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Amino Acid Requirements and Proteolytic Activity of *Streptococcus sanguis*

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The growth response of *Streptococcus sanguis* groups 1:A and 1:B in a complete chemically defined medium was not influenced by the oxygen concentration of the growth atmosphere. All of the cultures required cysteine and arginine; tyrosine and branched-chain amino acids were frequently required. Proteolysis of casein, mucin, and the anionic proteins of germfree rat saliva by *S. sanguis* was demonstrated. Hydrolytic activity toward casein was found in the soluble contents of the cells and in the cellular debris after disruption of the cells, with the soluble fractions exhibiting greater proteolytic activity toward casein. The soluble fractions from *S. sanguis* did not hydrolyze mucin, but this substrate was hydrolyzed by the cell debris fraction. When the amino acid requirements and proteolytic activity of *S. sanguis* and *S. mutans* were compared, these two oral streptococcal species exhibited distinct and characteristic differences.

Streptococcus sanguis generally is considered in connection with subacute bacterial endocarditis (18, 23), but its natural habitat in humans is the oral cavity (9), where it is a prominent member of microbial plaque deposits on the teeth. *S. sanguis* in common with *S. mutans*, a more cariogenic inhabitant of plaque, grow better under CO_2 tension, synthesize extracellular polyglucans from sucrose, and ferment simple carbohydrates with lactic acid as the principal end product. These oral streptococci can be differentiated by their colonial characteristics on sucrose-containing media and particularly by the inability of *S. sanguis* to ferment either sorbitol or mannitol.

Carlsson has reported that one strain of *S. mutans*, of serotype *c*, and strains of *S. sanguis*, of his group 1:B but not group 1:A, can utilize ammonium sulfate as the sole nitrogen source in a medium also containing cysteine, glucose, vitamins, minerals, and accessory cofactors under strictly anaerobic conditions (8, 10). By using a number of *S. mutans* strains encompassing the recognized serotypes *a*, *b*, *c*, and *d* (2, 3), we found that reproducible growth from small inocula (1%, vol/vol) required cysteine and at least one additional amino acid for type *c* strains, and even more stringent requirements for type *a*, *b*, and *d* (13). In the present study the amino acid requirements and proteolytic activity of *S. sanguis* are described and compared with those described earlier for *S. mutans* (12, 13).

MATERIALS AND METHODS

Organisms and maintenance. *S. sanguis* strains ATCC 10556-R (streptomycin resistant), F90A (ATCC 12396), F90A-R (streptomycin-resistant substrain of F90A), 134N, and 167N were from I. L. Shklair, Naval Dental Research Institute, Great Lakes, Ill. Strain 804-C8 was from J. Carlsson, University of Umeå, Sweden. Strains 903-1600 and GF-189 (ATCC 10558) were from our laboratory collection, as was *S. mutans* K1-R. Stock cultures were maintained in Trypticase-glucose broth containing 0.5% calcium carbonate, as described by Cowman et al. (13).

Taxonomic classification tests. Fermentation of lactose, raffinose, glucose, mannitol, sorbitol, trehalose, maltose, melibiose, and cellobiose was determined, as described by Carlsson (7), using the medium of Jordan et al. (21) containing 0.018% phenol red as the basal medium. The carbohydrates were sterilized by filtration through 0.22-μm membrane filters (Millipore Corp.) and then added to the sterile basal medium. The production of ammonia from arginine and serine was determined as described by Carlsson (6). Growth in media containing 3, 4, or 6.5% sodium chloride was determined by using the medium of Jordan et al. (21) containing 1.0% glucose. The inoculated media were incubated under ambient aerobic conditions, under 90% N_2-10% CO_2 (1.5% O_2), and under 70% N_2-20% H_2-10% CO_2 (see below) at 35 C, and all tests were repeated three times.

Incubation atmospheres. Inoculated tubes of media were incubated under a "controlled aerobic" atmosphere consisting of 90% N_2-10% CO_2 (1.5% O_2). In certain experiments, an atmosphere of 70% N_2-20% H_2-10% CO_2 provided by a BBL GasPak anaerobic

system and catalyst also was used (4). The detailed procedures of incubation have been described previously (13).

