BACTERIA-AGGLUTINATING GLYCOPROTEINS
IN HUMAN SALIVA
An *in vitro* study with special reference to *Streptococcus mutans*

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ABSTRACT


The activity and specificity of salivary glycoproteins (agglutinins) aggregating various human indigenous microorganisms were studied in vitro. The agglutinin reacting with a serotype c strain of Streptococcus mutans was isolated and chemically characterized.

The results can be summarized as follows:

1. Saliva contains agglutinins against predominant oral microorganisms but also against some of the predominant intestinal bacteria. Agglutinin from one individual was active against microorganisms both from this individual and other individuals.

2. Saliva-induced agglutination of S. sanguis was inhibited by antisera to IgA, IgG, IgM, and secretory component while S. mutans agglutination was inhibited only by albumin and antisera to IgA. The differences in inhibition indicate the presence of different agglutinins for the two strains.

3. The agglutinin for an S. mutans serotype c strain was sensitive to a reduction in pH and to treatment with Concanavalin A while an S. mitior agglutinin was not. The tested S. mitior strain, however, adsorbed the S. mutans agglutinin without being agglutinated.

4. The agglutinin reacting with an S. mutans serotype c strain could be desorbed from the microorganisms in a neutral phosphate buffer. Calcium in the order of 10 million molecules per bacteria was needed to restore agglutination induced by the isolated agglutinin.

5. The agglutinin in parotid saliva responsible for aggregation of an S. mutans serotype c strain was characterized as a non-immunoglobulin glycoprotein with no blood group activity. The agglutinin was a polymer with a molecular weight exceeding 5 000 kdaltons while the monomeric agglutinin had a molecular weight of 440 kdaltons. The concentration of the agglutinin in parotid saliva was as low as 0.5 per cent of total protein but because 0.1 µg of the agglutinin caused rapid aggregation of as many as 100 million bacteria the agglutinin is considered highly active.

The results indicate the presence in saliva of different agglutinins with specificity for different bacterial species. High molecular weight glycoproteins seem to play a major role in saliva-induced agglutination of microorganisms and in mechanisms related to clearance and retention of oral microorganisms.

Key words: Agglutinin. Glycoprotein. Saliva. Streptococcus mutans.

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LIST OF ERRATA

Preface, V: 'Accepted' read 'Submitted'.

I. Page 250, line 10: 'mucosa' read 'surfaces'.
Page 254, line 6: 'II' read 'III',
   line 13: '(A) read '(A and C)'.

II. Page 244, line 1: 'Gratthall' read 'Bratthall',
   line 27: 'γ' read 'μ'.
Page 251, line 10: 'and anti-SC antisera' should be deleted.
Page 252, line 21: 'or SC' should be deleted.
Page 254, ref. 11: 'molecular' read 'high molecular'.
   ref. 14: 'dextransucrose' read 'dextransucrase',
   ref. 19: 'folin' read 'Folin'.

III. Page 268, line 7: '10,' read '10, 248-260.'.

V. Page 4, line 32: 'emploied' read 'employed'.
Page 10, line 31: '108 S' read '110 S'.
Page 20: Figure legends for figs. 10b and 11 should change place.
BACTERIA-AGGLUTINATING GLYCOPROTEINS IN HUMAN SALIVA

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**ACKNOWLEDGEMENTS**

**REFERENCES**
PREFACE

This thesis is based upon the following papers:

I  RUNDEGREN, J. and ERICSON, T.
Saliva-induced aggregation of microorganisms from skin, tooth surfaces, oral mucosa, and rectum.

II  ERICSON, T., BRATTHALL, D. and RUNDEGREN, J.
Bacterial agglutination induced by saliva. Inhibition studies with anti-Ig antisera and serum components.

III  RUNDEGREN, J. and ERICSON, T.
An evaluation of the specificity of salivary agglutinins.

IV  RUNDEGREN, J. and ERICSON, T.
Effect of calcium on reactions between a salivary agglutinin and a serotype c strain of Streptococcus mutans.

V  ERICSON, T. and RUNDEGREN, J.
Characterization of a salivary agglutinin reacting with a serotype c strain of Streptococcus mutans.
Accepted for publication in Eur. J. Biochem. (1982).

In the thesis the papers will be referred to by their Roman numerals.
INTRODUCTION

BACKGROUND

The environment in the oral cavity can be compared to those in a bacterial chemostat (CARLSSON, 1980). In the mouth, however, growth is also controlled by host-parasite interactions. Microorganisms invading the mouth meet several salivary defense systems which are modulators of attachment and early growth of microorganisms on oral surfaces. Some modulators have a bactericidal or bacteriostatic effect and others affect adhesion to or clearance of microorganisms from oral surfaces.

Lactoferrin and lactoperoxidase are host factors which can inhibit bacterial growth in vitro. They are both glycoproteins with a molecular weight of approximately 76,000 daltons (QUERINJAN et al., 1971; ROMBAUTS et al., 1967; CARLSTRÖM, 1969). They are present in milk and saliva (MASSON et al., 1966; BAGGIOLINI et al., 1970; HAMON and KLEBANOFF, 1973; REITER et al., 1976) and their antimicrobial activity is based on inhibition of metabolic processes in bacteria. Lactoferrin has the ability to withhold iron which is required for bacterial metabolism (KOCHAN, 1973) and lactoperoxidase catalyzes the oxidation of salivary thiocyanate (SCN-) by bacterial hydrogen peroxide, a process which leads to the formation of active hypothiocyanite (OSCN-). This ion inhibits bacterial growth and metabolism (HOOGENDORN et al., 1977) by oxidizing protein sulfhydryl groups (THOMAS and AUNE, 1978). Lysozyme is another example of a secretory antibacterial substance. It can attack the bacterial cell wall enzymatically, disrupting the ionic balance within the cell.

Aside from substances which affect metabolism, saliva also contains substances which react with microorganisms without changing metabolism. Lysozyme can exert such an effect and cause aggregation (POLLOCK et al., 1976). Another example is secretory IgA which also can agglutinate bacteria and thereby contribute to their clearance from the mouth (WILLIAMS and GIBBONS, 1972). A similar role has been ascribed to some high molecular weight glycoproteins in saliva (GIBBONS and SPINELL, 1970; HAY et al., 1971; KASHKET and DONALDSON, 1972; ERICSON et al., 1975, 1976; EGGERT, 1980 a). The salivary agglutinins are all characterized by being surface active. They have a high affinity for the tooth surface and are therefore also natural members of the dental pellicle. This property also makes them potentially important for bacterial adherence to tooth surfaces and they may play a role in the selection mechanisms which lead
to a differentiation in the distribution of the microflora between different oral surfaces. Early studies of some conditions related to adsorption of salivary glycoproteins to apatite surfaces was presented by ERICSON (1968) and RÖLLA and MATHIESEN (1970), and to tooth surfaces in vivo by SÖNU et al. (1974).

Several properties of *Streptococcus mutans*, a bacterial species linked with the etiology of dental caries, including adherence to hydroxyapatite coated with salivary substances, have been extensively studied (e.g. GIBBONS and VAN HOUTE, 1975; MAGNUSSON and ERICSON, 1976; CLARK et al., 1978).

The potential biological activity of the agglutinating factors warrants more detailed information concerning the structure and function of these substances.

**NOMENCLATURE**

*Agglutination and aggregation* are used synonymously to describe processes by which cells form a three-dimensional matrix of particles.

*Agglutinin* is used for all types of substances with agglutinating activity.

