THE NUTRITION OF STREPTOCOCCUS SALIVARIUS

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Received for publication September 30, 1942

Streptococci are exacting in their nutritive requirements and have always been grown in complex media containing extracts or digests of tissues, such as meat, plant materials, or yeast. Woolley and Hutchings (1940) were the first to succeed in developing a medium consisting of chemically defined components that would support the growth of hemolytic members of the Lancefield groups D and B. This work has been extended by Schuman and Farrell (1941) who showed that a similar medium, differing somewhat in amino acid content, would support growth of *Streptococcus fecalis*, a non-hemolytic group D streptococcus. Considerable additional progress has been made in the study of the nutritive needs of other hemolytic streptococci but, aside from the work of Schuman and Farrell, little attention has been directed toward the requirements of the various non-hemolytic streptococci.

The subject of the present study, *Streptococcus salivarius*, a member of the so-called viridans group, occurs in large numbers in the human throat and is sometimes obtained from pathologic specimens. It has been found in this laboratory that members of this species represent a remarkably homogeneous physiological group, especially marked by the apparently unique ability to synthesize a levan from sucrose and raffinose (Niven, Smiley and Sherman, 1941; Sherman, Niven and Smiley, 1943).

CULTURES AND TECHNIQUE

The strain used in the development of the synthetic medium was *Streptococcus salivarius* (S2OB). Later, other typical strains of this species, isolated from human throats and intestines, were used.

The medium under test was inoculated from a 24-hour yeast-extract tryptone glucose broth with a fine wire needle. In all cases where growth occurred, a second transfer was made into another tube of the same medium. In all critical tests at least five serial transfers were made at 24-hour intervals, each with a fine wire needle, in order to eliminate the possibility of carrying over unknown growth factors from the original inoculum. Whenever the medium was made up for a long period of time before use, it was heated in flowing steam for approximately 10 minutes just prior to inoculation. This was found to be highly desirable in order to avoid anomalous results.

Growth was estimated after 24 and 48 hours by measuring the developed turbidity with the aid of a photoelectric densitometer, a slight modification of that described by Stier, Arnold and Stannard (1934). The densitometer scale is such that the greater the reading, the more turbid is the culture. Each unit on the scale corresponded to about six micrograms of bacterial nitrogen per 10 ml. of

medium. All measurements were made in specially cleaned, new Kimball glass culture tubes of uniform diameter.

GROWTH FACTOR REQUIREMENTS

The basal medium used in the first experiments was composed of 0.5 per cent acid-hydrolyzed, vitamin-free casein,[1] 3 mg. per cent tryptophane, 0.6 per cent K_2HPO_4, 0.5 per cent glucose, 10 mg. per cent sodium thioglycollate, and salts; pH 7.5. The salt mixture of Woolley (1941) was used (see table 1). All ingredients were mixed together, distributed into culture tubes in 10 ml. quantities, and sterilized by autoclaving.

No perceptible growth occurred in the basal medium alone, but, when the known vitamins of the B-complex were included, luxuriant growth occurred within less than 24 hours; and could be successfully transferred serially for an indefinite number of times. By eliminating the growth factors one by one, it was found that five vitamins were indispensable: riboflavin, pantothenic acid, nicotinic acid, biotin, and thiamin.

The lack of need for pyridoxine (vitamin B_6) seems to be somewhat unique to this organism, and it would be desirable to know if this character holds true for the other species in the viridans group of streptococci. Woolley and Hutchings (1940) and Schuman and Farrell have reported that, in addition to riboflavin and pantothenic acid, pyridoxine is indispensable for the growth of the group D streptococci; also, McIlwain (1940) and Pappenheimer and Hottle (1940) showed that pyridoxine is essential for group A streptococci.

That the medium for *Streptococcus salivarius* was not being contaminated unintentionally with pyridoxine was demonstrated by the fact that it would not support the growth of a strain of *Streptococcus zymogenes*, but when pyridoxine was included prompt growth resulted. The growth of *Streptococcus salivarius* was not altered by the presence or absence of this vitamin.

On the other hand, the necessity of thiamin for *Streptococcus salivarius* should be noted. Neither thiamin nor nicotinic acid has been described as essential for other streptococci, except by Pappenheimer and Hottle (1940) for the culture of the group A streptococcus investigated by them. The need for biotin parallels the finding of Hottle, Lampen and Pappenheimer (1941) for group A streptococci.

