A Biochemical and Immunological Investigation of the Extracellular Polysaccharides Produced from Sugars by the Oral Streptococci

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DECLARATION

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Abstract

The development of a method for the measurement of cell-associated extracellular polysaccharides produced by five species of oral streptococci indicated quantitative differences in polysaccharide synthesis. S. mutans, S. sanguis and S. salivarius produced extracellular polysaccharides (EPS) from sucrose but not from other sugars, nor from an equimolar mixture of glucose and fructose. Cells of S. mitior and S. milleri produced EPS from none of the sugars investigated. Maltose was shown to act as a pre-cursor for the synthesis of large amounts of intracellular iodophilic polysaccharide by S. mutans.

The EPS produced by sucrose-grown cells of S. mutans, S. sanguis and S. salivarius was shown to be synthesised during the logarithmic phase of growth, but was rapidly degraded after cells entered the stationary phase. These polysaccharides were predominantly α1-6 and α1-3 linked glucose homopolymers, and the relative proportions of these linkages varied between species. The glucans produced by S. mutans and S. salivarius showed evidence of a branched structure, whereas those produced by S. sanguis exhibited a "dextrin"-like structure of short, linear α1-6 glucosidic linkages with no evidence of branching.

Agglutination studies performed on mouse sera indicated that glucan-coated cells of S. mutans and S. sanguis elicited a lower antibody response to cell-wall determinants than did those lacking cell-associated glucans. The relevance of these data is discussed in terms of the aetiology of streptococcal endocarditis. An Enzyme-Linked Immunosorbent Assay was developed for the quantification of the antibody response of mice to the EPS of oral streptococci. Data are presented which indicate the quantity, specificity and class of this antibody response. The potential application of an immunological approach to the elucidation of the structure and composition of streptococcal glucans is discussed.

CONTENTS

INTRODUCTION

1. Introduction

The synthesis, by certain oral streptococci, of extracellular polysaccharides is known to be of importance in the aggregation, adherence and pathogenicity of these species. The implication of these polysaccharides in the pathogenesis of both dental caries and bacterial endocarditis has ensured that the synthesis, structure and to a lesser extent the immunology of these polymers have been extensively studied.

1.1. The Oral Streptococci

The bacterial population of the oral cavity in man contains a wide variety of species. The predominant species differ in different sites within the mouth, but the streptococci, Gram positive bacilli and veillonellae make up the majority of the total oral bacterial flora. The streptococci form the largest component of this flora and most isolates can be identified as one of the following species: Streptococcus mutans, Streptococcus sanguis, Streptococcus salivarius, Streptococcus mitior and Streptococcus milleri (Hardie and Bowden 1976). S. mutans can be isolated from the oral cavities of both man and animals. This organism preferentially colonises the tooth surfaces and is found in only low numbers at other sites within the mouth. Like S. sanguis, this organism only appears in significant numbers after the eruption of the deciduous teeth. Strains of S. mutans are known to produce extracellular glucose polymers and fructose polymers when grown in sucrose-containing media. (Guggenheim 1970a).

S. sanguis was originally isolated from the blood of patients with bacterial endocarditis, though it is now known to be a common and numerous inhabitant of the oral cavity. It constitutes a high proportion of the streptococci found in dental plaque, where it is capable of the production of extracellular glucans from sucrose.

S. salivarius is also found in large numbers in the mouth, but it is predominantly an inhabitant of the saliva and tongue surface. Growth of S. salivarius on sucrose-containing media results in the production of large quantities of an extracellular, water-soluble fructan, together with smaller amounts of a glucose polymer (Kelstrup 1981).

The species S. mitior is physiologically diverse, and it has been proposed (Colman 1976) that some strains might more accurately be assigned to the species S. sanguis.

Extracellular glucan synthesis from sucrose by strains of S. mitior is a variable characteristic. The species is widely distributed in the mouths of man and animals and is also commonly isolated from the blood of dental bacteraemias. The final species normally incorporated in the oral streptococci is S. milleri, an organism almost totally restricted within the oral cavity to the gingival crevice (Hardie and Marsh 1978). This species is a physiologically homogeneous but a serologically heterogenous group of organisms and their growth on sucrose-containing media does not result in the production

of extracellular polysaccharides (Colman 1976).

1.2. Pathogenicity of the Oral Streptococci

Members of the five species of oral streptococci have been implicated in the aetiology of dental caries, bacteraemia, infective endocarditis and purulent disease. It was recognised, even during the last century that certain oral bacteria could produce sufficient acid in vitro to decalcify tooth enamel and that bacteria accumulated at the tooth surface in the form of bacterial plaque, which contributed to the aetiology of caries (Miller 1890).

Orland *et al* (1955) indicated that dental caries production was dependent on dietary carbohydrate and on the presence of certain organisms. These workers showed that gnotobiotic rats remained caries-free when fed a high-sucrose diet, whereas animals inoculated with a coccus and a bacillus developed caries. It was later reported that this disease could be induced by infecting animals with certain streptococci, and most of the strains of streptococci which have been shown to be highly cariogenic in experimental animals belong to the species *S. mutans*. The pathogenicity of *S. mutans* and its implication in dental caries was reported to be directly related to the ability of these organisms to produce insoluble extracellular polysaccharides from sucrose, since mutant strains lacking the ability to synthesise such polymers showed diminished cariogenicity in animal experiments (De Stoppelaar *et al* 1971).

Although S. mutans and to a much lesser extent S. sanguis, S. milleri, S. salivarius and S. mitior have been shown by Gibbons and Van Houte (1975a) to possess caries-inducing activity, the evidence for a causative role is inconclusive. S. mutans in particular, however, is thought to play a major role in the aetiology of this disease. Supporting evidence for this view came from the work of Lehner (1975) who presented immunological evidence to suggest that antiobody levels to S. mutans antigens varied in subjects with high and low caries experience.

These findings stimulated investigations in various laboratories of the possibility of immunising against dental caries with S. mutans vaccines based on different antigenic determinants (Bowen et al 1975, Lehner et al 1976; Caldwell et al 1977; Russell 1979; Russell and Colman 1981).

Such an approach would be valuable since dental caries is a ubiquitous disease, expensive and difficult to treat. The sucrose-dependent cariogenic potential of S. mutans will be further discussed in later sections.

Since the early investigations of Okell and Elliott (1935) it has been recognised that a transient bacteraemia is produced whenever teeth are extracted. Unfortunately, many early workers investigating the organisms isolated from cases of dental bacteraemia did not fully identify them, referring to them instead as "viridans streptococci". The significance of a transient

dental bacteraemia with these organisms is that patients with predisposing conditions may develop infective endocarditis. In this condition, bacteria colonise the surface of heart valves giving rise to infected vegetations. Patients at risk include those who have rheumatic heart damage, those with congenital heart abnormalities of various types and following cardiac surgery, especially after the insertion of prosthetic heart valves. Although many micro-organisms have been known to cause infective endocarditis, the oral streptococci are organisms commonly involved.

A survey of the streptococci and aerococci associated with systemic infection in man (Parker and Ball, 1976) considered the relative frequency with which different types of streptococci were isolated from endocarditis and from other systemic diseases. These authors produced an "endocarditis index", defining for each species its association with cases of endocarditis. They found that the streptococci having the highest association with clinical endocarditis were S. sanguis, S. bovis, S. mutans and dextran-producing strains of S. mitior. The non-dextran producing species had lower endocarditis indices, and S. milleri had the lowest index of the species surveyed. These findings confirmed the suggestion by Elliot (1973) that dextran-production may be a determinant for the ability of S. sanguis to adhere to heart valves. Parker and Ball (1976) suggested that if dextran aids the establishment of streptococci on the heart valves by its adherent properties, this must be by

virtue of pre-formed polysaccharide, since the organism would be unlikely to produce more after entering the bloodstream. Other authors, using endocarditis models and dextran-negative mutants of S. sanguis, have confirmed the association between dextran production and endocarditis index (Pelletier et al 1978: Ramirez-Ronda 1978). The detailed mechanisms of streptococcal adherence, and their role in the disease process will be discussed in later sections.