Growth in minimal medium. In these studies, the minimal medium described by Carlsson (10) was used. The medium was freshly prepared and filter sterilized (0.22-μm membrane filter; Millipore Corp.) for each experiment.

Amino acid requirements. The composition and preparation of the complete defined amino acid medium used in this study have been described previously (13). The specific amino acid requirements of each strain were determined by making single or multiple omissions of amino acids from the medium, in accordance with procedures described earlier for *S. mutans* (13), with 1% (vol/vol) inocula. The growth response in an omission medium was expressed as a percentage of that attained in the complete amino acid medium. In accordance with the definitions of Reiter and Oram (25), an omitted amino acid was designated: (i) "essential" if growth was less than 50% of the control medium after 48 h, (ii) "non-essential" if growth was within 75% of the control, (iii) "stimulation" if growth was 55 to 73% of the control, or (iv) "inhibitory" if growth in the absence of a particular amino acid exceeded 126% of the growth in the control medium.

Preparation of cellular fractions. Cells grown in Trypticase-glucose medium under the controlled aerobic atmosphere for 16 h at 35 C were collected by centrifugation (10,000 × *g*), and the cell pellet was washed three times with 0.04 M sodium phosphate buffer, pH 7.0. After the final wash, the pellets were resuspended in 10 ml of buffer, placed in an ice bath, and sonically disrupted for 10 min at 50 W with a model W-140 Branson Sonifier (Heat Systems-Ultrasonics, Inc.). The cell lysates were centrifuged at 27,000 × *g* for 20 min after which the supernatant fluid, consisting of the soluble contents of the cell, was decanted and designated the "soluble" fraction. The remaining sediment, referred to as the "cell debris" fraction, was resuspended in 5.0 ml of buffer.

Preparation of casein and mucin substrates. A 10-g amount of casein (Hammersten) was solubilized in 0.1 N NaOH by stirring overnight in the cold. A 10-g amount of crude porcine mucin (type II, Sigma Chemical Co.) was solubilized in 0.01 N NaOH with stirring for 48 h in the cold. The substrate solutions were neutralized to pH 6.6 or 7.4 by slow addition of 0.1 N HCl and diluted with 0.2 M sodium phosphate buffer of the desired pH to give a final concentration of 1% casein or mucin. Toluene was added as a preservative and the substrate solutions were stored at 3 C.

Standardized enzyme assays. Protein hydrolysis was followed by measuring the increase in absorbancy at 280 nm of trichloroacetic acid filtrates after precipitation of the protein (26, 27). The reaction mixture for casein hydrolysis contained 1 volume of substrate in 8 volumes of 0.2 M phosphate buffer, and 1 volume of enzyme solution (cell fractions or spent medium), which were tempered separately at 37 ± 1 C. At zero hour and subsequent intervals, samples were removed from incubation and mixed with an equal volume of 11% trichloroacetic acid with shaking to stop the reaction. After 10 min the mixture was filtered through Whatman no. 4 filter paper, and the absorbance of the filtrates was measured at 280 nm. After subtraction of the zero-hour value, one unit of proteinase activity was defined as the amount of enzyme that caused a 0.01 optical density increase at 280 nm in 3 h. During this incubation period, the increase in absorbance due to enzyme action was linear. Difficulty in measuring the protein content of the cell debris fractions was encountered with the Lowry protein procedure; therefore, aliquots of the cell fractions, in triplicate, were dried to constant weight in vacuo, and the enzymatic activity was expressed as units of proteinase per milligram of residue weight. Appropriate controls were included in all experiments.

Mucin hydrolysis (mucinolytic activity) was measured in a similar manner, except that portions of the reaction mixture were mixed with 24% trichloroacetic acid and centrifuged at 27,000 × *g* for 20 min before filtration.