Snell and Mitchell (1941) reported that the purine bases were important in the nutrition of the lactic acid bacteria, including *Streptococcus lactis*. Although *Streptococcus salivarius* would grow satisfactorily without the addition of these substances, it was found that the pyrimidine, uracil, greatly stimulated the rate

[1] A 10 per cent acid hydrolyzed casein stock solution was prepared by refluxing 100 g Labco vitamin-free casein in 8 N H_2SO_4 for 24 hours. The acid solution was neutralized to pH 3.0 with hot, saturated $Ba(OH)_2$, after which the $BaSO_4$ was filtered off. The filtrate was stirred with 20 g norit for 30 minutes and then filtered again by suction. Hot $Ba(OH)_2$ was further added to bring the pH to 5.0, and once again filtered. The filtrate was finally adjusted to pH 7.4 with NaOH and the volume adjusted to 1 liter. This was distributed into small lots and sterilized by autoclaving.

of growth and in most cases afforded a greater maximum growth. This was found to be particularly true when the casein hydrolysate was replaced with an amino acid mixture. When this was accomplished, growth was greatly retarded, usually requiring 48 hours before visible turbidity became evident, if uracil was omitted.

AMINO ACID REQUIREMENTS

After the growth factor requirements were established using the casein hydrolysate medium, it was found that the hydrolyzed casein could be replaced succesfully with a mixture of 19 amino acids. This afforded an opportunity to determine the essential amino acids for *Streptococcus salivarius*. However, it was soon realized that in this simplified medium 0.6 per cent phosphate buffer exerted an inhibiting effect upon the organism, growth oftentimes not being initiated. When the phosphate buffer was lowered to 0.4 per cent, prompt growth occurred but maximum growth was naturally reduced as compared to that obtained in the casein hydrolysate medium. That the buffering capacity of the medium limited further growth was indicated by the fact that the pH was usually lowered to about 4.2, the final pH attained by this organism (Safford, Sherman and Hodge, 1937).

By eliminating the amino acids, singly or in groups, from the medium it was soon learned that satisfactory growth could be obtained when only seven amino acids were present: glutamic acid, leucine, arginine, methionine or cystine, lysine, tyrosine, and iso-leucine. The growth was of approximately the same magnitude as when the 19 amino acids were present.

Thus, the simplest effective medium for satisfactory growth of *Streptococcus salivarius* contained five vitamins and seven amino acids, as shown in table 1. Growth could be maintained in this medium for an indefinite number of transfers.

The most striking feature in connection with the amino acid requirements of *Streptococcus salivarius* is that tryptophane is not essential. This observation contrasts with the findings of Woolley and Hutchings (1940) in that tryptophane was indispensable in the nutrition of groups D and B streptococci. Enterococcus strains tested in this laboratory have also been found to need this amino acid. Further evidence of the lack of need for tryptophane is that *Streptococcus salivarius* grew luxuriantly in a casein hydrolysate medium with adequate vitamins present, whereas a group D strain failed to grow in the same medium unless it had been replenished with tryptophane.

In agreement with the findings on group D organisms, glutamic acid was found to be indispensable for *Streptococcus salivarius*. No growth would occur in the synthetic medium when this amino acid was omitted, even if all other 18 amino acids were present. However, glutamine could replace glutamic acid. Fildes and Gladstone (1939) studied several species of streptococci and other bacteria with respect to their glutamine requirements. They found that *Streptococcus fecalis* and group B streptococci did not need glutamine, although group A strains required the addition of glutamine. This has been confirmed by Bernheimer and Pappenheimer (1942) with respect to group A streptococci.

The growth of *Streptococcus salivarius* was in no way altered by the addition of glutamine, when in the presence of glutamic acid.