The survey of Parker and Ball (1976) emphasised the importance of isolates of S. milleri from purulent lesions in internal organs. This species was associated primarily with brain abscesses, meningitis, pleural empyema and a variety of intra-abdominal abscesses.

1.3. Sugar Metabolism by the Oral Streptococci

The uptake of fermentable sugars by members of the oral streptococci has been shown to be dependent on environmental factors.

Schachtele and Mayo (1973) identified the operation of a phosphoenol pyruvate (PEP) phospho-transferase system of sugar uptake in oral streptococcal species, and found that this system was dependent on growth rate. In a series of continuous culture experiments on S. mutans, it was shown that PEP phosphotransferase activity was high in cells grown at low rates, but that this activity fell as the growth rate was increased. It is now thought that multiple sugar transport systems operate in members of the oral streptococci (Slee and Tanzer 1982). These authors suggested the existence of at least three discrete transport systems for sucrose in S. mutans, two of which are PEP dependent, and a third which is not. They further suggested that under ecological conditions of excess carbohydrate, as occurs intermittently in the oral cavity, the conservation of energy facilitated by PEP dependent transport systems may not be a physiological necessity, and that a non-PEP dependent system may be utilised to support rapid cell growth and metabolism. The discovery of membrane-associated invertase activity in S. mutans by Bozzola et al (1981) led them to speculate that the presence of this invertase activity in the cell membrane could facilitate the entry of sucrose hydrolytic products into the cell cytoplasm for subsequent fermentation. It has been shown (Robrish and Krichevsky 1972) that most of

the glucosyls of sucrose are converted into lactic acid and that only a small portion of sucrose is diverted to extracellular polysaccharide synthesis.

Sugar uptake by members of the oral streptococci other than S. mutans has been less extensively studied, but it is likely that similar mechanisms operate in other species (Hamada and Slade 1980a).

All the species of oral streptococci have been reported as homofermentative lactic acid bacteria (Drucker and Melville 1968). Whilst it is true that organisms grown in conditions of glucose excess produce lactate almost exclusively as the end product of metabolism, it has been noted that, in continuous culture experiments, under conditions of glucose limitation, formate, acetate and ethanol are the major metabolic end products (Carlsson and Griffith 1974).

It is clear, therefore, that the metabolism of sugars and subsequent production of acidic metabolic products by members of the oral streptococci are intimately and directly related to growth conditions. This indicates the importance of microenvironmental conditions on the production of acid metabolic products, which are known to be implicated in the pathogenesis of dental caries (Guggenheim 1970).

1.4. Biosynthesis of Extracellular Polysaccharides

The extracellular synthesis of streptococcal glucans and fructans from sucrose is mediated by the action of two enzyme complexes i.e. by glucosyltransferases (GTase) and by fructosyltransferases (FTases) respectively (Carlsson and Erlander 1973; Kuramitsu 1975). Both of these enzyme complexes have been shown to be produced constitutively in cells of S. mutans (Wenham et al 1979), and also to be produced during active cell growth. Janda and Kuramitsu (1976) have proposed that these enzyme complexes are unique amongst extracellular bacterial enzymes in this respect and also in their dual catabolic and biosynthetic functions. Glucosyltransferases catalyse the transfer of a glucosyl moiety from sucrose to a terminal site on a growing, cell-associated glucan molecule.

$$n\text{-sucrose} \xrightarrow{\text{GTase}} (\text{glucose})^{n} + n.\ \text{fructose}$$

The present study will be largely concerned with the synthesis of streptococcal glucans and fructosyltransferase systems which act to polymerise the fructosyl moiety of sucrose to fructans, will not be reviewed in detail. The heterogeneity of glucans synthesised by the oral streptococci in terms of structure and water-solubility prompted investigations into the complex nature, site of action and regulation of streptococcal glucosyltransferases. Early workers were able, using various chromatographic techniques to separate the GTase enzyme complex from the supernatant of an S. mutans culture, into two fractions on the basis of molecular weight. The two fractions were

separated and partially characterised by Fukui et al (1974). These workers showed that the higher molecular weight enzyme fraction of S. mutans GTase synthesised water-insoluble glucans from sucrose, whereas the lower molecular weight fraction synthesised predominantly water-soluble glucans. This finding has since been amply confirmed by other workers (Mukasa and Slade 1974a; Chludzinski et al 1974; Ciardi et al 1977).

More recently, Mohan et al (1979) have suggested that both the soluble and insoluble glucans of S. mutans may be synthesised by the action of interconvertible forms of the same enzyme complex. They postulated a system whereby an aggregated, high molecular weight enzyme complex synthesises water insoluble "mutan" whereas disaggregation of the enzyme complex results in the production of water soluble "dextran". These activities have been termed mutansucrase and dextransucrase respectively. The synthesis of increased quantities of insoluble "mutan" by S. mutans GTase from sucrose in the presence of high concentratrations of NH^{4+} was observed by Kuramitsu and Wondrack (1983). These observations accorded well with the suggestion of Mohan et al (1979) that conditions favouring the aggregation of the low molecular weight GTase fraction into high molecular weight complexes result in the production of increased levels of insoluble glucans.

The work of Montville et al (1977) demonstrated that the addition of exogenous soluble dextran to the sucrose-containing media of S. mutans cells causes a decrease in mutansucrase activity and an accompanying increase in

dextransucrase activity in both a cell associated and cell-free fraction. Similarly Robyt and Corrigan (1977) observed a decrease in insoluble glucan production, and an increase in soluble glucan production, on the addition of increasing amounts of soluble dextran to the sucrose-GTase system.

Montville et al (1977) suggested that these findings could be explained if exogenous primer dextran promoted the conversion of mutansucrase activity into dextransucrase activity. Koga et al (1983) demonstrated that soluble glucan produced by dextransucrase acts as an intrinsic primer for "mutan" synthesis by mutansucrase, indicating the importance of autopriming in insoluble glucan production. Further insight into the regulation of "mutan" and "dextran" synthesis by the oral streptococci has recently been given by Fukui and Moriyama (1983). These workers investigated the original observation of Kunuttila and Makinen (1972) that exogenously applied maltose inhibits the insoluble glucan synthesis of oral streptococci. Fukui and Moriyama (1983) concluded that the effect of maltose is as follows:-

In the presence of maltose, dextransucrase initially synthesises 4-D-isomaltodextrinylglucose of various degrees of polymerisation from sucrose and these preformed isomaltodextrinylglucoses above hexasaccharides are used as glucosyl acceptors for mutansynthetase in insoluble glucan synthesis. Increase of maltose concentration results in the increased formation of tri- and tetrasaccharides together with decreased synthesis of

higher molecular weight oligosaccharides and leads to an inhibition of insoluble glucan synthesis.

Bozzola (1981), investigating the localisation of the GTase complex in cultures of S. mutans used immunological staining techniques to identify the relative positions on the cell surface of the two GTase fractions. It was shown that the GTase fraction responsible for insoluble glucan synthesis was localised as aggregates attached to the cell surface or to the extracellular polysaccharides of S. mutans. In contrast, they noted that the GTase activity synthesising primarily water-soluble glucans was distributed uniformly over the cell-surface or in association with the extracellular polysaccharides. The inter-relationships involved in the regulation of the GTase fractions responsible for soluble and insoluble glucan synthesis, and the factors involved in the inter-conversion of these enzyme activities can be seen to be complex. Work is at present in progress in several laboratories to elucidate the relationship between enzyme regulation, glucan synthesis and pathogenicity of the oral streptococci.

1.5 Sucrose-Dependent Adherence of the Oral Streptococci

Bacterial deposits forming on the surfaces of teeth are referred to collectively as "plaque", and it is known that the cariogenic potential of strains of oral streptococci is related directly to their ability to form such accumulations (Gibbons 1974). Plaque formation by S. mutans was found to be related to this organism's ability to synthesise extracellular polysaccharides from sucrose.

