Nutritional Requirements of *Streptococcus salivarius*

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**SUMMARY**

The nutritional requirements of *Streptococcus salivarius* ATCC 13419 were studied. Ammonia could serve as the major nitrogen source in a medium containing glucose, cysteine, nicotinic acid, biotin, thiamin, riboflavin, pantothenic acid and inorganic salts. *Streptococcus salivarius* NCTC 8618 and 11 oral isolates of *S. salivarius* grew in this medium. For growth of *S. salivarius* ATCC 9759 the medium had to be supplemented with glutamic acid. The cysteine requirement of *S. salivarius* ATCC 13419 could be replaced by cystine, homocysteine, homocystine or thiosulphate. Urea could be used as nitrogen source by *S. salivarius* ATCC 13419, *S. salivarius* ATCC 9759 and by five of the 11 oral isolates of *S. salivarius*.

**INTRODUCTION**

The predominant streptococcus in ruminants, *Streptococcus bovis*, has been considered as the only species of the genus *Streptococcus* which is capable of using ammonium salts as the major source of nitrogen for growth (Prescott, Williams & Ragland, 1959; Wolin, Manning & Nelson, 1959). Recently, however, it was found that when growing anaerobically the human oral streptococci *S. sanguis* and *S. mutans* may also utilize ammonia as major source of nitrogen (Carlsson, 1970a, b, 1971). This paper reports similar simple nutritional requirements of the predominant streptococcus of human saliva, *Streptococcus salivarius*.

**METHODS**

**Micro-organisms.** *Streptococcus salivarius*, American Type Culture Collection (ATCC) strain 13419 was used for the development of the media. Subsequently, the following strains were tested: *S. salivarius* ATCC 9759, *S. salivarius* National Collection of Type Cultures (NCTC) 8618 and 11 strains isolated from the oral cavity in man and considered to be *S. salivarius* in a numerical taxonomic study (Carlsson, 1968).

**Chemicals.** DL-Homoserine, DL-homocystine and DL-homocysteine thiolactone HCl were from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Thiouracil was from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Sodium thioglycollate as well as ingredients for *Mitis Salivarius* agar and *Brain Heart Infusion* agar were from Difco Laboratories, Detroit, Michigan, U.S.A. All other ingredients in the chemically defined media and other chemicals were of the purest grade available from the British Drug Houses Ltd, Poole, Dorset. Distilled water was used after filtration through mixed-bed ion-exchange resins (Crystalab Deeminizer, Crystal Research Laboratories Inc., Hartford, Connecticut, U.S.A.).

**Culture media.** Amino acids and salts were dissolved according to Williams (1955) and vitamins according to Leslie (1961). All ingredients were sterilized by filtration (membrane filter, type GS, Millipore Corporation, Bedford, Massachusetts, U.S.A.). The media were
distributed in 5 ml. amounts into screw-capped tubes (16 × 110 mm.) and were kept frozen (−20°C) until used.

**Assays.** Bacterial growth yield was estimated by determining the extinction (E) at 420 nm. in a double-beam spectrophotometer with the uninoculated medium as control. The culture was diluted in water to give an extinction less than 0.8.

The dry weight of the three reference strains was determined using two independent cultures of each strain. The bacteria were harvested by centrifugation, suspended in 10 ml. of water, recentrifuged and then dried for 2 days at 110°C on pre-weighed pieces of aluminium foil. The dried bacteria were weighed on an electrobalance (model G, Gram electrobalance, Cahn Instrument Co., Paramount, California, U.S.A.). The dry weight of bacteria in cultures with an extinction of 1.0 at 420 nm. was 0.131 to 0.136 mg./ml.