Hydrolysis of rat saliva. Whole, mixed, pilocarpine-stimulated saliva was collected from anesthetized germfree Spraque-Dawley male rats as described by Benarde et al. (1). The collected saliva was centrifuged at 27,000 × *g* for 20 min, and the supernatant fluid was decanted, sterilized by filtration through 0.2-μm Gelman membrane filters, and frozen. The saliva supernatants, in 1.0-ml quantities, were mixed with 0.3 ml of soluble or cell debris fractions and incubated at 37 ± 1 C for 0 and 24 h. Controls consisting of substrate and buffer were incubated simultaneously with the experimental preparations. At each time interval, 500-μl portions of assay mixtures were removed and centrifuged to remove particulate material from those samples containing the cell debris material. The density of each sample was then increased by adding solid sucrose, and 50-μl portions were layered on the surface of polyacrylamide disc-gel columns containing both a separation and a stacking gel (11). The applied sample contained either a 40-μg soluble fraction or a 25-μg cell debris fraction, and 500 μg of rat saliva (7% protein).

Electrophoretic analysis of the anionic salivary proteins was performed in 7.0% acrylamide gel columns with a continuous electrode buffer of tris(hydroxymethyl)aminomethane-glycine at pH 8.3 [2.88 g of glycine and 0.6 mg of tris(hydroxymethyl)aminomethane per liter]. After sample layering, 2 ml of 0.1% bromophenol blue dye was added to the upper electrode chamber, and a current of 1.0 mA per column was applied and maintained until the tracking dye migrated through the stacking gel. The amperage was then increased and maintained at 4.0 mA per column until the dye had migrated 10 cm toward the anodal end of the gel.

After electrophoresis, the gels were stained 30 min with 0.25% Coomassie blue in 12% trichloroacetic acid and, after destaining with 10% trichloroacetic acid, the gels were scanned at 525 nm with a model 240 Gilford spectrophotometer equipped with a gel scanning attachment and linear recorder. The distance that any protein band migrated from the origin was measured in centimeters.

RESULTS

S. sanguis strains F90A, F90A-R, 134N, and 167N were placed in group 1:A of Carlsson (7) on the basis of their fermentation of raffinose and melibiose and of their inability to grow in a medium containing 3% NaCl or to produce ammonia from arginine (Table 1). Strains 10556-R, 903-1600, GF-189, and 804-C8 were classified as members of group 1:B because of their inability to ferment raffinose or melibiose and their ability to grow in the presence of 3% NaCl and to produce ammonia from arginine but not serine. None of the cultures fermented sorbitol or mannitol.

Growth in defined media and amino acid requirements. Although the growth response of the *S. sanguis* cultures in the complete amino acid medium was not greatly influenced by the atmosphere of incubation (Fig. 1), cultures F90A-R, GF-189, and 804-C8 grew moderately better under the more anaerobic 70% N_2-20% H_2-10% CO_2 atmosphere. This difference was maintained even after these cultures were incubated for 48 h. Cultures 10556-R and 167N exhibited limited growth in the medium under both atmospheric conditions. However, when the medium was supplemented with 0.5% Casamino Acids, the growth response of these two cultures was markedly improved.

S. sanguis, of group 1:B, reportedly can utilize ammonium ions as a source of nitrogen for growth (10). However, none of our cultures (group 1:A or 1:B), including strain 804-C8, grew in the filter-sterilized minimal medium of Carlsson (10) under either of the atmospheric conditions used in this study. Growth densities of less than 0.15 optical density unit were observed in the first passage into minimal medium from either the complete amino acid medium or complex media after 4 days of continuous incubation. No transferable growth was obtained in the second passage even after 5 days of incubation. These observations indicated that the *S. sanguis* cultures, under the conditions of this study, required amino acids for growth, and therefore the specific amino acid requirements were determined.

Under the controlled aerobic atmosphere (90% N_2-10% CO_2-1.5% O_2), all of the cultures required cysteine and arginine except strain 10556-R, which was inhibited by arginine (Table 2). The requirement for cysteine could not be replaced by either dithiothreitol or cystine. Additional amino acid requirements of the cultures ranged from relatively simple for strain 134N to complex for strain 167N. Several of the cultures exhibited an interchangeable requirement for the pair glutamic acid-aspartic acid or for one of the branched-chain amino acids. When tested under the more anaerobic growth condition (70% N_2-20% H_2-10% CO_2), the amino acid requirements of the cultures were not greatly altered.