S. mutans was shown to form adherent microbial deposits on the walls of culture vessels when grown in sucrose-containing media and was found to initiate large bacterial plaques in experimental animals fed diets containing sucrose. Since this organism produces extracellular polysaccharides specifically from sucrose, it was suggested that both polysaccharide synthesis and plaque formation by this organism are specifically dependent upon this sugar (Keyes 1968). Several studies have shown that sucrose facilitates the colonisation of S. mutans in the mouths of both experimental animals and human subjects. Early work by Krasse (1965); and by Edwardsson and Krasse (1967) indicated that S. mutans could be established more easily when animals were fed on diets containing sucrose. It was found that de novo synthesis of extracellular polysaccharides from sucrose led to far stronger adherence to the tooth surface than that which occurs in the presence of dextran pre-coated onto either tooth or streptococcal cell surfaces (Van Houte and Upeslacis 1976).

However, as Fukushima et al (1982) have pointed out, the adherence of S. mutans to smooth surfaces is independent of water-insoluble glucan synthesis.

Hamada et al (1978) showed that sucrose-dependent adherence of members of the oral streptococci in vitro was mediated by the glucosyltransferase complex. These workers suggested that this adherence was directly related to the ability of certain streptococci to bind extracellular GTase to their surfaces. It was proposed that glucan on these bacterial surfaces acts as a binding site for these enzyme complexes (Hamada and Slade 1979). It was apparent, however, from earlier work that such surface glucan may not be a specific GTase binding site, and that other cell wall components may be involved in the binding of enzyme e.g. other complex polysaccharides and proteins (Mukasa and Slade 1974).

More recent studies by Staat et al (1980) have shown that bacterial accumulation at the tooth surface is a two stage phenomenon. These workers proposed that the first stage is attachment to the tooth pellicle which is mediated by cell-surface proteins rather than by glucans or teichoic acids. The second stage is a cellular accumulation mediated by sucrose-derived glucans and cell-surface lectins. Hamada and Torii (1980) demonstrated that the increased synthesis of water-soluble glucans by S. mutans in the presence of primer water-soluble dextrans inhibits in vitro adherence, suggesting a rôle for the more water-insoluble glucans in streptococcal adherence. It is

known that the production of extracellular glucans and subsequent adherence by members of the oral streptococci is an important factor in the pathogenicity of these organisms. Wenham *et al* (1981) investigated this relationship in *S. mutans* and concluded that the ability to catalyse insoluble glucan synthesis is a major determinant of the cariogenicity of these organisms. The implication of glucan-producing strains of *S. sanguis* and *S. mitior* in bacterial endocarditis has been reported (Ramirez-Ronda, 1978; Pelletier *et al*, 1978). Glucan synthesis aids cell accumulation and cardiac vegetation formation but also inhibits clearing of bacteria from the blood stream.

Meddens *et al* (1984) investigated the role of granulocytes in the induction of an experimental endocarditis in rabbits with a dextran producing strain of *S. sanguis* and with its dextran negative mutant. They found that the number of colony-forming units of *S. sanguis* needed to colonise the endocardial vegetations in 50% of the rabbits was significantly lower for the parent strain than for the dextran negative mutant. At serum concentrations of 5% and lower, they found that in vitro granulocytes phagocytosed the dextran negative mutant more rapidly than the dextran producer. It was concluded that an impaired phagocytic removal of attached bacteria from the vegetational surface could be a factor in the induction of endocarditis by dextran-producing streptococci.

1.6 Structure of Extracellular Polysaccharides

The clinical significance of the extracellular polysaccharides produced by the oral streptococci has ensured that their composition and structure have been extensively studied.

The water-soluble polysaccharides have been obtained by direct ethanol precipitation of streptococcal culture media, or by ethanol precipitation from aqueous cell washings. The more insoluble glucans may be obtained by extraction in alkaline solution, followed by ethanol precipitation. These precipitates have been separated into structurally different water soluble and insoluble fractions (Nisizawa *et al* 1976; Freedman *et al* 1978). Such crude preparations have been deproteinised by boiling in 4.3M KOH for 90 minutes, followed by neutralisation dialysis and subsequent lyophilisation.

Insoluble glucans have been more conveniently obtained by incubating cell-free GTase and sucrose. The glucan was obtained by centrifugation and washed extensively in water before lyophilisation (Guggenheim 1970). Using such techniques, early workers determined, by thin-layer chromatography of hydrolysed samples, that streptococcal glucans are glucose homopolymers and that streptococcal fructans are fructose homopolymers (Guggenheim 1970a). Early investigations of the structure of these glucans, using periodate oxidation, followed by Smith degradation indicated that they were predominantly α1-6 glucosidic linked glucose homopolymers or "dextrans" (Gibbons and Banghart 1967). Subsequently, Long and Edwards (1972)

showed that soluble glucans from S. mutans consisted of an α1-6 linked glucose polymer with α1-3 glucosidic branch linkages. Applying similar techniques to elucidate the structure of insoluble S. mutans glucan, obtained in a cell-free system, Guggenheim (1970b) showed that this glucan contained a high proportion of α 1-3 glucosidic linkages (< 90%). This proportion of α 1-3 linkages in "mutan" compares with results obtained for the analysis of S. sanguis glucan, which was found by Guggenheim to contain equal quantities of α 1-3 and α 1-6 glucosidic linkages.

Subsequent periodate oxidation and Smith degradation studies confirmed these findings (Ceska et al 1972; Baird et al 1973; Ebisu et al 1974; Nisizawa et al 1976). Soluble and insoluble streptococcal glucans were found to differ in their proportions of the α 1-3 glucosidic linkage, with insoluble glucans having a far higher α 1-3 linked component than the soluble glucans. Specific structural studies on insoluble glucans have shown them to consist of consecutive α 1-3 glucosidic linkages forming long chains as the backbone of a highly branched insoluble polymer (Hare et al 1978). It has been reported that the proportion of α 1-3 linkages in streptococcal glucans varies from 0.5% to 60% depending on the origin of the glucan (Walker 1978).

Work by Trautner et al (1982) and by Inoue and Koga (1979) on the structural characterisation of glucans obtained from different serotypes of S. mutans indicated that type 'd' strains synthesised significantly larger amounts of glucan

than did type 'c' strains. They also found a higher α 1-3 linked component in glucans from type 'd' strains and this, it was proposed, explained the higher proportion of insoluble material from these strains. Kuramitsu and Wondrack (1983) confirmed that type 'c' S. mutans strains produce low levels of insoluble glucan and demonstrated that this was the result of low levels of mutan-synthetase activity associated with these strains. A comparison of the glucans of the five serotypes of S. mutans (Trautner et al 1982) revealed that glucans of type b,c, and e form one group of polysaccharides characterised by a greater mean percentage of α 1-6 linkages and branching sites than the other group consisting of glucans of type a and d. Further it was pointed out that strains of S. mutans seemed capable of synthesising an almost continuous series of glucans with variable proportions of different types of linkages.

Much of this work was performed on glucans obtained in cell-free systems by incubating streptococcal GTases with sucrose, and then by analysing the product glucans by methylation analysis (Lindberg 1972).

Nisizawa et al (1977) suggested that the detailed analysis of glucans obtained in such cell-free systems may not accurately reflect the structure of glucans produced at the streptococcal cell surface. Hence these workers undertook the analysis of glucans extracted from S. mutans cells grown on sucrose. They fractionated the resultant glucans on the basis of water solubility into two fractions i.e. a water soluble and a water insoluble fraction. The

soluble glucan was found, by methylation analysis to contain 47%α1-6 linked units and 33%α1-3 linked units. Branched 1,3,6 linked units and terminally linked units each comprised 10% of the total. In contrast, the insoluble glucan contained 36%α1-6 units 46%α1-3 units, 9% branch units and 9% terminal residues. These authors noted that glucans obtained from streptococcal cells were more difficult to methylate than samples obtained from cell-free systems because of their more complex structure and larger molecular weight. Another problem with methylation analysis was said to be the possible over-estimation of α1-3 linked components by this method because of the resistance of the 3 hydroxyl group of the α-D-glucopyranosyl residues to complete methylation (Seymour et al 1980).

1.7. The Immunology of Extracellular Polysaccharides

The immunology and immunochemistry of glucans synthesised from sucrose by several members of the oral streptococci have been poorly investigated. Anti-glucan antibodies which may inhibit the interaction of glucans with glucosyltransferases and non-enzymatic glucan-binding proteins have not been investigated in any depth. Such antibodies would not only compete with various proteins in dental plaque for sites on glucan, preventing bacterial accumulation but also yield valuable information on the antigenic determinants of these glucans.