**Table 1. Composition of media for Streptococcus salivarius**

0.1 M Potassium phosphate buffer (pH 7.0) in all media.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration of components (mg./l.) in medium</th>
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<tbody>
<tr>
<td></td>
<td>C4</td>
</tr>
<tr>
<td>Glucose</td>
<td>10,000</td>
</tr>
<tr>
<td>L-Cysteine HCl</td>
<td>50</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>12</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2.3</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.006</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>0.05</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.2</td>
</tr>
<tr>
<td>Ca-pantothenate</td>
<td>1.2</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1,320</td>
</tr>
<tr>
<td>NH₄HCO₃</td>
<td></td>
</tr>
<tr>
<td>Salts B</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>10</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>10</td>
</tr>
<tr>
<td>NaCl</td>
<td>10</td>
</tr>
</tbody>
</table>

Experimental procedure. Streptococcus salivarius ATCC 13419 grew in a medium (C4, Table 1) previously developed for S. mutans (Carlsson, 1970b). After 15 daily transfers, 0.1 ml. of a 24 h. culture was inoculated into a number of media each of which lacked one of the ingredients of medium C4. However, none of the salts (B) were omitted. The organism was subcultured daily for 10 days with 0.1 ml. inocula in each of these media. If the growth had ceased before that time, the procedure was repeated with 0.5 ml. inocula. The growth in each tube was followed for 5 days by scoring −, + or ++ and finally the extinction of the culture at 420 nm. was measured on the fifth day. When the qualitative composition of a minimal medium for growth had been elaborated, the concentration of each component necessary for optimal yield was determined. Strain ATCC 13419 was subcultured in media with one component in such a low concentration that the yield was 20 to 50% of maximal. A series of media with various concentrations of this component was inoculated with 0.1 ml. from such a limited culture grown for 24 h. The growth yield in these media was determined after 72 h. by measuring the extinction at 420 nm.

When a minimal medium with optimal concentration of each component had been developed for strain ATCC 13419, the growth yields of the other strains were tested in that medium. If any strain failed to grow, the medium was supplemented with various compon-
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ents until transferable growth was obtained. A strain was considered to be able to grow in a medium if it could be subcultured ten times in the medium with 2% (v/v) inocula.

Preparation of media, daily transfers and sampling from culture tubes were done in a sterile bench with horizontal laminar air flow (Munktell, Gryksbo, Sweden). The purity of the cultures were checked at least once a week by streaking one drop of the culture on the surface of a Mitis Salivarius agar plate and a Brain Heart Infusion agar plate.

All media were incubated in the dark at 37°C. For incubations in atmospheres other than air, the culture tubes were placed in a jar, the air evacuated to a pressure of $-1.0$ kg./cm.$^2$ and replaced by the required gas. When the gas contained hydrogen the jar was provided with a palladium-asbestos catalyst in brass net. If not otherwise stated, anaerobic growth refers to growth in an atmosphere of 95% H$_2$ and 5% CO$_2$.

![Fig. 1. Growth yield of Streptococcus salivarius ATCC 13419 in media containing various concentrations of one of the components. The complete medium contained (per ml.): glucose, 10 mg.; (NH$_4$)$_2$SO$_4$, 1.32 mg.; l-cysteine HCl, 59 μg.; biotin, 2.4 μg.; Capantothenate, 4.8 μg.; nicotinic acid, 0.23 μg.; riboflavin, 0.19 μg.; thiamin HCl, 0.34 μg.; MgSO$_4$·7H$_2$O, 0.2 mg.; FeSO$_4$·7H$_2$O, 10 μg.; MnSO$_4$·H$_2$O, 10 μg.; NaCl, 10 μg., and potassium phosphate buffer (pH 7.0) 0.1 m mole. Cultures grown in 95% H$_2$+5% CO$_2$. The mean extinction of three cultures (± s.o.) in each medium after 72 h. is given.](image)

RESULTS

Growth of Streptococcus salivarius ATCC 13419. The organism grew in medium C 4 (Table I) when incubated in an atmosphere containing 95% H$_2$ and 5% CO$_2$. Only pyridoxine and p-aminobenzoic acid could be omitted from this medium without interfering with the growth yield. The effect of omitting each of the salts (B) was not tested.