Casein and mucin hydrolysis. Preliminary studies had indicated that whole cells of *S. sanguis* 903-1600 and 10556-R, as well as the soluble and cell debris fractions from these cultures, possessed hydrolytic activity toward casein at pH 7.0. The relative specific activity of the cellular fractions from the *S. sanguis* cul-

TABLE 1. *Biochemical tests applied for the differentiation of S. sanguis types*

Character tested	*S. sanguis* strain						
	F90A	F90A-R	134N	167N	10556 R	GF-189	903-1600
Fermentation of:							
Lactose	+	+	+	+	+	+	+
Raffinose	+	+	+	+	−	−	−
Glucose	+	+	+	+	+	+	+
Mannitol	−	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−
Trehalose	+	+	+	−	+	+	+
Maltose	+	+	+	+	+	+	+
Melibiose	+	+	+	+	−	−	−
Cellobiose	+	+	+	−	+	+	+
Ammonia from:							
Arginine	−	−	−	−	+	+	+
Serine	−	−	−	−	−	−	−
Growth in:							
3% NaCl	−	−	+	−	+	+	+
4% NaCl	−	−	−	−	−	−	+
6.5% NaCl	−	−	−	−	−	−	−

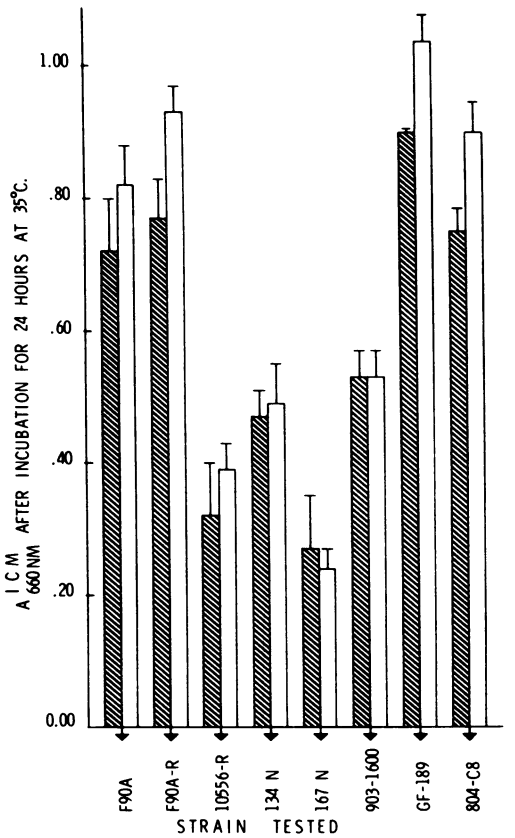

FIG. 1. *Influence of atmosphere on growth of S. sanguis representing grouping 1:A and 1:B in reference defined medium after 24 h at 35 C. Cross-hatched bars represent growth under 90% N_2-10% CO_2 (1.5% O_2); open bars represent growth under 70% N_2-20% H_2-10% CO_2. T, Standard deviation based on ten replicate experiments.*

tures and *S. mutans* K1-R were compared by using casein and mucin substrates. The soluble fraction of *S. sanguis* 10556-R had a higher relative specific activity toward casein at pH 6.6 than at pH 7.4, whereas a similar fraction from strain 903-1600 possessed a high relative specific activity at both pH values (Table 3). Conversely, the cell debris fractions of the *S. sanguis* cultures possessed relative specific activities toward casein only about half that noted for the soluble fractions at either pH 6.6 or 7.4. When compared to the respective fractions of *S. mutans* K1-R, the soluble fractions of the *S. sanguis* cultures were more proteolytically active at pH 6.6, but the cell debris fraction of *S. mutans* exhibited much higher proteolytic activity than the *S. sanguis* fractions at pH 7.4.