Most immunological studies have concerned themselves with the immunochemistry of dextrans. Much information on the immunology of these polymers was yielded by the long-term investigations of Kabat, who, working with others, elucidated some of the major characteristics of antidextran antibodies (Cisar and Kabat 1976). This work revealed that human antidextrans are very persistent and that, for example, serum from one subject after immunisation with dextran B1255 contained 18μg antidextran Nitrogen.ml^{-1}. Serum obtained from this individual 18 years later still had 4μg antidextran Nitrogen.ml^{-1}, and the specificity of these antibodies had remained unchanged. It was also shown that a dextran must have a minimum molecular weight of approximately 70,000 before it is capable of acting as an immunogen (Howard et al 1975a). BALB/C mice raised α 1-6 linked glucose specific antibodies in response to an optimal dose of a dextran fraction of 70,000 molecular weight and were tolerised by a high dose of this dextran,

whereas a dextran fraction of 20,000 molecular weight lacked both immunogenicity and tolerogenicity.

These workers also found that mitogenicity was dependent on the presence of phagocytic cells, which, they suggested, implied that macrophages play a role in the presentation of dextran to lymphocytes.

Different antigenic determinants on the same dextran molecule were found to differ in their ability to induce immunity and tolerance. This was shown by the work of Howard et al (1975b) that BALB/C mice responded to dextran B1355 by forming α 1-6 and α 1-3 specific antidextrans. Inoculation of large amounts of this dextran induced stable tolerance to the α 1-6 determinant, and, at the same time caused only a transient depression in the response to the α 1-3 determinant.

Cross-reaction studies with dextran were shown to require antibodies having complementarity for structures larger than a single linked glucopyranosyl unit (Kabat 1960). This was confirmed by studies with rabbit antibodies directed against conjugates of bovine serum albumin and isomaltonic, isomaltotrionic and isomaltohexanoic acids, (Arakatsu et al 1966). Subsequent oligosaccharide inhibition assays (Torii et al 1982) indicated that the maximum size of the α 1-6 specific antibody combining site corresponded to isomaltopentaose. It was found that antibody response to dextrans was predominantly IgG mediated and that antibodies were mostly directed to linear, non-terminal glucosidic linkages.

Various antigenic determinants were demonstrated by Howard et al (1975a and 1975b), who observed antibody populations specific for α1-6, α1-3, and α1-6 and α1-2 linked glucopyranosyl units. They found that dextrans with a high content of α1-6 linkages contain two types of antigenic determinant. The first is represented by the terminal, non-reducing ends of α1-6 linked branches and the second was expressed by non-terminal groups of the α1-6 linked chain.

Several types of antidextrans were found not to precipitate with α1-6 linked dextrans, but which would precipitate with dextrans rich in non α1-6 linkages, in addition to α1-6 residues. Evidence from this work suggested that an α1-2 linked determinant could involve the glucosyl branch together with a portion of the main α1-6 linked chain. Antibodies with an α1-4 specificity were found in a human antiserum after immunisation with a clinical dextran containing only 4% α1-4 linkages. Immunisation of human subjects with a dextran containing 34% α1-3 linkages induced a population of antibodies which precipitated with the homologous dextran but not with dextran rich in α1-6 linkages. The terminal or non-terminal locations of the α1-4 and α1-3 linked determinants were not established.

Immunochemical studies of similar depth have not been done on streptococcal cell-derived glucans but various diverse reports have appeared on the immunology of these bacterial polysaccharides.

Studies by Inai et al (1976) demonstrated that streptococcal

glucans derived from a cell-free system of S. mutans GTase and sucrose were capable of activating the alternative complement system in human sera. These workers investigated which components of streptococcal glucans were responsible for this activity and found that both the backbone α 1-3 linked chain and the branched α 1-6 linked chains of insoluble glucans were necessary for the operation of the alternative complement pathway. It was suggested that this ability was due to either the polyanionic nature of these glucans, or to their stereochemical conformation.

Genco et al (1976) investigated the immune response of rabbits and monkeys to immunisation with a cell-associated glucan extracted from the surface of sucrose-grown S. mutans cells. These glucans, when administered in Freund's complete adjuvant were weakly immunogenic in rabbits, the response being quantitated using indirect immunofluorescence techniques. They also found that Irus monkeys injected via the parotid duct showed a weak but definite IgG, IgM and IgA response. Absorption experiments demonstrated that cell-associated glucan (CAG) induced anti-glucan antibodies which cross-reacted with sephadex G25, and others which reacted with unique determinants on the cell-associated glucan. Antisera to this glucan reacted with whole cells of S. mutans, and antisera to S. mutans reacted with the cell-associated glucan.

Investigations of adherence inhibition by Scheld et al (1979) implicated anti-dextran antibodies as inhibitors of S. sanguis adherence. These authors, studied the

influence of anti-whole organism sera on the development of S. sanguis endocarditis both in vitro and in an animal model. It was found that such sera prevented endocarditis in rabbits and also prevented the adherence of S. sanguis to constituents of non-bacterial thrombotic lesions. Pre-absorption of sera with whole S. sanguis cells removed the adherence inhibiting activity of these sera and it was shown that pre-absorption of sera with dextran markedly removed the inhibiting activity. These results led the authors to suggest a role for antidextran antibodies in adherence inhibition of S. sanguis. No correlation was observed between levels of antidextran antibodies and caries inhibition in experimental animals, (Russell et al 1980), though these experiments were hampered by the intrinsic anticomplementary activity of the insoluble glucans used in complement fixation tests.

Novel methods of detecting anti-polysaccharide antibodies including radioimmunoassay and enzyme-linked immunosorbant assay (Elisa) procedures have been reported. Elisa techniques have been found by Melville-Smith and Sheffield (1980) to be a practical and more economic alternative to RIA for the estimation of the antibody responses of humans to the polyvalent pneumococcal polysaccharide vaccine, using purified pneumococcal polysaccharides as plate-coating antigens. Gray (1979) reported that polysaccharide antigens, which adhere only poorly to polystyrene assay plates could be much more effeciently absorbed to these plates if they were previously conjugated to the synthetic protein poly-l-lysine. Poly-l-lysine, coupled to the

polysaccharide by cyanuric chloride, adsorbs strongly to polystyrene. Hamada et al (1983) used dextran as a plate-coating antigen in an ELISA test for antidextran antibodies and found the technique to be reliable. These workers detected very low levels of antidextran antibodies in sera raised against cells of S. mutans. However, the inoculating cells were grown on glucose and so could not be expected to elicit high levels of antiglucan activity. Similarly Czerkinsky et al (1983) and Challacombe et al (1984) using an RIA technique attempted using glucose-grown cells of S. mutans as an immunogen, to characterise the antibody response to such cells using antibody inhibition studies. These inhibition studies showed that antibodies to whole S. mutans cells could be inhibited by purified cell surface antigens GTase and proteins I and II. However, they were only minimally inhibited by lipoteichoic acid or dextran, indicating only low levels of anti-dextran activity.

1.8. Aims of the Present Study

The aims of the present study are three-fold:-

Firstly it is proposed to quantify the cell-associated polysaccharides produced by the oral streptococci. The ability of these organisms to produce such polysaccharides has normally been measured qualitatively by noting altered colonial morphology on sucrose-containing media, or by the ethanol precipitation of medium-soluble polysaccharides from culture supernatants (Hehre and Neill 1946)

It is proposed in the present study to monitor the quantity of polysaccharide produced by five species of oral streptococci from nine mono- and disaccharides, during growth in batch cultures. The possible production of extracellular polysaccharides by these organisms from sugars other than sucrose will be investigated in order to clarify the findings of Colman *et al* (1977) that there was no significant difference in caries development in monkeys fed on sucrose and those fed on an equimolar mixture of glucose and fructose. Despite the fact that species other than *S. mutans* are not implicated to the same extent in the aetiology of dental caries, they have sufficient importance as causes of infective endocarditis to make them worthy of study. The study of their extracellular polysaccharides is of particular relevance in this context because of their implication in the pathogenesis of this disease (Meddens *et al* 1984).