When the amino acids were confined to the seven previously mentioned, leucine was found to be indispensable; but when 19 amino acids were included,

TABLE 1

A chemically defined medium for Streptococcus salivarius

	micrograms
Riboflavin	10
Calcium pantothenate	10
Nicotinic acid	10
Biotin methyl ester (crystalline)	0.01
Thiamin HCl	1.0
Uracil	50
	milligrams
d-glutamic acid	2.5
dl-leucine	1.0
d-arginine HCl	1.0
dl-methionine	1.0
dl-lysine 2HCl	2.0
l-tyrosine	0.7
dl-iso-leucine	1.0
Glucose	50
K_2HPO_4	40
Sodium thioglycollate	1
Salts*	
	ml.
Water to make	10

* Salts composed of 20 mg. NaCl, 0.8 mg. $MgSO_4 \cdot 7H_2O$, 40 micrograms $FeSO_4 \cdot 7H_2O$, and 12 micrograms $MnCl_2$ per 10 ml. medium.

TABLE 2

Effect of leucine, nor-leucine and aspartic acid upon growth of Streptococcus salivarius (S2OB)

MEDIUM	DENSITOMETER READING*
Basal†	0
Basal + leucine	40
Basal + nor-leucine	0
Basal + aspartic	0
Basal + nor-leucine + aspartic	41

* Determined 48 hours after second transfer.

† Basal medium contained the necessary growth factors, glucose, phosphate buffer, sodium thioglycollate, salts, glutamic acid, arginine, methionine, lysine, tyrosine, and iso-leucine.

growth was not altered by omitting leucine. It was found that in the restricted medium leucine could be replaced with a combination of nor-leucine and aspartic acid, neither of which alone would suffice. This is shown in table 2.

Of the other five amino acids: arginine, methionine (or cystine), lysine, tyrosine, and iso-leucine, any one could be omitted from the medium and growth would occur that could be serially sub-cultured in the same medium for an in-

definite number of transfers. However, the resulting growth was notably slower and not so abundant. No growth would occur when glutamic acid and leucine were the only amino acids present in the medium. The addition of ammonium chloride, ammonium carbonate or ammonium citrate failed to narrow the limits of the amino acid requirements.

Even though it would be safe to assume that all of the seven amino acids are utilized by *Streptococcus salivarius*, it is possible that had a different course been taken in eliminating the original amino acids, a different confined group would have sufficed, with the exception of glutamic acid. This is exemplified by the fact that the omission of arginine did not alter the growth when the other 18 amino acids were present. But when the number of amino acids was limited to seven, a pronounced decrease in growth resulted when arginine was not included. That *Streptococcus salivarius* can utilize arginine in its growth and metabolism is interesting since this organism, in contrast to the majority of the other species of streptococci, is unable to attack arginine with the production of ammonia, carbon dioxide, and ornithine (Niven, Smiley and Sherman, 1942).

GROWTH OF OTHER STREPTOCOCCI IN THE SYNTHETIC MEDIUM

Twenty-one typical mucoid-colony-producing strains of *Streptococcus salivarius* were tested for growth in the simplest effective medium containing seven amino acids. The inoculation was made from glucose broth cultures directly into the medium with a fine wire needle. Under these conditions, 14 of the 21 cultures grew well within 24 hours and were successfully transferred serially. When casein hydrolysate was substituted for the seven amino acids, growth of all strains took place readily. This indicates that the seven amino acids may not be sufficient for all strains of *Streptococcus salivarius*.

In addition to *Streptococcus salivarius*, there exist in the normal human throat non-hemolytic streptococci that make up a rather poorly defined and heterogeneous group. Other than their inability to synthesize a carbohydrate from sucrose or raffinose they differ from the typical *Streptococcus salivarius* in many respects (Sherman, Niven and Smiley, 1943). These may be appropriately designated as the *Streptococcus mitis* of Andrewes and Horder (1906). From a collection of 20 cultures of this group only three grew in the medium containing casein hydrolysate and none grew in the medium containing the seven amino acids.

A few strains of *Streptococcus bovis*, another species within the viridans group of streptococci, seem to have somewhat similar nutritive requirements to those of *Streptococcus salivarius*. Of the 12 cultures tested, three were able to grow serially in the medium having casein hydrolysate as an amino acid source. Of these three, two cultures grew well in the amino acid medium. Upon investigating the nutritive requirements of one of these strains, it was found that only thiamin, nicotinic acid, and biotin were needed for prompt growth, thus indicating the diverse nature of the growth factor requirements for different streptococci. It may be recalled that the growth factor requirements for the enterococci are riboflavin, pantothenic acid, and pyridoxine, no one of which is common

to the vitamin needs of this strain of *Streptococcus bovis*. Whether the lack of need for riboflavin is characteristic for all members of this species is not known at the present time.