When mucin served as the substrate, low and quite variable hydrolytic activity was observed in the soluble fraction of either the *S. sanguis* or *S. mutans* cultures (Table 3). The cell debris fractions from the three cultures hydrolyzed mucin at both pH 6.6 and 7.4. At pH 7.4, the cell debris fractions of the *S. sanguis* cultures were more proteolytically active toward mucin than casein.

The proteolytic activity of the cellular fractions from *S. sanguis* 903-1600 and *S. mutans* K1-R were further compared by using germ-free, fasting-rat saliva as substrate for the enzymes. Electrophoresis of the saliva-cellular fraction mixtures at zero hour (solid lines) generally revealed the presence of protein zones at 2.0, 2.5, 3.4, 5.0, and 6.5 cm from the origin (Fig. 2 and 3). These zones were attributed to proteins present in the rat saliva since, under the conditions used in this study, electrophore-

TABLE 2. *Amino acid requirements[a] of S. sanguis strains representing groups 1:A and 1:B*

Omission of:[b]	Group 1:A strains				Group 1:B strains			
	F90A	F90A-R	134N	167N	903-1600	10556-R	GF-189	804-C8
Cysteine	E	E	E	E	E	E	E	E
Arginine	E	E	E	E	E	I	E	E
Histidine	—	—	S	S	—	S	—	—
Methionine	—	—	—	E	—	—	—	—
Lysine	—	—	—	E	—	—	—	—
Tryptophan	—	—	—	S	—	—	—	—
Tyrosine	S	—	—	E	E	—	E	E
Glutamic	S	R-1	R-1	E	R-1	R-1	E	E
Aspartic	E	R-1	R-1	E	R-1	R-1	E	E
Leucine	R-1	—	—	E	—	I	R-1	—
Isoleucine	R-1	—	—	E	—	E	R-1	—
Valine	R-1	—	—	E	—	E	R-1	—

[a] E, Essential; S, stimulatory; I, inhibitory; —, no exogenous requirement; R-1, requirement for one of the designated amino acids.

[b] Amino acids not listed were not required for all organisms tested.

TABLE 3. *Hydrolysis of casein and porcine crude mucin by cellular fractions of S. sanguis and S. mutans at pH 6.6 and 7.4*[a]

Hydrolysis of:	U of proteinase/mg of residue weight			
	Soluble fraction		Cell debris fraction	
	pH 6.6	pH 7.4	pH 6.6	pH 7.4
Casein				
S. sanguis 10556-R	124 ± 12	76 ± 6	46 ± 7	52 ± 7
S. sanguis 903-1600	163 ± 15	156 ± 9	70 ± 10	66 ± 14
S. mutans K1-R	74 ± 17	65 ± 20	76 ± 17	104 ± 18
Crude mucin				
S. sanguis 10556-R	0 ± 0	0 ± 0	68 ± 15	133 ± 25
S. sanguis 903-1600	0 ± 0	7 ± 6	100 ± 23	104 ± 18
S. mutans K1-R	0 ± 0	12 ± 13	81 ± 18	96 ± 19

[a] Each value represents the mean ± standard error for data from 6 replicated experiments for *S. sanguis* cultures and 15 replicated experiments for *S. mutans* K1-R.