The second objective of the study is a biochemical investigation of the composition and structure of cell-associated glucans synthesised from various sugars by species of oral streptococci. Previous investigations of this nature have been largely confined to the polysaccharides produced from sucrose by strains of S. mutans. Other species have been less extensively studied. The composition of streptococcal glucans in the present study will be investigated by thin-layer chromatography and subsequently by the more sensitive techniques of silylation and g.l.c. analysis. Structural investigations of these substances have normally been performed on glucans obtained by the action of isolated glucosyltransferases on sucrose in a cell-free system. Because of possible anomalies between the glucans produced by streptococci in vitro and in vivo, the present study will attempt the structural characterisation of glucans extracted from bacterial cells. The methylation, fragmentation and subsequent gas-liquid-chromatographic analysis of these glucans should provide information on the detailed structure of these polymers at the cell surface.

The third aspect of the current work will be an immunological investigation of the cell-associated polysaccharides synthesised from various sugars by the oral streptococci. Despite the recommendations of Cisar and Kabat (1976) that there was an urgent need for immunochemical information on these polymers, little work in this field has been reported.

It is proposed to immunise animals with whole streptococcal cells grown on various sugars, and to monitor the production of anti-glucan antibodies by agglutination tests and by the development of a novel enzyme-linked immunosorbent assay (ELISA) technique. The specificity and immunoglobulin class of anti-glucan antibodies produced will be determined by cross-reaction and cross-absorption studies. It is hoped that these investigations may elucidate immunochemical differences and similarities between the glucans studied.

Agglutination and immunoassay techniques will also be employed to investigate an observation made in this laboratory (Hammonds 1980; pers. comm.) that sucrose-grown cells of S. mutans induce a slower and weaker antibody response in rats to the cell-wall antigens than do glucose-grown cells.

It may be that this is significant in the survival of oral streptococci during dental bacteraemia and hence in the pathogenesis of infective endocarditis.

MATERIALS AND METHODS

2. Materials and Methods

2.1. Organisms

2.1.1. Origin

Five species of oral streptococci, obtained from the National Collection of Type Cultures, were used in the study. They were;-

(i)	Streptococcus mutans	(NCTC 10449)
(ii)	Streptococcus sanguis	(NCTC 7864)
(iii)	Streptococcus salivarius	(NCTC 7366)
(iv)	Streptococcus mitior	(NCTC 10712)
(v)	Streptococcus milleri	(NCTC 10708)

2.1.2. Maintenance of Cultures

Lyophilised bacterial cultures, obtained from the NCTC were grown aerobically at 37°C for 18 hours in 20ml Tryptone Soya Broth (Difco). After incubation, cells were harvested by centrifugation at 10,000xg for 10 minutes at 10°C, and resuspended in 2ml 10% (w/v) sterile skimmed milk (Difco). 0.1ml volumes of this cell suspension were dispensed aseptically to glass ampoules and lyophilised using an Edwards model EF03 Freeze Drier.

2.1.3. Storage

Lyophilised bacterial cultures, sealed in glass ampoules under vacuum were stored in the dark at 4°C until required.

2.1.4. Quality Control Procedures

Before using lyophilised organisms, viability and purity of cultures were checked by streaking onto Tryptone Soya Agar plates which were incubated aerobically and anaerobically at 37°C for 48 hours. Additionally, smears

from reconstituted cultures were stained by a modification of the Gram stain and examined microscopically for contaminants. Catalase activity of cultures was determined using 10% H_2O_2 in capillary tubes.

2.2. Culture Media

2.2.1. Media and Growth Conditions

Batch cultures (10ml or 1l volumes) of streptococci were grown aerobically in 0.1M phosphate buffered, 2% (w/v) proteose peptone broth (Difco), pH 7.6 supplemented with AnalAR grade sugars (BDH) to a final concentration of 2.5% (w/v). The phosphate buffered proteose peptone broth was previously sterilised for 15 minutes at 121°C, and the sugar solutions by millipore filtration (pore size 0.2μm diameter). The sugars incorporated into the growth medium, which were previously analysed by thin-layer chromatography (t.l.c.) and shown to contain no detectable contaminating sugars were sucrose, glucose, maltose, fructose, lactose, cellobiose, trehalose and an equimolar solution of glucose and fructose. The proteose peptone broth was similarly shown to contain no detectable mono- or disaccharides.

2.2.2. Inoculation Procedures

Ampoules containing lyophilised streptococcal cultures were opened and the cultures reconstituted and grown overnight at 37°C in 10ml growth medium containing 2.5% glucose. After incubation these cultures were used as inocula for experimental cultures. The inoculum/culture ratio was 1:100 (vol/vol.). Static batch cultures were

grown aerobically at 37°C.

2.3. Harvesting of Cells and Cell-Associated Polysaccharide (C.A.P.) Measurements

2.3.1. Procedure for Growth Curve Experiments

10ml cultures of the five species of streptococci, grown on eight sugar substrates were grown as described. Triplicate cultures were harvested at 12 hour incubation intervals over a total of 60 hours at 37°C.

Cells adhering to the walls of the growth flasks were suspended in the medium by scraping with a sterile glass rod. Cultures were then harvested by centrifugation (10,000xg for 10 minutes at 10°C), and washed in sterile deionised water (3 x 10ml). The washed cell pellet and associated polysaccharides were resuspended in sterile 1N NaOH (10% of original culture volume) and the cell-associated polysaccharides extracted by gentle shaking at 25°C for 30 minutes. Streptococcal cells were removed from the NaOH extract by centrifugation as before and retained. The NaOH extract was diluted appropriately and assayed for total carbohydrate by the anthrone method (see Section 2.4.1). Extraction conditions were optimised by a series of experiments varying both extraction temperature and NaOH concentration. Results obtained (Section 3.1.3.) indicate the effect of varying these parameters during the extraction of cell associated polysaccharides from an overnight culture of *S.mutans* grown on sucrose.

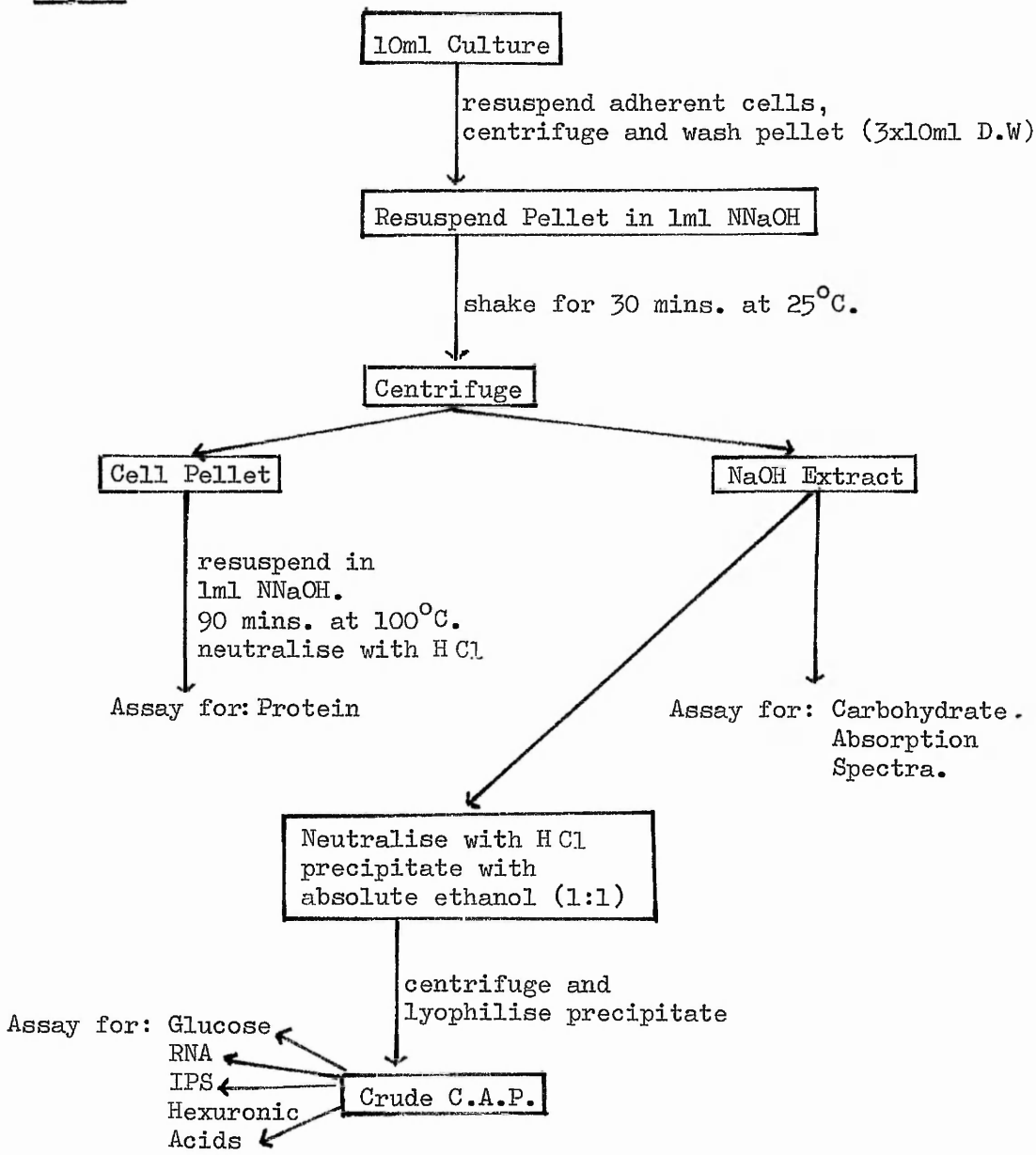
The retained cell pellet obtained after NaOH extraction of polysaccharides was resuspended in sterile 1N NaOH