In general, aggregates can be formed by the influence of several soluble bridging components such as calcium ions, immunoglobulins, enzymes, or various types of glycoproteins. The term agglutinin was originally applied only when immunoglobulins caused the cross-linking reactions with cell surface antigens. Examples of non-immunoglobulin agglutinins are a yeast agglutinating substance from rabbit lung lavage (LA FORCE et al., 1979) and an agglutinin for Corynebacterium ovis from sera of lamb (BURRELL, 1978). SCHOUSBOE (1979) described a β2-glycoprotein which aggregated mitochondria. Thus the term agglutinin is no longer reserved for immunoglobulins alone but is used to describe all types of substances with agglutinating activity.

In the papers upon which this dissertation is based, the term agglutinin is used to describe a saliva glycoprotein which aggregates microorganisms. It is also referred to as bacteria-agglutinating glycoproteins (BAGP).
A lectin is a protein with at least two receptor sites which bind to specific glycoses.

The term was introduced by BOYD (1954) to describe plant proteins which were blood group specific and caused erythrocyte aggregation by a reaction with glycoses on the cell surface. Plant lectins were referred to as phytohemagglutinins or phytoagglutinins (LIS and SHARON, 1973). Later, proteins with high specificity for glycoses were found also in man and animal. The term lectin then came to include all such proteins regardless of their reaction with erythrocytes. The term hemagglutinin (HA) was adopted to designate all blood group specific substances in plants, invertebrates, and vertebrates (KABAT, 1956; PROKOP et al., 1968).

Lectins of non-plant origin are exemplified by the HA isolated from the albumin gland of Helix pomatia (PROKOP et al., 1965; HAMMARSTRÖM and KABAT, 1969) and from the hemolymph of the horse shoe crab, Limulus polyphemus (MARCHALONIS and EDELMAN, 1968). In vertebrates, α-galactosylbinding lectins have been found in the ova of certain fishes by PARDOE and UHLENBRUCK (1970), and in mammals a membrane bound lectin in rabbit liver has been described by STOCKERT et al. (1974). Because of the presence of erythrocyte aggregating substances in a variety of sources, the wider definition used for lectins suggested by GOLDSTEIN et al. (1980) is used in the present study.

Bacterial nomenclature after the recommendations of the AD HOC COMMITTEE of the Judicial Commission of the ICSB (1976) was used. The strain isolated in (I) and later used in (III) is called S. mitior. The terms S. mitior and S. mitis have been used for the same type of bacteria.

AGGLUTININS IN SECRETIONS

Lysozyme

Structure and occurrence. Human lysozyme is a basic protein with an isoelectric point of pH 11, a pH optimum of 6.3, and a molecular weight of
Lysozyme was first described by Flemming (1922) who found the enzyme in most body fluids. He showed that saliva and serum contain lower levels of lysozyme than lacrimal fluid, sputum, and nasal secretion which have concentrations approximately 15 times higher than that of saliva. Later investigations (Goldman and Smith, 1973) have shown that human milk contains the highest concentration of lysozyme (300-400 μg/ml) of all extracellular fluids. Stimulated whole saliva contains 60 to 70 μg/ml (Hoerman et al., 1956; Van PALENSTEIN HELDERMAN, 1976). Parotid saliva, not contaminated with leucocyte lysozyme, in contrast to whole saliva, contains about 40 μg per ml (Van PALENSTEIN HELDERMAN, 1976; Skurk et al., 1979). The blood level is approximately 20 μg/ml (Van PALENSTEIN HELDERMAN, 1976) and the serum level about 10 μg/ml (Brandtzæg and Mann, 1964). Salivary lysozyme is secreted by some striated duct cells (Kraus and Mestecky, 1971).

Biologic function. Lysozyme can interact with bacteria in two ways; via its antimicrobial activity and through its bacteria-agglutinating activity. Lysozyme exerts its antimicrobial effect by breaking the glycosidic (1→4) bond between N-acetylmuramic acid and N-acetylglucosamine in the murein peptidoglycan of bacterial cell walls (Salton, 1961). The lysis of gram-positive bacteria having naked cellmembranes (i.e. protoplasts), is enhanced by osmotic breakage induced by NaCl or detergents (Bleiweis et al., 1971; Metcalf and Deibel, 1969; Kruse and Hurst, 1972). Gram-negative microorganisms have a more complex cell wall. Their surface layers contain lipoproteins and lipopolysaccharides which render the murein peptidoglycans less accessible and consequently the cells less sensitive to lysozyme (Miller, 1969). Gram-negative cells can be lysed by lysozyme under acid conditions (Grula and Hartsell, 1957) or in the presence of a mixture of hydrogen peroxide and ascorbic acid (Miller, 1969). Complement and antibody in combination with lysozyme have also been reported to cause lysis of gram-negative bacteria (Adinolfi et al., 1966; Bladen et al., 1973). It seems that the antimicrobial action of lysozyme is enhanced in combination with other antibacterial systems.

The bacteria-agglutinating effect of lysozyme was studied by Metcalf and Deibel (1969) who showed that suspended S. faecium cells incubated for more than 10 minutes with lysozyme clumped to aggregates containing from a few to several hundred cells. Purified hen egg white lysozyme was used by Pollock et al. (1976) in aggregation experiments with S. mutans. Different serotypes of S. mutans agglutinated at different
rates. Strains AHT and BHT, serotypes a and b respectively, showed the fastest agglutination rates and these strains also bound the enzyme at a faster rate than other strains. Both aggregation and enzyme binding were partly inhibited by the saccharides, N-formyl-D-glucosamine and N-acetyl-D-glucosamine. However, a recent report (CHERUKA et al., 1981) indicates that the concentration of salivary lysozyme under normal conditions may be too low to play a role in saliva-induced aggregation.

Secretory immunoglobulins

Occurrence. In 1961 HANSON published data showing immunochemical differences between serum and milk antibodies. CHODIRKER and TOMASI (1963) showed that IgA was the predominant antibody in exocrine secretions and TOMASI et al. (1965) described physico-chemical differences between serum and secretory IgA (sIgA).

The techniques for collection of both breast milk and saliva as well as for IgA analysis have a large impact upon the analytical result and a lack of reproducibility makes many early values difficult to evaluate. Normally values in the order of 100-300 µg/ml of sIgA are reported for whole unstimulated saliva but the individual variations are great (cf. BRANDTZAEG et al., 1970; BRANDTZAEG, 1971 b). In unstimulated parotid saliva sIgA concentrations of 50-150 µg/ml (OON and LEE, 1972; BRANDTZAEG, 1971 b) are found but in stimulated parotid saliva the values are considerably lower (TOMASI and ZIEGELBAUM, 1963; BRANDTZAEG et al., 1970; BRANDTZAEG, 1971 b; OON and LEE, 1972) and inversely correlated to secretion rates (MANDEL and KHURANA, 1969; BRANDTZAEG, 1971 b). The lower concentration in stimulated saliva is compensated by the large volume secreted. The amounts secreted per minute are not lower and often increase (BRANDTZAEG, 1971 b). The same effect is reported for sIgA in human breast milk (HANSON and WINBERG, 1972). The relatively wide variations reported for salivary sIgA are partly due to the use of different IgA-standards in different studies.