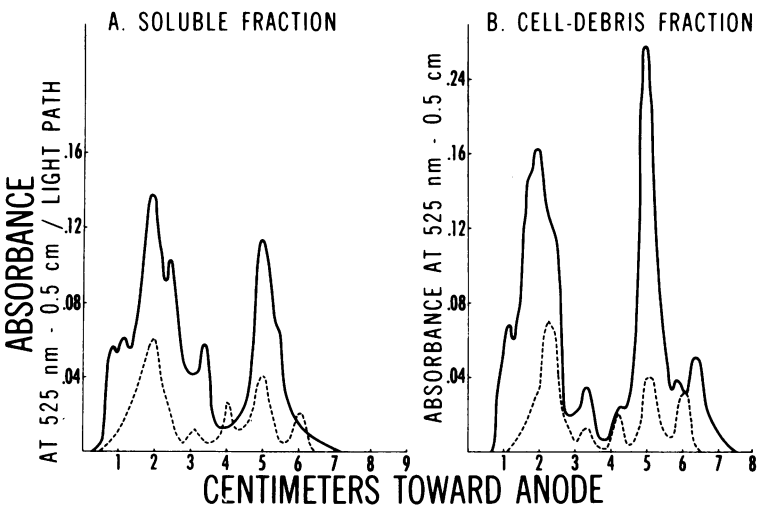

FIG. 2. *Tracing of densitometric scans of germfree rat salivary anionic proteins incubated with the cellular fractions of S. sanguis 903-1600 in 0.1 M sodium phosphate buffer at pH 6.6 and 37 C. Solid lines denote zero-hour incubation, and dashed lines represent the 24-h incubation of saliva-fraction mixtures at 37 C.*

sis of the cellular fractions alone resulted in no detection of zones from this source. The variation observed in relative absorbancies and zonal positions was due to variations in the different preparations of rat saliva used.

Incubation of the saliva alone for 24 h revealed that no major alterations had occurred in the protein patterns. When saliva was incubated with the soluble fraction of *S. sanguis* for 24 h, extensive degradation of the saliva proteins occurred, as indicated by the minor amounts of protein-staining material present (Fig. 2A). Similarly, only minor amounts of protein-staining material were detectable after saliva had been incubated with the cell debris fraction of the *S. sanguis* culture (Fig. 2B). Incubation of the rat saliva with either the soluble or cell debris fraction of *S. mutans* K1-R for 24 h resulted in less-extensive degradation of the proteins present, with particular reference to the protein zone at 5.0 cm (Fig. 3A, B).

DISCUSSION

The purpose of this study was to investigate certain aspects of the nitrogen metabolism of *S. sanguis* and to compare them with those of *S. mutans*.

It was shown earlier (13) that the growth of *S. mutans* in a complete defined amino acid medium was related to the relative anaerobicity of the growth environment and that the requirements for specific amino acids were

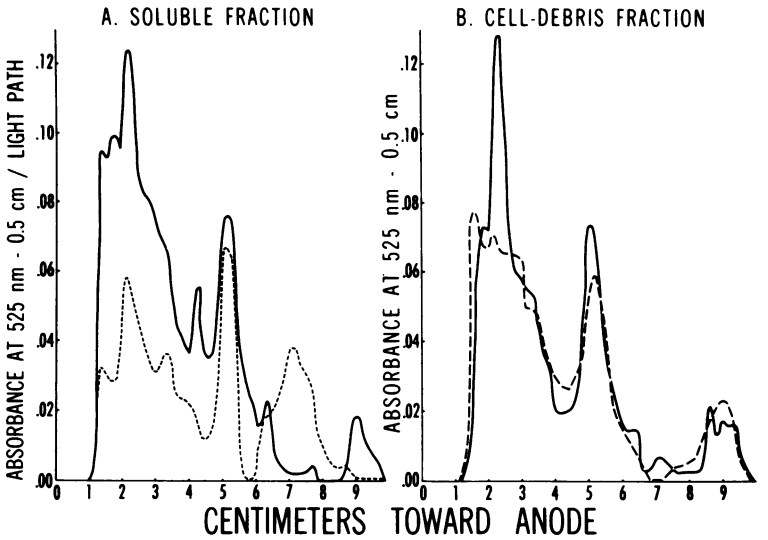

FIG. 3. *Tracing of densitometric scans of germfree rat salivary anionic proteins incubated with the cellular fractions of S. mutans K1-R in 0.1 M sodium phosphate buffer at pH 6.6 and at 37 C. Solid lines denote zero-hour incubation, and dashed lines represent the 24-h incubation of saliva-fraction mixtures at 37 C.*