(10% of original culture volume) and kept at 100°C for 90 minutes to dissolve the cells. Dilutions of the cooled, neutralised extract were assayed for total protein by the method of Lowry et al (Section 2.4.2.). Results obtained from these growth curve experiments (Section 3.1.5.), expressed as μg carbohydrate. mg^{-1} Cell Protein were used to illustrate the relationships between incubation time, sugar substrate and CAP yield.

The NaOH CAP extract remaining after total carbohydrate estimations was neutralised, and a crude CAP preparation obtained by precipitation with absolute ethanol (1:1). This crude CAP preparation was subjected to analysis for glucose (2.4.3.), hexuronic acids (2.4.6.), intracellular polysaccharides (IPS) (2.4.5.), RNA (2.4.6.) and absorption spectra were produced for the extracts between 200nm and 700nm (2.4.7.).

These methods described for estimation of cell-associated polysaccharides are illustrated schematically in Fig. 1.

Fig. 1.



All Centrifugation Steps: 10,000xg 10 minutes at 10°C.

2.3.2. Procedure for Analytical Investigations

11 cultures of the five species of streptococci, grown on eight sugar substrates were grown as described.

Cells adhering to the walls of the growth flasks were suspended in the medium by scraping with a sterile glass rod. Cultures were then harvested by centrifugation (10,000xg for 10 minutes at 10°C) and washed in sterile deionised water (3 x 200ml).

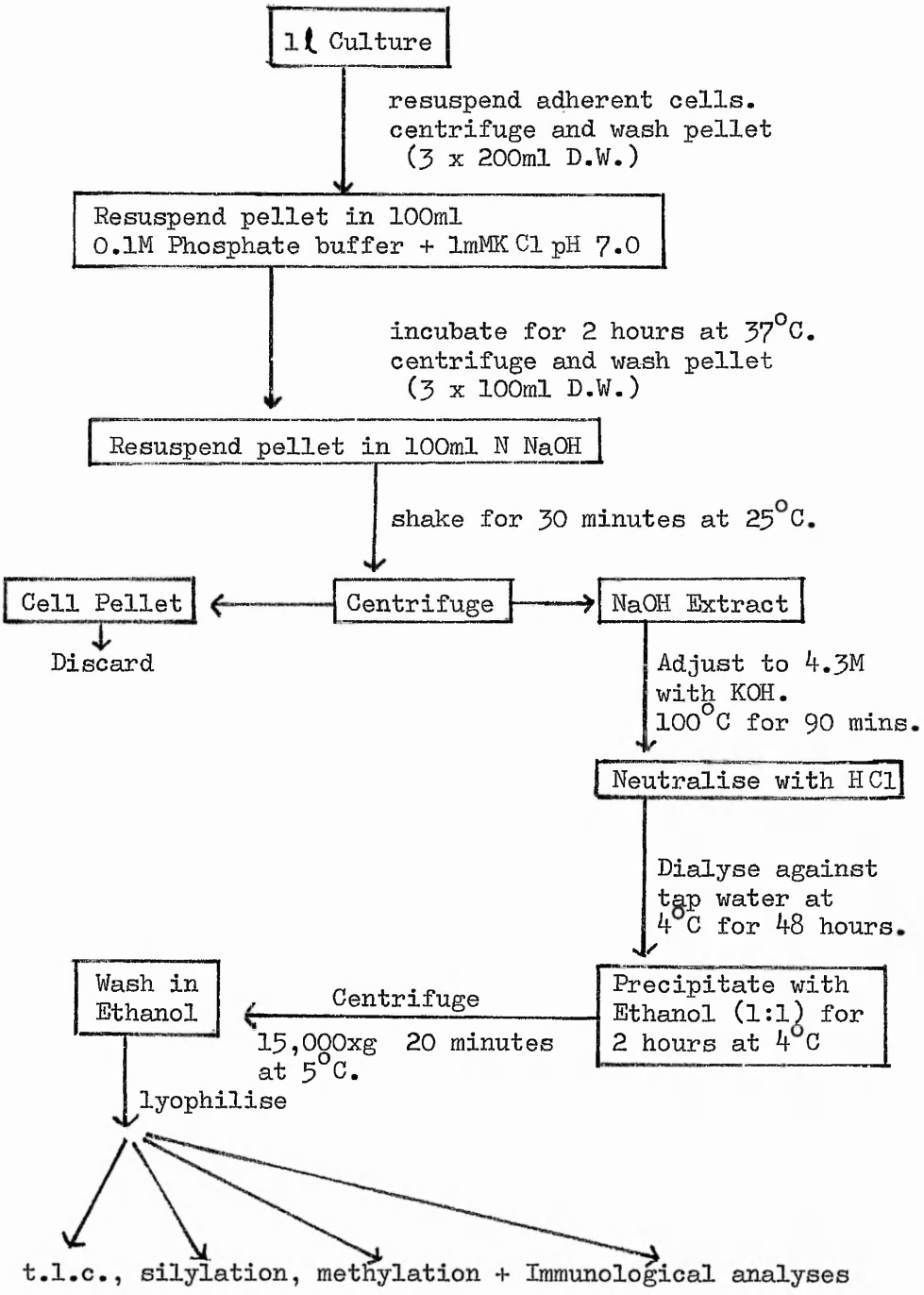
To minimise possible interference with C.A.P. analyses by intracellular polysaccharides (IPS) liberated by cell lysis during extraction, the following procedure was employed. Washed streptococcal cells were incubated in 100ml sterile 0.1M phosphate buffer + 1mM KCl (pH 7.0) at 37°C for 8 hours. After incubation, the cells were centrifuged (10,000xg at 10°C) for 15 minutes and washed in sterile deionised water (3 x 100ml).

The optimum incubation time necessary for reduction of IPS was determined as follows; triplicate 10ml cultures of S. mutans grown overnight at 37°C in sucrose broth were harvested, washed and resuspended in 0.1M phosphate buffer + 1mM KCl as described. The cell suspension was incubated at 37°C and duplicate 0.5ml samples removed at 1 hr. intervals over 8 hours. The cells were washed in sterile distilled water (3 x 1ml) and their "surface" and "total" carbohydrate contents measured. The "surface" carbohydrate was measured as μgCHO/mg Protein (Section 2.3.1.) Total cell carbohydrate was measured by dissolving the washed streptococcal cells in 1ml N NaOH at 100°C for 90 minutes. NaOH extracts were assayed

for carbohydrate and protein as described. Results are described in Section 3.1.6.