Structure and biosynthesis of sIgA. Secretory IgA has a molecular weight of about 380,000 daltons and consists of two monomeric IgA molecules joined by a glycoprotein called the J (joining) chain and a glycoprotein called the secretory component (SC). The J chain has a molecular weight of 15,600 daltons (BAENZIGER, 1979) and a carbohydrate content of 7.5 % (w/w). The SC is produced by secretory epithelial cells and has a molecular weight in the range of 83,000 daltons (BRANDTZAEG,
of which the carbohydrate content constitutes about 19% (w/w) (SLETTEN et al., 1975). Dimeric IgA with incorporated J chain is mainly formed in plasma cells adjacent to glandular epithelium (BRANDTZAEG, 1976). The incorporation of J chain is a prerequisite for complexing of IgA with SC at the surface of secretory epithelial cells (BRANDTZAEG and SAVILAHTI, 1978). The SC probably serves to stabilize the sIgA composite molecule (BRANDTZAEG, 1971a) and to protect it from proteolytic degradation (TOMASI and BIENENSTOCK, 1968; SHUSTER, 1971; PARKIN et al., 1973).

**Biological role of sIgA.** Secretory IgA antibody is ascribed a role in mucosal resistance to infection, referred to as local immunity (PIERCE, 1959; TOMASI and BIENENSTOCK, 1968). Secretory IgA is often referred to as "antiseptic paint" because of its tendency to accumulate in mucus as a complex with other proteins (HEREMANS, 1968). Due to the relative accessibility and high concentrations of sIgA in colostrum, it has received much attention and is attributed a major importance in the immunologic defense of the newborn before they acquire a competent immune system (HANSON and BRANDTZAEG, 1972). Specific antibodies to neonatal pathogens, e.g. *Escherichia coli*, have been found in colostrum (HANSON and WINBERG, 1972; GOLDBLUM et al., 1975; OGRA et al., 1978).

WILLIAMS and GIBBONS (1972) suggested, after observing that a partially purified sIgA fraction from saliva could reduce adsorption of oral bacteria to epithelial cells, that sIgA contributes to the clearance of bacteria from the mouth by the formation of non-adhering aggregates.

The bacterial enzyme IgA protease can cleave the IgA1 subclass into intact Fab and Fc fragments. It has been suggested to serve as a virulence factor for several pathogenic bacteria (PLAUT et al., 1975; MULKS and PLAUT, 1978; KILIAN et al., 1979) involved in e.g. meningitis and gonorrhea. Some strains of *S. mitior* and *S. sanguis*, which are the first bacterial species to colonize cleaned tooth surfaces, produce IgA1 protease (KILIAN and HOLMGREN, 1981). The enzyme production may give these streptococcal species an ecological advantage on the tooth surface.

**High molecular weight secretory glycoproteins**

**Nomenclature.** Glycoproteins are defined as proteins with covalently bound oligosaccharide units, glycoconjugates (cf. REID and CLAMP, 1978). The use of the term "mucous glycoproteins" was suggested for the secretion from
the mucous cell in order to distinguish it from the secretion from the serous cell in e.g. salivary glands.

**General structure.** Glycoproteins from all epithelial secretions have the same general structure. The oligosaccharide units, normally containing only 2-10 glucose molecules, are covalently attached to a polypeptide backbone. Galactose, fucose, N-acetylg glucosamine, N-acetyl-galactosamine, sialic acid and sometimes mannose take part in the formation of the units (for review see MARSHALL and NEUBERGER, 1972). Sialic acid appears in two forms: N-acetyl- or N-glycolyl-neuraminic acid (CLAMP et al., 1978). When sialic acid and/or fucose (CLAUSER et al., 1972) are members of the oligosaccharide chains they always occur in terminal positions. The carbohydrate content constitutes up to 60-70% of the glycoprotein molecule. The oligosaccharide contains no repetitious sequences (GOTTSCHALK, 1972) and is bound to the protein core with either N-acetyl-galactosamine, linked to the protein core with an oxygen atom, or N-acetylg glucosamine linked with a nitrogen atom (MORRIS and REES, 1978). The protein core of most mucus glycoproteins have a high content of glycoseconjugate-binding threonine and serine (BAIG et al., 1973; OEMRAWNSINGH and ROUKEMA, 1974; SACHDEV et al., 1980), but the glycoconjugates are also linked to aspartic acid and glutamic acid in ester linkages (SPIRO, 1973).

In mucus, native glycoproteins have molecular weights of up to several millions and the oligosaccharide units are attached to about every third amino acid in the polypeptide chain (REID and CLAMP, 1978). The glycoprotein structure has been compared to a test-tube brush, where the bristles represent carbohydrate side chains.

**Biosynthesis.** The biosynthesis and secretion of glycoproteins are now fairly well understood (for review see PALADE, 1975; PHELPS, 1978; DAVIS and TAI, 1980). Synthesis begins in the ribosomes attached to the endoplasmic reticulum. The nascent polypeptide chain moves through the endoplasmic membrane and is released into the cisternal space of the endoplasmic reticulum. Here the first step of glycosylation of the polypeptide chain occurs mediated by glycosyltransferases. The embryonic glycoprotein is further transported to the Golgi apparatus, where the final glycosylation occurs. Within the Golgi complex a concentration of the macromolecules takes place that ends in a budding off of a part of the Golgi wall to form the mature secretion granules. These granules function as storage compartments until the secretory proteins are discharged into the glandular lumen.
Biological function in general. Glycoproteins have a long evolutionary history of protecting organisms such as snails and worms from desiccation and fish from damage caused by changes in osmotic pressure (Jones, 1978).

In the gastrointestinal tract mucus glycoproteins play an important role as lubricants of the intestinal walls and as protectors of the gut epithelium from microorganisms and proteolytic enzymes. An impaired production of mucus favours the development of gastric and duodenal ulcer (Velican et al., 1970; Clamp et al., 1978). Glycoproteins are relatively resistant to the action of proteolytic enzymes (Gottschalk and Fazekas de St Groth, 1960).

Apart from these general roles of secretory glycoproteins, there are also glycoproteins with specific biological activities. In this group are found e.g. blood group substances, enzymes, hormones, and SIgA.

GLYCOPROTEINS MEDIATING ADHESION AND AGGLUTINATION IN OTHER IN VIVO SYSTEMS

Glycoproteins in vertebrate tissues are recognized as factors in the mechanism of cell-cell recognition. Some have been called "endogenous lectins" and ascribed functional roles in recognition processes (for review see Simpson et al., 1978). Thus they are potentially important for the in vivo adhesion and aggregation of cells.

Some other examples of glycoproteins active at cell surfaces are given in a review of Ashwell and Morell (1977). Since the discovery of sialic acid by BLIX (1936) the role of sialic acid in receptor mechanisms at cell surfaces has been recognized (for review see Burnet, 1951). In the aggregation of oral streptococci neuraminidase treatment of saliva eliminates the saliva induced aggregation of S. sanguis (McBride and Gisslow, 1977; Levine et al., 1978).

Fibronectin is an affinity glycoprotein found in soluble form in blood and in a less soluble form in connective tissue (for review see Vaheri and Mosher, 1978) and at epithelial cell surfaces. Loss of fibronectin is suggested to result in an increased susceptibility to Pseudomonas aeruginosa infection of the upper respiratory tract (Woods et al., 1981).
SALIVA-INDUCED AGGLUTINATION OF BACTERIA

Saliva-induced aggregation of oral microorganisms was first reported by GIBBONS and SPINELL (1970). They found agglutinating activity in whole, parotid and submaxillary secretions. The agglutinating system in whole saliva for a coccobacillus strain 26 was studied. It was stable after heating at 60°C for 30 minutes and when the activity was studied between pH 4.5-9, activity was found between pH 5 and pH 7.5. The agglutinating factor was inactivated by treatment with EDTA and reactivated by calcium ions at a concentration of 0.3 mM. During bacterial agglutination, the aggregating factor was bound to the cell surface but could be recovered after EDTA treatment. HAY et al. (1971) continued to analyse the agglutinating system in whole saliva for coccobacillus strain 26. They reported the active material to be a high molecular weight glycoprotein, high in serine and threonine, and with an isoelectric point below pH 3. Analytical ultracentrifugation revealed a major component with a sedimentation coefficient of 13 S, which corresponds, for globular proteins, to a molecular weight of approximately 300,000 daltons. However, during filtration in agarose gel with an exclusion limit of 150 x 10^6 daltons, the active material was eluted in the void volume. The discrepancy in the molecular size was suggested to result in part from random coil properties of the molecule, which would attribute to aberrant chromatographic behaviour.