more complex under highly anaerobic conditions ($\leq$0.0006% O_2) than in an environment containing approximately 1.5% O_2. In the present study, no difference was noted in the growth of the various strains of *S. sanguis* in the complete defined medium or in specific amino acid requirements which were relatable to the two growth environments. In this connection, Carlsson (9) reported that *S. sanguis* 804 required arginine, cysteine, glutamic acid, leucine, methionine, and valine when grown under normal atmospheric oxygen tension. Under strictly anaerobic conditions, this strain could be adapted to grow in a minimal medium containing cysteine with ammonium sulfate as the major nitrogen source (10). Although the conditions may not be strictly comparable, we were unable to adapt strain 804-C8 (a substrain of 804) or any of the other *S. sanguis* strains tested to grow in Carlsson's minimal medium under the anaerobic conditions used in this study. In the case of strain 10556 with respect to amino acid requirements our findings and Carlsson's (10) are in good agreement with one exception. On the basis of single amino acid omissions, Carlsson concluded that *S. sanguis* 10556 required neither glutamic acid or aspartic acid, whereas we found that no growth occurred when both amino acids were simultaneously omitted. When either amino acid was present growth occurred. Therefore, this strain required one or the other of these amino acids, but not both.

S. sanguis strains exhibited a general requirement for arginine and frequently for tyrosine and at least one of the branched-chain amino acids. Arginine and tyrosine were rarely required by *S. mutans*, and many of the strains were inhibited in growth by the branched-chain amino acids, particularly strains of serotype *c* (13). Considering the number and types of amino acids required for growth, *S. sanguis* appears to be more fastidious nutritionally than *S. mutans*.

Proteolytic activity of *S. sanguis* strains 903-1600 and 10556-R was demonstrated both in the fraction consisting of the soluble contents of the cells and in the cellular debris resulting from disruption of the cells. The enzymatic activities of these two fractions were distinguishable on the basis of their relative specific activity toward casein and mucin. The soluble fraction of the cells exhibited a high proteolytic activity on casein but showed no hydrolytic activity toward mucin at pH 6.6 or 7.4. Conversely, the cell debris fractions, while possessing hydrolytic activity for both casein and mucin, had an apparent greater specificity for mucin.

The soluble fraction of the *S. sanguis* cultures appeared to be quantitatively more hydrolytic toward casein than the respective fraction from *S. mutans* K1-R, but on this substrate the enzyme(s) present in the cell debris fraction of *S. mutans* was more hydrolytic than that of *S. sanguis*. When the hydrolytic activity of the cellular fractions of *S. mutans* and *S. sanguis* was examined with germfree rat saliva as the substrate, the fractions from *S. sanguis* were more hydrolytic and less specific than the respective fractions from *S. mutans*. The action of

these two streptococci on the rat salivary proteins appeared to be different and distinguishable. Similar observations have been made previously regarding the hydrolytic action of these oral streptococci on human salivary proteins (12).

S. sanguis and *S. mutans*, which represent the major oral streptococci that colonize tooth surfaces and dental plaque, possess many similar biochemical characteristics (7). Yet these two species differ notably in their ability to cause caries in experimental rodent systems (15, 16, 22), and together with human epidemiological studies (14, 20, 21) *S. mutans* is now generally regarded as a cariogenic microorganism, with *S. sanguis* at best being considered as weakly cariogenic. Although many studies have been concerned with the carbohydrate metabolism and adherence properties of these organisms (17, 24), our studies have focused on the nitrogen metabolism of these organisms. As shown in this and a previous study (13), *S. sanguis* and *S. mutans* require certain amino acids for growth, but there is a definite species differentiation regarding specific requirements. The free amino acids present in saliva are inadequate, in types and amounts, to support growth of either of these oral streptococci (5). Alternate sources of amino acids such as the salivary and dietary peptides and proteins would require further degradation by enzymes associated directly with the organisms or by other exogenous enzymes. Proteolytic activity has been demonstrated in both *S. sanguis* and *S. mutans*, but the specificity of the enzymes from these organisms appears, quantitatively and qualitatively, to be quite different and distinguishable. The relative importance of these findings in the ecology and relative cariogenicity of these organisms remains to be determined.

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