The growth yield at various concentrations of the components in a minimal medium is
shown in Fig. 1. From these results medium C 31 (Table 1) was composed. In medium C 31 *Streptococcus salivarius* ATCC 13419 grew luxuriantly with a yield of about 0.8 mg. dry wt of bacteria/ml. of medium, when incubated in an atmosphere containing 95% H₂ + 5% CO₂ or 95% N₂ + 5% CO₂. The organism failed to grow in this medium when incubated aerobically or in an atmosphere containing only H₂ or N₂. When (NH₄)₂SO₄ in medium C 31 was replaced by urea or NH₄HCO₃ the organism grew anaerobically (Fig. 2) as well as aerobically. The carbon dioxide requirement of *S. salivarius* ATCC 13419 in aerobic culture in medium C 31 could be replaced by 10 mM-glutamic acid or oxaloacetic acid. The addition of aspartic acid, α-oxoglutaric acid or succinic acid to medium C 31 gave inconsistent growth aerobically.

![Fig. 2. Growth yield of *Streptococcus salivarius* ATCC 13419 in medium C 31 (Table 1) containing different concentrations of ammonium sulphate (●), urea (□) or ammonium bicarbonate (■) substituting for the ammonium sulphate. Cultures grown in 95% H₂ + 5% CO₂. The mean extinction of three cultures (± S.D.) in each medium after 72 h. is given.](image)

The cysteine requirement of *Streptococcus salivarius* ATCC 13419 in anaerobic culture in medium C 39 could be replaced by 0.5 mM-L-cystine, DL-homocystine, DL-homocysteine thiolactone or sodium thiosulphate (Fig. 3), but not with 0.5 mM-DL-homoserine, L-methionine, thioglycollic acid, mercaptoethanol, thiourea, thiouracil, sodium sulphite or sodium sulphide. Only L-cysteine supported growth aerobically.

**Growth of other strains of *Streptococcus salivarius*.** *Streptococcus salivarius* NCTC 8618 grew in medium C 39. *Streptococcus salivarius* ATCC 9759 did not grow in medium C 31 nor C 39 unless these media were supplemented with glutamic acid. The growth yield when medium C 31 was supplemented with different concentrations of glutamic acid is shown in Fig. 4. Succinic acid, oxaloacetic acid and α-oxoglutaric acid in a concentration of 10 mM could not substitute for the glutamic acid requirement of *S. salivarius* ATCC 9759. All 11 oral strains of *S. salivarius* grew anaerobically in medium C 39. Three of the 11 strains also grew
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Five of the 11 oral strains grew anaerobically in medium C39 when NH₄HCO₃ was replaced by 20 mM urea. *Streptococcus salivarius* ATCC9759 could also use urea instead of NH₄HCO₃ in medium C39, when this medium was supplemented with 10 mM glutamic acid. *Streptococcus salivarius* NC18618 could not utilize urea. When L-cysteine in medium C39 was replaced by 0.5 mM sodium thiosulphate, *S. salivarius* NC18618 and six of the oral strains grew in this medium. *Streptococcus salivarius* ATCC9759 could also use sodium thiosulphate instead of L-cysteine in medium C39, when this medium was supplemented with 10 mM-glutamic acid.

**DISCUSSION**

*Streptococcus salivarius* was among the first streptococci in which the nutritive requirements were studied (Smiley, Niven & Sherman, 1943). A medium for transferable growth of 14 of 21 strains of *S. salivarius* contained inorganic salts, glucose, sodium thioglycollate, glutamic acid, leucine, arginine, isoleucine, lysine, methionine, tyrosine, riboflavin, nicotinic...
acid, pantothenic acid, biotin, thiamin and uracil. Two oral strains of *S. salivarius* studied by Paul (1961) required glutamic acid, cystine, alanine, and lysine, and were stimulated by aspartic acid or asparagine and by histidine or isoleucine or tyrosine. The present study shows that the nutritional requirements of *S. salivarius* are much simpler, if the oxidation-reduction potential of the medium is lowered and if the culture is provided with carbon dioxide. In the presence of glucose and five B-group vitamins, ammonia could serve as sole nitrogen source.