Another type of calcium-dependent agglutinin, named conglutinin-like factor, has been suggested to exist in saliva (EGGERT, 1980 ab). Originally, conglutinin was the name given to substances in bovine serum that caused excessive clumping of erythrocytes, which had reacted with antibody and complement (BORDET and STRENG, 1909). Conglutinin has a carbohydrate content less than 1% and a molecular weight of 750,000 daltons. In the presence of sodium dodecyl sulphate (SDS) and mercaptoethanol (ME) the molecular weight was around 100,000 daltons (LACHMANN, 1967). Conglutinin is also reported to be heat stable (COOMBS et al., 1961). Lately, conglutinin-like factors (CLF) have been found both in human amniotic fluid and saliva (EGGERT, 1980 abc). Conglutinin-like factors react with and are inhibited by C3 but unbound salivary CLF exists in excess and may agglutinate some oral streptococcal strains (EGGERT, 1980 c). However, a very weak distinction is made between CLF and other salivary glycoproteins with bacteria-agglutinating properties.
ERICSON et al. (1976) reported data on a partly purified parotid glycoprotein which agglutinated a serotype c strain, KPSK 2, of _S. mutans_. The agglutinin was isolated by different chromatographic procedures and by preparative centrifugation. They reported an apparent molecular weight of 106-107 daltons based on gel filtration data and low penetrability in the spacer gel of a 7.5 % polyacrylamide gel during electrophoresis. The carbohydrate content was estimated to be approximately 60 % (w/w). When the glycoconjugate was removed by β-elimination the aggregation activity was lost. The free glycoconjugate did not block receptor sites on the bacterial surface because aggregation could be initiated when the complete agglutinin was added in the presence of the glycoconjugate.

EGGERT (1980 b) used phenol-water partitioning of whole saliva to extract the fraction containing the non-immunoglobulin bacterial aggregating factors. These factors were found to have an apparent sedimentation coefficient of 60-90 S indicating molecular weights of several million daltons. The aggregating factors lost their activity after EDTA-treatment and had no blood group activity.

WILLIAMS and GIBBONS (1975) isolated an agglutinin-containing fraction from whole unstimulated saliva using a Bio-Gel A-15 column. The material in the fraction was active against some strains of _S. salivarius, S. sanguis_ and _S. mitior_. The fraction also contained blood group (BG) activity as judged by the reduction in adhesion of bacteria to epithelial cells after addition of BG-antisera. Similar data were presented by GIBBONS and QURESFI (1978). FALKLER et al. (1979) studied a salivary material obtained by precipitation with ammonium sulphate. They found both agglutinin activity and hemagglutination inhibition activity in the isolate. KONDO et al. (1978) isolated a fraction containing glycoprotein by Sepharose 2B column chromatography of whole unstimulated saliva. They found both agglutinin and blood group activity in the material. In these four studies there are not sufficient data to show identity between agglutinating substances and the substances carrying blood group activity.

LEVINE et al. (1978) found that a 150,000 dalton glycopeptide fragment of a high molecular weight glycoprotein was unable to induce aggregation but could block the aggregation inducible with the glycoprotein. They also showed that removal of sialic acid from salivary glycoproteins resulted in a loss of aggregating activity for _S. sanguis_ but not for _S. mutans_.

Support for specificity of salivary agglutinins has been provided in several reports. Aggregating factors with different chemical characteristics have been demonstrated for _S. sanguis_ and _S. mitis_ (KASHKET and DONALDSON, 1972; KASHKET and HANKIN, 1977), and a specificity of agglutinins for different serotypes of _S. mutans_ has been shown (ERICSON et al., 1975; ERICSON and MAGNUSSON, 1976). Separate salivary aggregating factors for _S. sanguis_ and _S. mutans_ were found by HOGG and EMBERY (1979) who showed that a salivary component isolated by DEAE-cellulose and gel filtration chromatography aggregated _S. sanguis_ but not _S. mutans_ cells. The fraction containing the aggregating factor also contained blood group A activity and was devoid of IgA. The relatively insensitive double immunodiffusion technique was used to detect IgA. EVERHART et al. (1980) chromatographed saliva on a Sepharose Cl-2B column and found glycoprotein with agglutinin activity for _S. mutans_ in the void volume material and sIgA agglutinins in the second peak.

BRATTHALL and CARLEN (1978) measured the concentration of sIgA antibodies in parotid saliva of three individuals by ELISA technique. Saliva samples from the three subjects, who were of different blood groups, aggregated some _S. sanguis_ and _S. mutans_ strains to the same extent although the titers of specific sIgA antibodies to these strains clearly differed. ERICSON and MAGNUSSON (1976) adsorbed salivary _S. mutans_ agglutinins to hydroxyapatite. They found a difference in affinity of the agglutinins for different serotypes and 95% of the IgA remained in the saliva when all of the agglutinin for serotype _d_ strains of _S. mutans_ was removed. Non-immunoglobulin agglutinins were suggested to play an important role in saliva-induced aggregation of microorganisms.

β2-microglobulin which is a non-glycosylated protein (Mr 11,600), present in free form in epithelial secretions and constituting the light chain of the major histocompatibility antigen (BERGGÅRD et al., 1980), cannot cause bacterial aggregation in the monomeric form in vitro but can affect aggregation when the monomers are aggregated (ERICSON et al., 1981). It is suggested that when β2-microglobulin forms complexes with secretory macromolecules aggregation may be induced.
AIMS

The aims of the present investigation were:

to compare patterns for saliva-induced agglutination of microorganisms from different human biosurfaces

to study the influence of serum components and antibodies to immunoglobulins on bacterial agglutination induced by saliva

to evaluate the specificity of salivary agglutinins

to develop a method for isolation of the salivary agglutinin reacting with a *Streptococcus mutans* serotype c strain

to isolate and characterize chemically the salivary agglutinin responsible for aggregation of a *Streptococcus mutans* serotype c strain
METHODS

Microorganisms

All bacterial strains used in this thesis, except for the \textit{S. sanguis} strain DB 401 isolated by Bratthall (II) and the \textit{S. mutans} strain KPSK 2 (CARLSSON, 1967) used in (II), were isolated anaerobically according to FULGHUM (1971). The isolation procedure is described in detail in (I). All strains were kept frozen at -80°C in an anaerobic atmosphere. Before use they were thawed and plated on blood agar in an anaerobic glove box. Broth cultures of microorganisms were obtained with pre-reduced media in the anaerobic box except for experiments carried out with \textit{S. mutans} strain TH 16 in (IV) and (V).

\textit{S. mutans} has lately been classified as an anaerobic species (HOLDEMAN et al., 1977) but it is capnophilic and the strain TH 16 grew well aerobically in a trypticase broth supplemented with 0.5 \% glucose (JORDAN et al., 1960).