Of four cultures tested, one strain of *Streptococcus equinus*, also a member of the viridans group, was able to grow in the hydrolyzed casein medium but could not in the amino acid medium.

Of the representatives of other species tested, including *Streptococcus lactis* and members of all the Lancefield groups, none was found that was able to grow in either medium employed (table 3).

TABLE 3

The specificity of the Streptococcus salivarius medium

SPECIES OR GROUP	NUMBER OF STRAINS TESTED	NUMBER OF STRAINS GROWING IN	
		Casein hydrolysate + growth factors*	7 amino acids + growth factors*
Streptococcus salivarius	21	21	14
Streptococcus mitis	20	3	0
Streptococcus bovis	12	3	2
Streptococcus equinus	4	1	0
Streptococcus lactis	2	0	0
Lancefield group A	2	0	0
Lancefield group B	2	0	0
Lancefield group C	2	0	0
Lancefield group D	8	0	0
Lancefield group E	2	0	0
Lancefield group F	1	0	0
Lancefield group G	2	0	0
Lancefield group H	2	0	0

* Growth factors: riboflavin, calcium pantothenate, nicotinic acid, biotin methyl ester, thiamin HCl and uracil.

HEAT TREATMENT OF MEDIUM AS AFFECTING GROWTH

In a few experiments during the earlier part of this study glucose was added aseptically to the simplified medium after autoclaving in order to avoid caramelization of part of the sugar during the heat treatment. Whenever this was done, growth was greatly delayed or was never initiated. This peculiarity was noticed in either the casein hydrolysate or amino acid medium, but was not apparent unless a *small* inoculum was used. A number of experiments were performed in an attempt to find the cause of this peculiar effect.

To demonstrate the necessity of heating the medium, a series of tubes of medium containing glucose (same as in table 1) was autoclaved at 15 pounds pressure for different periods of time. After cooling they were inoculated with one drop of a 1:100 dilution of a 24-hour culture of *Streptococcus salivarius*. After different time intervals the growth was recorded by measuring the developed turbidity. The results are shown in table 4. Not only does a prolonged heat-

ing insure a more prompt development of growth but also a higher maximum level.

In order to determine the responsible substance, or substances, different fractions of the synthetic medium were heated individually and in combination. The results were variable and confusing, with the exception that whenever glucose and phosphate were heated together and then added to the remaining sterilized fractions, prompt growth always resulted.

It is well known that when glucose is heated under slightly alkaline conditions, especially when phosphates and other salts are present, some of the sugar is broken down into a number of substances. Therefore, it would seem logical to assume that some degradation product, or products, of glucose made growth possible in the medium. Following this assumption, a 10 per cent solution of glucose in N/10 NaOH was autoclaved for 30 minutes at 15 pounds pressure. The deeply colored solution, which had become acid, was neutralized with NaOH. One-tenth ml. of this solution was added aseptically to a tube of the casein hydrolysate medium to which no sugar had been added before sterilization.

TABLE 4

Growth response after autoclaving for different periods of time at 121°C.

AUTOCLAVING TIME	DENSITOMETER READING AFTER		
	16 hours	24 hours	48 hours
minutes			
7	0	1	23
20	9	33	35
40	32	43	43
60	61	61	61

Growth resulted which equalled in intensity and promptness the growth occurring in the medium which had been sterilized in the presence of glucose.

Ammonium hydroxide was just as effective as NaOH in producing the degradation product. Also, arabinose, which is not attacked by *Streptococcus salivarius*, proved to be just as effective as glucose when heated in NaOH and included in a medium to which glucose had been added aseptically.

Further experiments showed that the degradation product was not associated with the caramel color in the alkali-treated glucose. Decolorization with norit did not remove its activity. When some of the caramelized glucose was acidified with H_2SO_4 to pH 2.0 and distilled under vacuum, part of the activity was found in the distillate. This indicated that some of the active material is volatile.

When glucose is oxidized under alkaline conditions, over a hundred compounds are known to be formed (Evans, 1929). Among these products there are many acids and aldehydes, along with other products, which are found to be intermediate or final products in carbohydrate metabolism. Pyruvic and lactic acids and acetaldehyde appear in appreciable quantities.