After reduction of IPS the washed streptococcal cells were resuspended in 100ml sterile 1N NaOH and the cell-associated polysaccharides extracted by gentle shaking at 25°C for 30 minutes. Cells were removed from the NaOH extract by centrifugation and discarded. The NaOH extract was deproteinised by adjusting the molarity to 4.3M with KOH, and then boiling the extract at 100°C for 90 minutes. After cooling, the extract was neutralised with concentrated HCl, re-cooled and dialysed (Visking tubing) against running tap water at 4°C for 48 hours. After dialysis, the C.A.P. was precipitated with an equal volume of absolute ethanol for 120 minutes at 4°C. The precipitated C.A.P. was removed by centrifugation (15,000xg for 20 minutes at 5°C), washed in ethanol (10ml), lyophilised and stored in the dark at 4°C, prior to their chromatographic and immunological analysis. Extraction and purification procedures, which were based on those of Nisizawa et al (1976) are summarised in Fig. 2.

Fig. 2


1ℓ Culture
resuspend adherent cells.
centrifuge and wash pellet
(3 x 200ml D.W.)
Resuspend pellet in 100ml
0.1M Phosphate buffer + 1mMK Cl pH 7.0
incubate for 2 hours at 37°C.
centrifuge and wash pellet
(3 x 100ml D.W.)
Resuspend pellet in 100ml N NaOH
shake for 30 minutes at 25°C.
Cell Pellet
Centrifuge
NaOH Extract
Discard
Adjust to 4.3M
with KOH.
100°C for 90 mins.
Neutralise with HCl
Dialyse against
tap water at
4°C for 48 hours.
Wash in
Ethanol
Centrifuge
15,000xg 20 minutes
at 5°C.
Precipitate with
Ethanol (1:1) for
2 hours at 4°C
lyophilise
t.l.c., silylation, methylation + Immunological analyses

2.4. Analytical Techniques

2.4.1. Total Carbohydrate

Measured by the method of Scott and Melvin (1953) with glucose as the standard. A typical standard curve and experimental details are shown in Fig. 3.

2.4.2. Protein

Measured by the method of Lowry *et al* (1951) with bovine serum albumin as the standard. A typical standard curve and experimental details are shown in Fig. 4.

2.4.3. Glucose

Measured by the glucose oxidase method of Morley *et al* (1968) with glucose as the standard. A typical calibration curve and experimental details are shown in Fig. 5.

2.4.4. Hexuronic acids

Measured by the orcinol-sulphuric acid reaction of Dische (1963), with glucuronic acid as the standard. A typical calibration curve and experimental details are shown in Fig. 6.

2.4.5. Intracellular Polysaccharides

Measured by the method of Dipersio (1974) with glycogen as the standard. A typical standard curve and experimental details are shown in Fig. 7.

2.4.6. RNA

Measured by Bial's Reaction (Dische 1963) with RNA as the standard. A typical calibration curve and experimental details are shown in Fig. 8.

2.4.7. Absorption Spectra

Absorption spectra were produced for 1N NaOH extracts of crude C.A.P.'s between wavelengths of 200 and 700nm using a Unicam SP1800 U.V. Spectrophotometer with a Unicam AR 25 linear recorder, and peaks identified by reference to standards.

Fig. 3

Estimation of Total Carbohydrate by the Anthrone Method (Scott and Melvin 1953)

Standard solutions of glucose were prepared in duplicate over a range of 0-100 $\mu g.ml^{-1}$ in 1ml.

To each solution was added 2ml of a cooled 0.2% solution of anthrone in concentrated H_2SO_4. The tubes were shaken vigorously and heated at $90^{o}C$ for exactly 16 minutes. Absorbance was read immediately at 625nm against a distilled water blank.

Total carbohydrate determinations on cell extracts were prepared in triplicate.

Fig. 3.

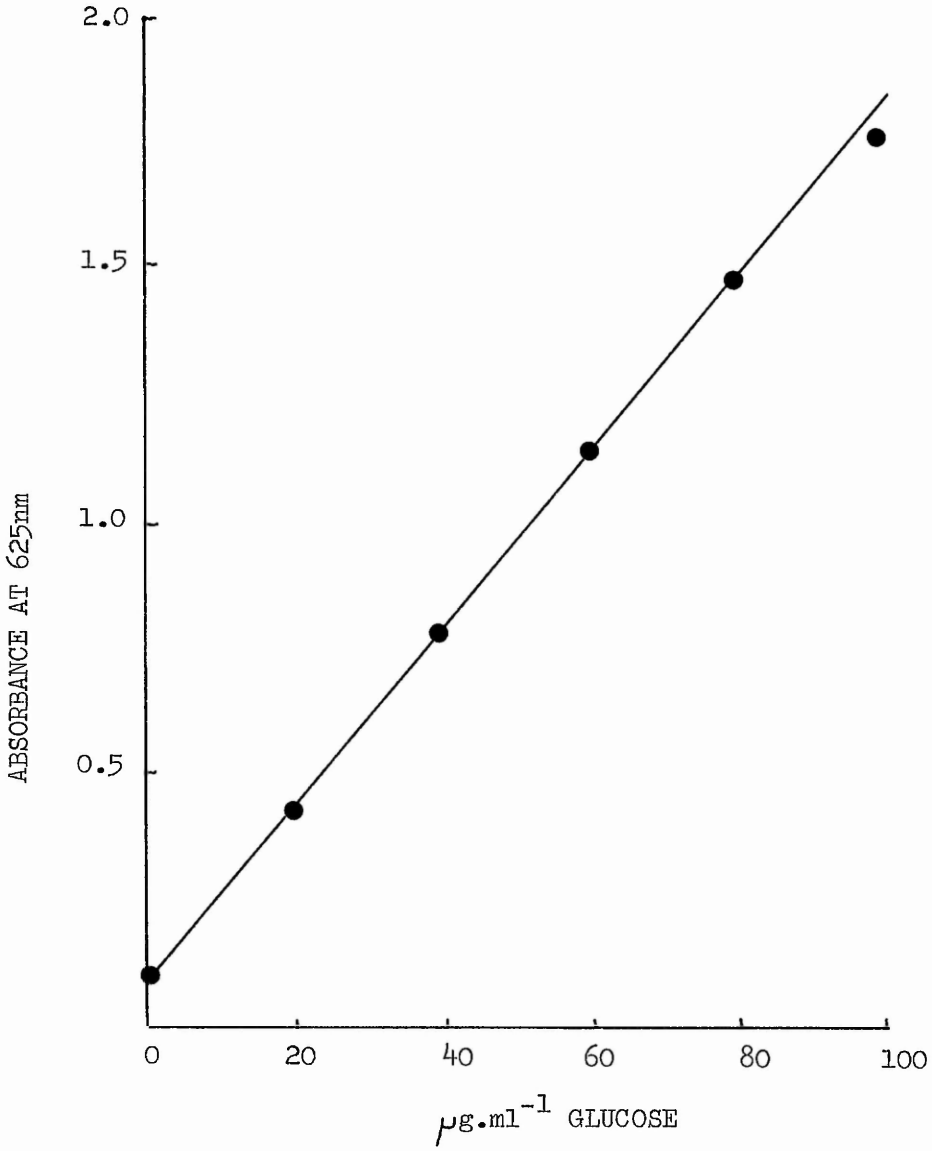


Fig. 4

Estimation of Protein by the Method of Lowry et al (1951)

Standard solutions of bovine serum albumin were prepared in duplicate over a range of 0-250 $\mu g.ml^{-1}$ in 1ml. To each solution was added 5ml of a mixture of 2% (w/v) $Na_2 CO_3$ in 0.1N NaOH (100 volumes), 1% $CuSO_4.5H_2O$ (1 volume) and 2% sodium potassium tartrate (1 volume). After 10 minutes 0.5ml of Folin-Ciocalteau reagent (diluted 1:1 with distilled water) was added to each tube and shaken vigorously. Absorbance was read after 30 minutes at 750nm against a distilled water blank.

Protein determinations on cell samples were prepared in triplicate.

Fig. 4.