Saliva

Individual variations, circadian variations and degree of stimulation are important factors to be considered when collecting saliva samples (FERGUSON and BOTCHWAY, 1980). To standardize the collection procedure in the present study all saliva samples were collected in the morning one hour after breakfast. Resting whole saliva was used in the experiments in (II) and some experiments in (III). In all other experiments resting parotid saliva was used. During the resting conditions no motoric activity of tongue or lips was allowed and the person was sitting with the head bent forward keeping the face in a horizontal position to allow saliva to flow passively from the mouth. The parotid saliva was collected with Lashley cups. It contained less then 900 colony forming units (CFU) per ml when grown on blood agar in the anaerobic glove box. This amount is negligible compared to the number of microorganisms, \(10^8\) CFU/ml, present in the saliva-bacteria mixtures studied. The parotid saliva used for isolation of purified BAGP (V) was collected from one person by pooling portions collected on different occasions. Each portion (7-8 ml) was diluted with one volume of 0.01 M potassium phosphate buffer containing 0.154 M NaCl (PBS) and then shell-frozen in round bottom flasks. All saliva collections were made into ice-chilled tubes and secretion rates were recorded.
Measurement of saliva-induced agglutination

Bacterial aggregation was determined by means of a spectrophotometrical method (ERICSON et al., 1975). The method is based upon the decrease in absorbance with time in a bacterial suspension when aggregation occurs. A volume of 1.2 ml of a bacterial suspension with an optical density of 1.5 at 700 nm measured in a Beckman DB-GT spectrophotometer was mixed with a 0.6 ml sample containing agglutinin. The bacterial suspension and the agglutinin-containing sample were first incubated separately at 37°C for 5 minutes, then mixed quickly and placed in the spectrophotometer. The aggregation was followed continuously at 37°C for at least 60 minutes. Compared to the titration of agglutinins in 2-fold serial dilutions, the present method has the advantage of following the course of events during aggregation thus giving information on the rate and the pattern of aggregation.

In III and V a mathematical expression for the aggregation was used (ERICSON et al., 1975) to facilitate a quantitative comparison between different aggregation curves for a given strain. The aggregation was expressed as:

$$\ln\left(\frac{A_0 - A}{A}\right) = mt + b,$$

where $A_0$ is the absorbance at zero time and $A$ absorbance at time $t$ in minutes. The $m$ values, which can be calculated for a set of $(A, t)$ values, is related to the slope of the aggregation curve at the inflection point and describes the aggregation rate or the agglutinin activity.

To measure aggregation with isolated agglutinins for the S. mutans strain TH 16 (V) addition of calcium ions was necessary. The calcium-dependence of the TH 16 agglutinin was studied (IV).

Test of specificity of agglutinins in saliva

In order to reveal whether different salivary agglutinins were present, saliva samples were treated in different ways before measuring bacterial aggregation. Serum components or rabbit antisera against human IgA, IgG, IgM, and secretory component were added to aggregation mixtures (II) and saliva samples were adsorbed with one bacterial strain before measuring aggregation of another strain (III). In a second set of experiments (III)
inhibition of the agglutinins responsible for aggregation of an \textit{S. mutans} strain and an \textit{S. mitior} strain was tested by lowering pH or adding concanavalin A.

\textbf{Isolation of a salivary agglutinin}

Earlier work on isolation and purification of salivary agglutinins have utilized a number of different chromatographic procedures (HAY et al., 1971; ERICSON et al., 1976; LEVINE et al., 1978; HOGG and EMBERY, 1979) or rather insensitive precipitation procedures (EGGERT, 1980 b; FALKLER et al., 1979).

In the present study biospecific adsorption was used to isolate a salivary agglutinin reacting with a serotype \textit{c} strain of \textit{S. mutans} (IV, V). The agglutinin in a parotid saliva sample was first adsorbed to the microorganisms, and then desorbed in a 0.01 M PBS in the absence of Ca. The desorption was performed by a thorough mixing on a Whirlimixer\textsuperscript{R}. The microorganisms were removed by centrifugation and the agglutinin activity remained in the supernatant. This method has the advantage of selecting salivary substances reacting only with the homologous strain. In the next step the desorbed agglutinin was concentrated and separated from low molecular weight material by two consecutive centrifugations at 100,000 x g for 18 h at 4\textdegree C. The concentrated agglutinin collected from the bottom part of the centrifuge tubes was diluted 7-fold in PBS and the ultracentrifugation was repeated to further eliminate contamination of lower molecular weight material. The agglutinin was then filtered on an agarose column (Bio-Gel A 5.0 m). The active material which appeared in the void volume fractions was concentrated again by ultracentrifugation and ultrafiltration in an Amicon cell equipped with a PM 10 Diaflo membrane.

\textbf{Characterization of a salivary agglutinin}

The methods used for determination of molecular weight included gel filtration, sedimentation velocity measurements, and polyacrylamide gel-electrophoresis (PAGE) of native and sodium dodecyl sulphate (SDS) treated agglutinin. The analytical ultracentrifugation was made in a MSE Centriscan 75 centrifuge of native BAGP (18,000 x g) and BAGP treated with SDS (200,000 x g) at different temperatures. Eight measurements of boundary distances were made during each run. The partial specific volumes were calculated from values for the individual carbohydrates and amino
acids (COHN and EDSALL, 1943; GIBBONS, 1972). PAGE was conducted made in a Pharmacia GE-2/4 apparatus according to recommendations by the manufacturer. SDS-treatment was affected by heating BAGP in 1 % SDS-solution at 1000°C for 5 minutes.

Heat stability of BAGP was tested by heating samples at 37°C, 80°C, 90°C, and 100°C for 30 minutes. The aggregating activity of the heated BAGP was then tested. Blood group activity was determined by the method of PEREIRA et al. (1969) by measuring hemagglutination inhibition. Lysozyme was measured by the method of OSSERMAN and LAWLOR (1966) using the Quantiplate™ Lysozyme Test Kit of Kallestad (Chaska, Mn, USA). Protein was analysed by the method of LOWRY et al. (1951) or the Coomassie Brilliant Blue technique of BRADFORD (1976). The amino acid composition was measured after hydrolysis in 5.7 M HCl in N₂ atmosphere for 24 hours. The analysis was made in a Beckman 120 C amino acid analyser.

Fucose was determined with the method of GIBBONS (1955), sialic acid with the method of WARREN (1959), hexose with the anthrone method (JERMYN, 1975), and hexosamine according to Elson-Morgan after hydrolysis for 3 hours in a 6 M HCl (BLIX, 1948). The acid was then removed by freeze-drying. IgA was determined with the Immuno Bead™ method of Bio-Rad using FITC-conjugated antibodies. Readings were made in an Aminco Fluorometer, Type J4-7439.
RESULTS

I Saliva-induced aggregation of microorganisms from skin, tooth surfaces, oral mucosa, and rectum.

Microorganisms from one individual were aggregated by saliva from this individual and also by saliva from two other individuals. The great majority of strains collected from the tooth surfaces and from the oral mucosa were aggregated by saliva. The spectrophotometric recording of sedimentation as a measure of aggregation followed a sigmoid pattern. Strains collected from the skin of the upper lip, which were Propionibacterium acnes and Staphylococcus epidermidis, did not aggregate. Of the eight fecal strains tested, all but one were aggregated by saliva although five had an unusual pattern of aggregation; a fairly rapid initial decrease in optical density was followed by a slow decrease. The colonization pattern of the different surfaces could not be exclusively explained by the activity of the BAGP and the combination of several regulating mechanisms can be assumed. Furthermore in the case of the soft tissues, primary colonization could not be observed, only the composition of the established microflora.