When a simple medium such as that described in this communication is used, it would appear possible that a streptococcus would require a compound to be present that could serve as a hydrogen acceptor in the initial carbohydrate metabolism by the growing culture. If a substance of this nature were not present, even in minute traces, it is conceivable that the carbohydrate metabolism carried on by this organism might be blocked in its initial stages. Pyruvic acid and perhaps acetaldehyde might serve this purpose. An experiment was designed to test this hypothesis.

Approximately one mg. each of pyruvic acid and acetaldehyde, along with one ml. of a sterile, 10 per cent glucose solution, were added aseptically to culture tubes containing 10 ml. of the casein hydrolystate medium which had been autoclaved without glucose. Prompt growth resulted in both cases, but did not occur in the control tube. When lactic acid was added in a similar fashion there was no response. It would seem probable that only traces of a compound of this nature would suffice because, upon initiation of carbohydrate breakdown, similar or identical substances would be formed by the organism.

Pyruvic acid seemed to be more efficient than acetaldehyde in stimulating the prompt growth response. More luxuriant growth occurred as the pyruvate content was increased up to 0.5 per cent, the highest concentration tested. In the higher concentrations there was strong evidence of carbon dioxide liberation, sometimes in quantities sufficient to elevate a paraffin seal placed on the medium. This suggests that some of the pyruvate was possibly being dismutated (Krebs, 1937). No growth took place in the medium with pyruvate when glucose was omitted. It would be expected that other keto-acids and aldehydes might serve the same purpose provided the organism possesses the proper mechanism.

S. Orla-Jensen (1931) and A. D. Orla-Jensen (1933) reported a similar occurrence among various streptococci and lactobacilli. Using a yeast autolysate basal medium, they found that no growth (or a delayed growth) occurred when glucose was sterilized in distilled water before addition to the medium. This was true for all cultures studied with the exception of *Streptococcus liquefaciens*, which would grow promptly in either case. But, when the glucose was sterilized, either in tap water or the basal medium itself, prompt growth would occur. With reference to the tap water, it was concluded that the small amount of alkali present was "sufficient to transform the sugar molecule into a less stable condition." The authors found that when methylglyoxal, furfuraldehyde, or a pentose (which was not fermented by the test organism) was *sterilized* in the basal medium, prompt growth resulted when glucose was added aseptically. Acetaldehyde would not suffice.

A yeast growth factor has been reported by Nielsen and Dagys (1940), and Nielsen and Eistrup (1940), to be formed upon heating glucose and other sugars with ammonium salts of various organic acids, especially tartaric, malic, succinic and citric acids. This growth substance, needed in the simple medium to support the growth of the yeast cultures employed, could be replaced with β-alanine, thus leading them to believe that a similar substance was formed during the

heating process. Later, Hartelius and Nielsen (1941a, b) reported that the growth substance was formed in even greater concentrations when glucose was heated in the presence of ammonium hydroxide. It was claimed that glucose heated with sodium hydroxide did not yield the growth factor. It appears that the factor of Nielsen, *et al.* is different from that involved in the present study.

Since acetaldehyde was found to serve our purpose, it seems probable that *Streptococcus salivarius* possesses the alcohol dehydrogenase system, whereas the organisms used by Orla-Jensen possibly lacked this mechanism. Some streptococci have been shown to possess this enzymic system (Gunsalus and Wood, 1942).

Although the peculiarity discussed above is not apparent in ordinary bacteriological media nor when large inocula are used, it should be kept in mind by those investigating the nutritive requirements of microörganisms.

SUMMARY

A chemically defined medium has been devised which will support the growth of *Streptococcus salivarius* cultures. The simplest effective medium contained, in addition to inorganic salts, glucose and sodium thioglycollate, the following substances: glutamic acid, leucine, arginine, iso-leucine, lysine, methionine, tyrosine, riboflavin, nicotinic acid, pantothenic acid, biotin, thiamin and uracil.

No other streptococci were found that would grow in the medium with the exception of a few cultures of other species within the viridans group.

The necessity of heating the medium in order to obtain satisfactory growth response was noted, together with a possible explanation of this effect.

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