Assimilation of carbon dioxide has been demonstrated in other streptococci. When grown on complex nitrogen sources *Streptococcus bovis* (Wright, 1960), *S. anginosus* (Martin & Niven, 1960) and *S. faecium* var. *durans* (Lachica & Hartman, 1968) incorporate carbon dioxide mainly as the β-carboxyl group of aspartic acid. In *S. faecalis* var. *liquefaciens* carbon dioxide is fixed in oxaloacetic acid by a pyruvate carboxylase, and oxaloacetic acid is transaminated to aspartic acid (Hartman, 1970). In *S. bovis* grown in an ammonium salt medium exogenous carbon dioxide is the sole source of the α-carboxyl group of glutamic acid (Burchall, Niederman & Wolin, 1964) and the carboxyl and guanidine carbons of arginine (Niederman & Wolin, 1967). In addition *S. bovis* incorporates acetate mainly into glutamic acid (Prescott, Ragland & Hurley, 1965). These results indicate that a tri-carboxylic acid pathway to α-oxoglutaric acid may be present in streptococci.

The primary site of incorporation of ammonia into amino acids in *Streptococcus bovis* is a nicotinamide adenine dinucleotide phosphate (NADP)-linked glutamate dehydrogenase (Burchall, Niederman & Wolin, 1964). An asparagine synthetase has also been demonstrated in this organism (Burchall, Reichelt & Wolin, 1964) and ammonia as well as carbon dioxide may also be fixed by enzymes involved in carbamoyl phosphate synthesis (Niederman & Wolin, 1967).

The close overall similarity between *Streptococcus bovis* and *S. salivarius* (Carlsson, 1968) and the similarity in nutritional requirements of these species suggest that ammonia and carbon dioxide are assimilated in similar ways in these streptococci.

*Streptococcus salivarius* could utilize cysteine, cystine, homocysteine, homocystine and thiosulphate as sulphur source. This is in accordance with findings in *S. bovis* (Prescott, 1961). Unlike *S. bovis*, *S. salivarius* could not utilize thioglycollic acid, thiourea, thiouracil and sulphide. Both organisms failed to utilize methionine, sulphate and sulphite. It is not known how streptococci synthesize their sulphur-containing amino acids.

An interesting question is how *Streptococcus salivarius* satisfies its nutritional requirements in the natural habitat, the surface of the tongue (Krassé, 1954). Although a luxuriant growth of this organism in the mouth requires the presence of readily fermentable carbohydrates in the diet (Carlsson, 1965), *S. salivarius* can establish itself in the mouth of infants before they have consumed any milk (Carlsson, Grahnén, Jonsson & Wikner, 1970). The requirements for ammonia, carbon dioxide and vitamins may be provided by saliva or by the microbiota inhabiting the crypts of the tongue. Saliva contains 3 to 6 μmoles ammonia/ml. (Battistone & Burnett, 1961) and 0.1 to 5 ml. carbon dioxide/ml. of fluid (Jenkins, 1966). All the B-group vitamins have been demonstrated in saliva, but there are wide differences in the amounts reported by various workers (Mäkilä, 1968).

Urea may also be a valuable nitrogen source for *Streptococcus salivarius*. The concentration of urea in saliva is closely related to its plasma level (Nikiforuk, Jackson, Cox & Grainger, 1956). When urea is supplied in the diet the ammonia production in the mouth is greater than the rate of ammonia utilization (Stephan, 1943; Frostell, 1960; Kleinberg, 1967). Urea has been included in sweets in order to prevent the drop of pH in microbial aggregations on the teeth, thereby preventing dental caries (Frostell, 1967, 1970). Such
sweets will be adequate carbon and nitrogen sources for S. mutans and S. sanguis (Carlsson, 1970a, b, 1971). From the results of the present study these sweets cannot be recommended as caries-preventing agents.

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