the pantothenic acid concentrate for growth in the purified medium. Thus it seems that *Streptococcus zymogenes* can be grown in a synthetic medium.

Growth of other hemolytic streptococci in chemically-defined media

Representatives of the Lancefield groups A, B, C, E and F were tested for their ability to grow on the synthetic medium which supported growth of *S. zymogenes* (group D). The strains used were the same as previously described (Woolley and Hutchings, 1939). It was found that only the group B (*Streptococcus mastitidis*) would grow (table 5). This was of interest in view of the fact that it was previously shown by us (1939) that organisms of groups B and D appeared to be somewhat less exacting in their requirements than did those of the other groups. It

TABLE 5

Growth of various hemolytic streptococci in synthetic media

MEDIUM	COLORIMETER READING OBTAINED AFTER 72 HOURS WITH				
	S. pyogenes	*S. mastitidis*	Group C Strain F132	Group E Strain K129	Group F Strain H-60R
(1) Basal	98	98	98	100	100
(2) (1) + 19 amino acids	100	98	100	98	100
(3) (2) + thiamin (1 microgram per ml.)	100	96	98	100	100
(4) (3) + vitamin B_6 (0.5 microgram per ml.)	100	72	100	98	98

Basal same as in table 1.

should be mentioned here that in the synthetic medium *S. zymogenes* failed completely to grow in the absence of pantothenic acid, whereas in the alkali-treated medium previously used some growth occurred without this factor. No growth was obtained in the synthetic medium when β-alanine was substituted for the pantothenic acid concentrate.

SUMMARY

The essential nature of vitamin B_6 for two species of hemolytic streptococci has been demonstrated. It has been shown that *Streptococcus zymogenes* will grow luxuriantly in a solution which contains only known pure chemicals. These necessary substances were found to be glucose, inorganic salts, isoleucine, lysine,

tyrosine, cystine (or methionine or inorganic sulfide), arginine, tryptophane, glutamic acid, riboflavin, pantothenic acid, "reduced" iron, and vitamin B_6. Strains belonging to the groups B and D of Lancefield could be grown successfully in synthetic medium but members of other Lancefield groups could not. In the absence of glutamic acid or of tryptophane, *Streptococcus zymogenes* failed to grow. In the presence of these two, but in the absence of all other amino acids, no growth was obtained. The simplest effective amino acid mixture was found to be tryptophane, glutamic acid, isoleucine, lysine, arginine, tyrosine and cystine.

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