II Bacterial agglutination induced by saliva. Inhibition studies with anti-Ig antisera and serum components.

Further studies on the specificity of agglutination reactions were necessary and this was accomplished by testing the effect of serum components and antisera against human immunoglobulins on the BAGP induced aggregation of two S. mutans strains and one S. sanguis strain.

For the S. sanguis strain, inhibition of aggregation was obtained in the presence of an excess of anti-sIgA, anti-IgG, anti-IgM antisera and of anti-SC antisera. S. mutans aggregation was inhibited by anti-IgA antisera and by human albumin only. The results indicated differences in saliva-induced aggregation of S. sanguis and S. mutans. The inhibition of S. sanguis aggregation by all commercial antisera may point to a non-specific protein-protein interaction or a type of specific interaction with a component in common with all antisera. The interaction with the S. mutans agglutinin seemed more specific because the anti-IgM and anti-IgG did not interfere with aggregation while anti-IgA did. However, albumin and blood agar bacteriological medium resulted in in-
hibitation of aggregation. In relation to the concentrations of sIgA found in the saliva used for aggregation, the anti-IgA antisera had to be added in about 5-fold excess to fully inhibit aggregation. The antisera contributed to a 6-fold increase in protein concentration compared to that of saliva. It was also shown that only the most centrifugal parts from the 75,000 × g centrifugation of parotid saliva aggregated S. mutans, while IgA could be detected in all 4 fractions. The different effects of serum proteins on aggregation of the S. mutans and S. sanguis strains required further studies of the specificity of agglutinins reacting with strains from different surfaces.

III An evaluation of the specificity of salivary agglutinins.

The effect of salivary agglutinins on aggregation of selected bacterial strains was tested by using strains described in (I): S. mutans from tooth surfaces, S. mitior from the oral mucosa, Staphylococcus epidermidis from the skin of the lip, and Bacteroides amylophilus from rectum.

By absorbing salivary agglutinins from whole resting saliva from one person with one strain and reacting the supernatant with another strain, similarities and dissimilarities between agglutinating systems could be demonstrated. The Staph. epidermidis strain was not aggregated by any of the saliva samples and did not adsorb agglutinin reacting with the other strains. The B. amylophilus strain was aggregated initially but a relatively high absorbancy remained (cf.I). B. amylophilus clearly adsorbed the agglutinin responsible for S. mutans aggregation. S. mitior aggregation was not affected by S. mutans adsorption of saliva but S. mitior adsorption of saliva gave an inhibition of S. mutans aggregation.

The saliva-induced aggregation of the S. mutans strain was inhibited at a pH level below 5 and Con A concentrations above 0.1 mg/ml whereas aggregation of the S. mitior strain was not inhibited at low pH or in the presence of Con A. Thus the agglutinins reacting with the S. mutans strain and the S. mitior strain are chemically different. From the adsorption experiments it was evident that the S. mitior strain carried two receptor sites; one for the S. mitior agglutinin and one for the S. mutans agglutinin, while the S. mutans strain had only receptor sites for its own agglutinin. The pronounced sensitivity of the S. mutans agglutinin but not of the S. mitior agglutinin for Con A implies significant differences in the glycoconjugates of the agglutinin.
IV Effect of calcium on reactions between a salivary agglutinin and a serotype C strain of STREPTOCOCCUS MUTANS.

Further characterization of the agglutinin required a more purified material and a biospecific adsorption method for isolation of the agglutinin reacting with S. mutans was developed. Agglutinin from resting parotid saliva of one person was adsorbed by S. mutans strain TH 16. The adsorbed agglutinins could be desorbed by washing and centrifuging the agglutinin-coated bacteria in a 0.01 M phosphate buffer, pH 6.8, containing 0.15 M sodium chloride (PBS). The desorbed agglutinin was detected in the supernatant and required calcium ions at a concentration of 0.1 mM to regain most of the initial activity. Other divalent cations like cobalt, magnesium, and strontium could not restore agglutinin activity.

V Characterization of a salivary agglutinin reacting with a serotype C strain of STREPTOCOCCUS MUTANS.

The biospecific adsorption method developed in (IV) for isolation of the agglutinin reacting with S. mutans TH 16 was used as the first step of purification. It was followed by preparative centrifugation, filtration through Bio-Gel A-5.0 m, extensive dialysis, and positive pressure diafiltration in the Amicon system using PM 10 Diaflo membranes.

As little as 0.1 µg of the isolated agglutinin was required to obtain the aggregation activity present in 300 µl of parotid saliva. The purification protocol resulted in a 700-fold purification by the methods employed.

From the results of gel filtration and gel electrophoresis experiments it was shown that the agglutinin, in the native state, had a molecular weight exceeding 5 million daltons. This result was confirmed in the sedimentation velocity experiments which gave a value for $S_{20,w}^0$ of 110 S corresponding to about $10^7$ daltons for the isolated agglutinin. After treatment of the native agglutinin with SDS, subunits of $4.4 \times 10^5$ daltons were obtained. The isolated agglutinin was heat stable even after 30 min at 90°C. It had no detectable blood group activity and contained no lysozyme. Minor contamination with SIGA may occur. Analysis, using fluorescent antibody in the Immuno Bead system (Bio-Rad Laboratories, Calif.),
revealed 5.7 % IgA. This would translate to less than 1 slgA molecule per agglutinin complex of 107 daltons or approximately 15 subunits. The possibility that slgA and the agglutinin contain common antigenic determinants cannot be excluded.

The agglutinin contains 45 % (w/w) carbohydrate. The molar ratios of the constituent carbohydrates were hexosamine 2.5, hexose 4.5, fucose 4.0, and sialic acid 1.0. Aspartic plus glutamic acids constitute 21.1 % and serine plus threonine 17.6 %. Hexosamine may, therefore, be linked both with ether linkages to serine and threonine and ester linkages to aspartic and glutamic acid.
GENERAL DISCUSSION

Nature of salivary agglutinins

One or several agglutinins? The differences in rates of saliva-induced aggregation obtained for different bacterial species with the same saliva sample (I, II) indicate the involvement of different agglutinins for aggregation of the various strains and/or different receptor sites or numbers of receptor sites in the bacterial wall. The possibility that varying numbers of receptor sites on the bacterial wall can explain different rates of aggregation induced by saliva may be ruled out by the fact that the relative rates vary when saliva from different persons is used. This would not be the case if aggregation was induced by one salivary component and the variations in rates were obtained by variations in numbers of cell wall receptor sites.

There are several observations indicating that different substances are involved in the aggregation of different microorganisms. Some degree of specificity seems to occur.

Confirmatory evidence of different salivary aggregating substances reacting with different oral streptococci was presented in (III), where treatment of saliva with concanavalin A or lowering the pH of saliva showed that glycoproteins of different conformation and carbohydrate content aggregated an S. mitior strain and an S. mutans serotype c strain. MIRTH et al. (1977, 1979) also found that saliva-induced aggregation of S. mutans was inhibited by Con A. Con A alone (III) did not induce aggregation of a S. mutans serotype c strain or S. mitior during a one hour incubation period. This is in agreement with earlier findings for S. mutans serotype c strains (HAMADA et al., 1977; NONAKA et al., 1981) and some S. mitior strains (NONAKA et al., 1981). Using Con A for pretreatment of saliva samples KASHKET and GUILMETTE (1975) suggested that different salivary substances could aggregate different strains of S. sanguis whereas they, in agreement with the findings in (III), found virtually no influence of Con A on salivary substances aggregating S. mitis. In addition, it was shown (KASHKET and HANKIN, 1977) that differences exist between S. sanguis and S. mitis salivary aggregating factors. The S. sanguis factor was inhibited by SH-reacting compounds like iodoacetamid whereas the S. mitis factor was unaffected. The two factors also had different affinities for hydroxyapatite (KASHKET and DONALDSON, 1972).
Different rates of aggregation induced by whole saliva have been obtained for *S. sanguis*, *S. mutans* serotypes a, b, c, and *S. salivarius* (ERICSON et al., 1975). In a study by ERICSON and MAGNUSSON (1976) large variations in affinity of salivary agglutinins for hydroxyapatite for different strains of *S. mutans*, *S. sanguis* and *S. mitis* were found. The agglutinins for different serotypes of *S. mutans* had different affinities for hydroxyapatite but strains within a serotype showed similar adsorption isotherms for hydroxyapatite.

The exact nature of the agglutinins studied in the cited investigations is not revealed by the data presented but the findings indicate that salivary high molecular weight glycoproteins are responsible for the aggregation. In some studies where antisera have been used the conclusion was drawn that sIgA was responsible for aggregation. A distinction has therefore to be made between sIgA or BAGP being the active components.

**Secretory IgA or glycoprotein agglutinins?** Several experiments have been performed where anti-Ig antisera have been added to salivary aggregation systems. Inhibition has been taken to indicate that immunoglobulins are the active agglutinin (ARNOLD et al., 1976; LILJEMARK et al., 1979). However, some commercial antisera contain high concentrations of other serum proteins which may interfere with the aggregation in several ways. In experiments similar to the above, comparable results using commercial antisera were obtained (II). Treatment of saliva with anti-Ig antisera and other serum components could inhibit aggregation especially of an *S. sanguis* strain but also of *S. mutans* serotype *c* strains. However, it was also shown that human albumin caused inhibition and that strains of *S. mutans* freshly isolated from blood agar plates did not aggregate when parotid saliva was added (II). The later observations imply that it cannot be concluded that immunoglobulins were the active agglutinin in the saliva samples used. The effect of all tested antisera on *S. sanguis* agglutination rather suggest unspecific protein-protein interactions or the presence of a common impurity which can interfere with the aggregation of *S. sanguis*. The unpredictable effect of several serum proteins on cell aggregation should be recognized. Thus, albumin was also found to inhibit platelet aggregation (COLLIER and MCDONALD-GIBSON, 1980). It should be observed that antisera are often stabilized with albumin and that albumin in some studies was added to saliva samples in aggregation experiments.
when low or no activity was found (ARNOLD et al., 1976). Many observations in the literature cannot be properly interpreted until the relative binding of albumin to different salivary proteins e.g. sIgA and BAGP is known.

The observation by BRATTHALL and CARLEN (1978) that there was no correlation between titers of specific sIgA and rate of aggregation also seems to exclude a major influence of sIgA on aggregation.

Some studies of bacterial aggregation induced by saliva from immunodeficient persons have been undertaken. Parotid saliva collected from immunodeficient patients agglutinated S. mutans, strain KPSK 2 to the same extent as saliva collected from immunocompetent subjects (HANSON, L. Å. and ERICSON, TH., personal communication). In a report by BRATTHALL and BJÖRKANDER (1980) of a study of 11 immunodeficient patients the same observation was made.

There may still be some uncertainties concerning the involvement of sIgA in bacterial aggregation induced by salivary high molecular weight glycoprotein fractions. In an attempt to clarify this, and other points, we have purified an S. mutans agglutinin from unstimulated parotid saliva (V). The purification procedure which began with biospecific adsorption steps led to a 700-fold increase in specific activity of the isolated BAGP.

Several of the described properties of the purified BAGP are not congruent with the concept that antibodies are involved in aggregation. Also the PAGE pattern of SDS-treated BAGP is different from that of sIgA (cf. MESTECKY et al., 1972).

The content of sIgA in the isolated BAGP was tested by the Bio-Rad ImmunoBead method using fluorescent antibody for detection and sIgA as a standard. The sensitivity of this technique is high. Assay of the isolated BAGP indicated that 5.7% of the BAGP material was sIgA. This would mean that there would be 1 sIgA molecule for approximately 20 BAGP molecules. This would mean less than 1 sIgA molecule per BAGP complex, which contained 10-15 BAGP monomers. The possibility exists that the anti-chain antibody recognized an antigenic determinant common to BAGP and sIgA. Different glycoproteins have many structural similarities. The linking region between the protein and glycoconjugate would be a strong candidate for a determinant present in most glycoproteins.
Secretory IgA, in common with most other secretory substances is a glycoprotein. The majority part of the carbohydrate is located at the Fc terminal end of the heavy chains (TOMANA et al., 1972; BAENZIGER and KORNFELD, 1974), to which the glycoprotein molecules J-chain and SC are attached. The Fab-fragment of the IgA molecule contains different amino acid sequences and is called the variable region, while the hinge region and the Fc-fragment are called the constant region. The hinge region, however, has been shown to have a variable degree of glycosylation (PIERCE-CRETEL et al., 1981) which may influence the conformation of the IgA-molecule and possibly its biological role. Antisera raised against sIgA or the α-chain of IgA is commonly used for IgA-determinations. Because of the presence of oligosaccharides in 7 S IgA and in the other molecular subunits of sIgA it is likely that antisera made against IgA or sIgA may cross-react with oligosaccharide antigenic determinants in other epithelial glycoproteins as well. The 0-glycosidically linked oligosaccharides of the hinge region of sIgA also represent the precursors of oligosaccharides of glycoproteins which have blood group activity.

Consequently, the possibility exists that non-immunoglobulin glycoproteins can share antigenic determinants with sIgA. This could lead to false interpretations of results from antigen-antibody experiments performed with salivary antigens. A positive antibody reaction against sIgA or its subunits may therefore not necessarily reveal the presence of sIgA but could merely imply reactions with other glycoproteins. The difficulty in designing the proper experiment to rule out this possibility is great but the pool of data seem to indicate that the possibility of sIgA taking part in the aggregation reaction induced by the purified BAGP is small. In vivo, however, the situation may be quite different. A complex macromolecule like BAGP is likely to interact with a number of other molecules. The possibility of sIgA, lactoperoxidase, lactoferrin, lysozyme, glucosyltransferase and others to be intimately associated with BAGP is obvious. The possibility of complex formation between epithelial glycoproteins and biologically active proteins in secretions has been pointed out earlier (HEREMANS, 1968; HANSON and BRANDTZAEG, 1972; BROECKHUYSE, 1974; BOAT and WAN CHENG, 1980). The addition of an antiserum to complexes of this magnitude could well cause coprecipitation and enhance adverse protein-protein reactions creating confusing results in terms of lost biological activity.
Chemical nature of the isolated BAGP

A biospecific adsorption procedure was used as initial step in the purification of BAGP from parotid saliva (V). The saliva sample was mixed with the S. mutans strain under the conditions used for bacterial aggregation. The bound, active components could be desorbed in PBS in the absence of Ca. This fraction constituted 1% of the total protein in parotid saliva. From the purification protocol it appears that the specific activity expressed as m/mg protein increased close to 100 times in this single step. Further purification by several steps of preparative ultracentrifugation, gel filtration in Bio-Gel A-5.0, and filtration and concentration in an Amicon ultrafiltration cell using PM 10 Diaflo membranes led to a 700-fold increase in specific activity.

The product gave single peaks in PAGE and analytical ultracentrifugation. The native product was estimated to have an apparent molecular weight (Mr) of \( \approx 107 \) and an S-value of 110. SDS-treatment in the presence of mercaptoethanol gave considerably smaller fragments, Mr = 4.4 \( \times 10^5 \) and it was concluded that the isolated product appeared as a complex of 15 monomeric units. The nature of the linking bonds is not known but a large complex like this, containing close to 50% carbohydrate, would serve very well as a bridging substance in the formation of threedimensional aggregates. The biological activity is lost when pH is decreased to 5 or when 0.1 mg Con A is added to the aggregation mixture. Ca is required for aggregation to occur. It has been calculated that a large number of Ca ions are needed (107 per bacteria and 104 per agglutinin molecule when the molecular weight was estimated to 106) to support aggregation. Possibly the effect of Ca is to reduce the zeta-potential of bacteria to facilitate the approach of two bacteria to each other (IV). The purified agglutinin is very heat stable. It can be heated at 90°C for 30 min and still retain most of its activity.

Chemical analysis revealed 45% of the material to be carbohydrate. The fucose content was high; 33% of the glycoconjugate. The hexose was dominating, 38%, with hexosamine constituting 21% and sialic acid 8% of the glycoconjugate. A high fucose content in bacteria-agglutinating glycoproteins from saliva has been observed before (ERICSON et al., 1976; LEVINE et al., 1978). LEVINE et al. (1978) found blood group activity in their isolate whereas none was detected in the present study; the glycoconjugate of their material constituted a larger portion of the glycoprotein.
The variations in composition of various isolated BAGP are remarkable and the reasons for these variations are difficult to analyse. Different secretions and procedures were used for isolation of the glycoproteins, and different techniques were used for assaying BAGP activity. To avoid confusion by introducing the possibility of interindividual variations one person was used as donor in the present isolation of a salivary agglutinin.

Biological significance of agglutinins in saliva

The function of salivary aggregating factors in the oral cavity is complex. Initially these factors were considered to contribute to interbacterial adhesion in plaque formation (GIBBONS and SPINELL, 1970). Later theories for the action of salivary agglutinins include reducing bacterial adherence to oral surfaces, which would constitute a mechanism for bacterial clearance (GIBBONS and VAN HOUTE, 1975; ERICSON et al., 1976; MAND, 1976, 1979). In the oral cavity salivary components exist as solutes in saliva and adsorbed as a film of proteins on tooth and mucosal surfaces. This immobilized film on the tooth surfaces, called the acquired pellicle, contains biologically active proteins (ERICSON, 1968; RÖLLA and SÖNJU, 1972; SÖNJU et al., 1974; ÖRSTAVIK and KRAUS, 1974). Pellicle formed on hydroxyapatite surfaces in vitro will either promote or impair bacterial adhesion (LILJEMARK and SCHAUER, 1975; MAGNUSSON and ERICSON, 1976; CLARK et al., 1978). The properties of the adsorbed salivary proteins result in the selective adherence of bacteria to tooth surfaces. The initial bacterial selection is believed to be crucial in determining the composition of the ensuing bacteria colonizing the tooth surface (GIBBONS and VAN HOUTE, 1975; GIBBONS and QUERSHI, 1978). The specific composition of dental plaque is of great importance in the etiology of caries and probably also of periodontal disease (SLOTS, 1977; NEWMAN, 1979; BAENHNI et al., 1979).

The agglutinating factors may influence the bacteria differently, depending on the concentration of the factors, according to a hypothesis put forward by MAGNUSSON and ERICSON (1976). They speculated that at a low concentration of an agglutinin, free receptor sites on the bacteria will remain due to undersaturation with agglutinin and some of these receptor sites will be able to react with a corresponding salivary factor adsorbed to the tooth surface. Free reaction sites can induce a
'chain-reaction' causing multilayers of microorganisms to be built up and colonization to be enhanced. When, on the other hand, there is a high concentration of the agglutinin the receptor sites will be saturated. In this case the bacteria in saliva will either agglutinate or the receptors sites become blocked. Only monolayers will be deposited.

In (1) a tendency towards an inverse relationship between low agglutinin titers for a strain and the frequency of isolation of the strain from tooth surfaces was found. However, far-reaching conclusions concerning such a relationship cannot be drawn from a single sampling occasion and the small sample size.

WILLIAMS and GIBBONS (1975) found that treatment of human oral epithelial cells with blood group active, 'mucinous' glycoproteins from saliva inhibited attachment of indigenous oral streptococci to the cells and that the inhibition of adherence was correlated with the ability of the glycoproteins to aggregate the microorganisms. Support for the hypothesis that high concentrations of agglutinin inhibit plaque formation was also presented by MAGNUSSON et al. (1976), who, in resting whole saliva, found that high concentrations of the agglutinins reacting with a serotype c strain of S. mutans was related to a low rate of dental plaque formation during a 16 hour period while a fast rate of plaque formation was related to low concentrations of the agglutinin.

In a study on caries prediction based on parameters believed to be related to caries etiology, the measure of the agglutinin titer of a serotype c strain of S. mutans contributed to a concerted predictive ability of the parameters (RUNDEGREN and ERICSON, 1978).

The results of a recent study (LILJEMARK et al., 1981) on the effect of bacterial aggregation on the adherence of S. sanguis to hydroxyapatite agree well with the hypothesis of MAGNUSSON and ERICSON (1976). LILJEMARK et al. (1981) found that at low concentrations of the aggregating substance in saliva, an increase in bacterial adherence occurred and at high concentrations of the aggregating substance a reduced adherence was noted. In respect to the role ascribed to this type of substance a characterization of the molecules involved is warranted.
SUMMARY

The activity and specificity of salivary glycoproteins (agglutinins) aggregating various human indigenous microorganisms were studied in vitro. The agglutinin reacting with a serotype c strain of Streptococcus mutans was isolated and chemically characterized.

The results can be summarized as follows:

1. Saliva contains agglutinins against predominant oral microorganisms but also against some of the predominant intestinal bacteria. Agglutinin from one individual was active against microorganisms both from this individual and other individuals.

2. Saliva-induced agglutination of S. sanguis was inhibited by antisera to IgA, IgG, IgM, and secretory component while S. mutans agglutination was inhibited only by albumin and antisera to IgA. The differences in inhibition indicate the presence of different agglutinins for the two strains.

3. The agglutinin for an S. mutans serotype c strain was sensitive to a reduction in pH and to treatment with Concanavalin A while an S. mitior agglutinin was not. The tested S. mitior strain, however, adsorbed the S. mutans agglutinin without being agglutinated.

4. The agglutinin reacting with an S. mutans serotype c strain could be desorbed from the microorganisms in a neutral phosphate buffer. Calcium in the order of 10 million molecules per bacteria was needed to restore agglutination induced by the isolated agglutinin.

5. The agglutinin in parotid saliva responsible for aggregation of an S. mutans serotype c strain was characterized as a non-immunoglobulin glycoprotein with no blood group activity. The agglutinin was a polymer with a molecular weight exceeding 5,000 kdaltons while the monomeric agglutinin had a molecular weight of 440 kdaltons. The concentration of the agglutinin in parotid saliva was as low as 0.5 per cent of total protein but because 0.1 µg of the agglutinin caused rapid aggregation of as many as 100 million bacteria the agglutinin is considered highly active.

It is suggested that high molecular weight glycoproteins play a major role in the saliva-induced agglutination of microorganisms.
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