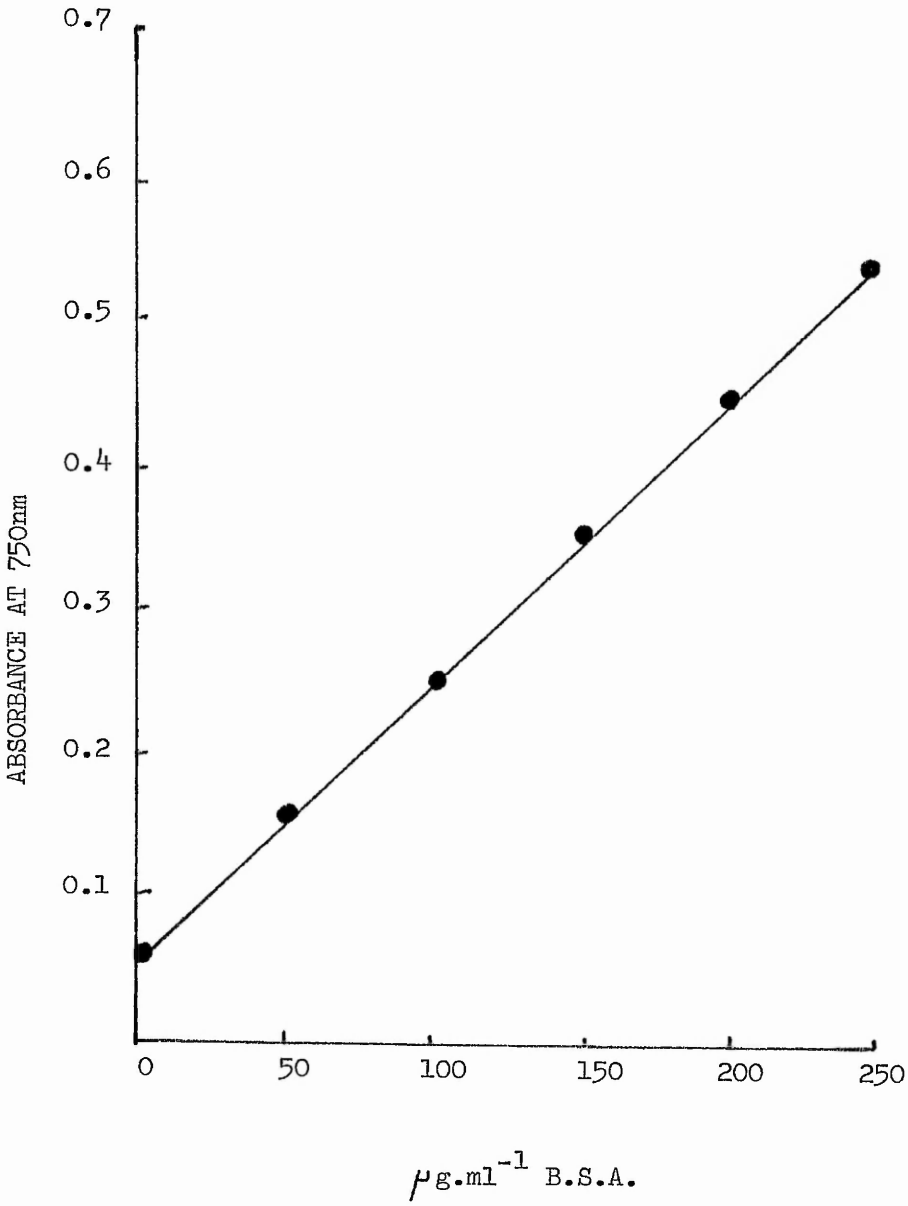

0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 750nm
0
50
100
150
200
250
μg.ml-1 B.S.A.

Fig. 5

Estimation of Glucose by the Glucose Oxidase Method

(Morley et al 1968)

Standard solutions of glucose were prepared in duplicate over a range of 0-1mM in 1ml.

To each solution was added 6ml of a mixture of peroxidase/guaiacum solution (10 volumes), acetate buffer pH5.0 (2 volumes), and glucose oxidase solution (80mg. l^{-1}) 2.5 volumes).

Solutions were incubated at room temperature for 15 minutes.

Absorbance was read at 600nm against a distilled water blank.

Glucose determinations on hydrolysed polysaccharide samples were prepared in triplicate.

Fig. 5.

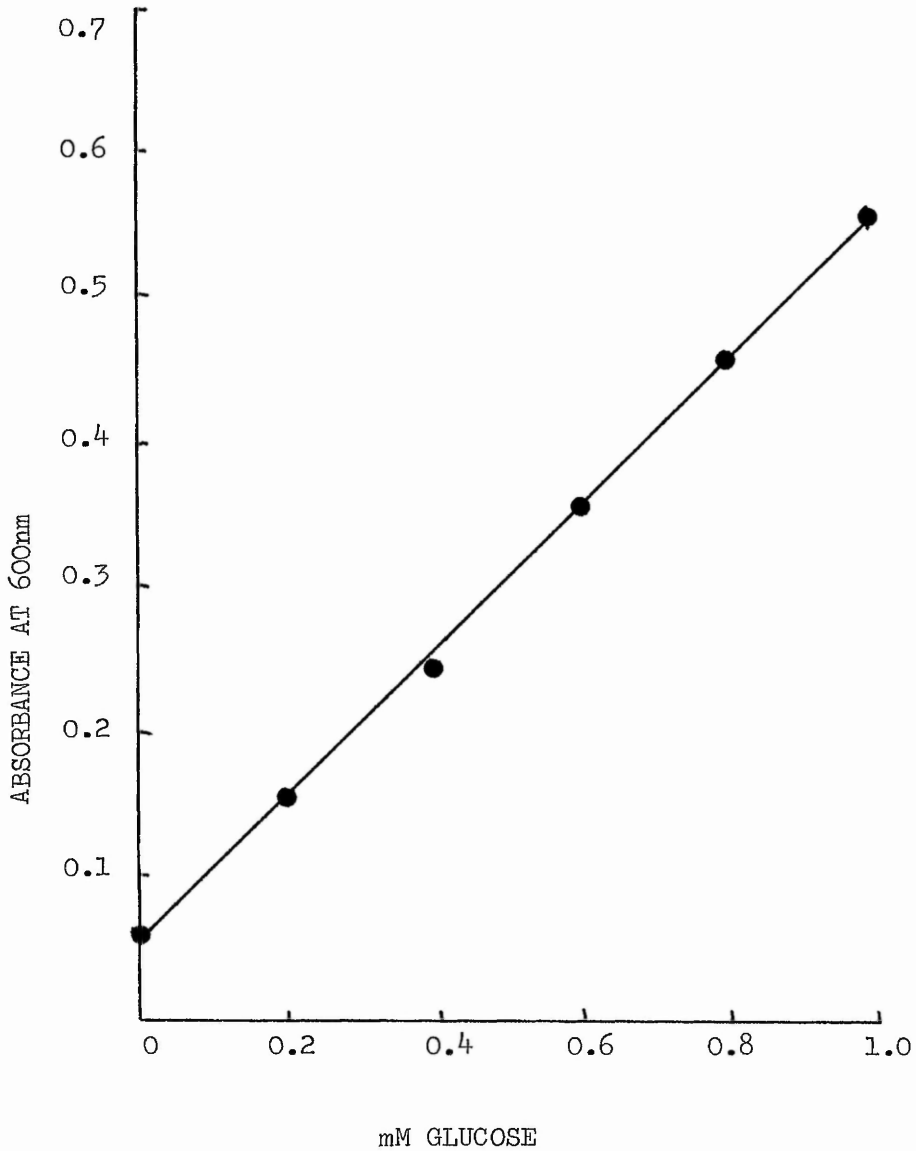

0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 600nm
0
0.2
0.4
0.6
0.8
1.0
mM GLUCOSE

Fig. 6

Estimation of Hexuronic acids by the Orcinol-Sulphuric Acid Reaction (Dische 1963)

Standard solutions of glucuronic acid were prepared in duplicate over a range of 0-100 μ g.ml^{-1} in 0.2ml.
To each solution was added 1ml concentrated HCl containing ferric chloride (10% w/v in distilled water) at 0.5% (v/v), and 0.1ml orcinol (10% w/v in 90% ethanol). After mixing the solution was heated at 80°C for 3 minutes, cooled to 4°C and the absorbance read at 505nm against a distilled water blank.
Hexuronic acid determinations on hydrolysed polysaccharide samples were prepared in triplicate.

Fig. 6.

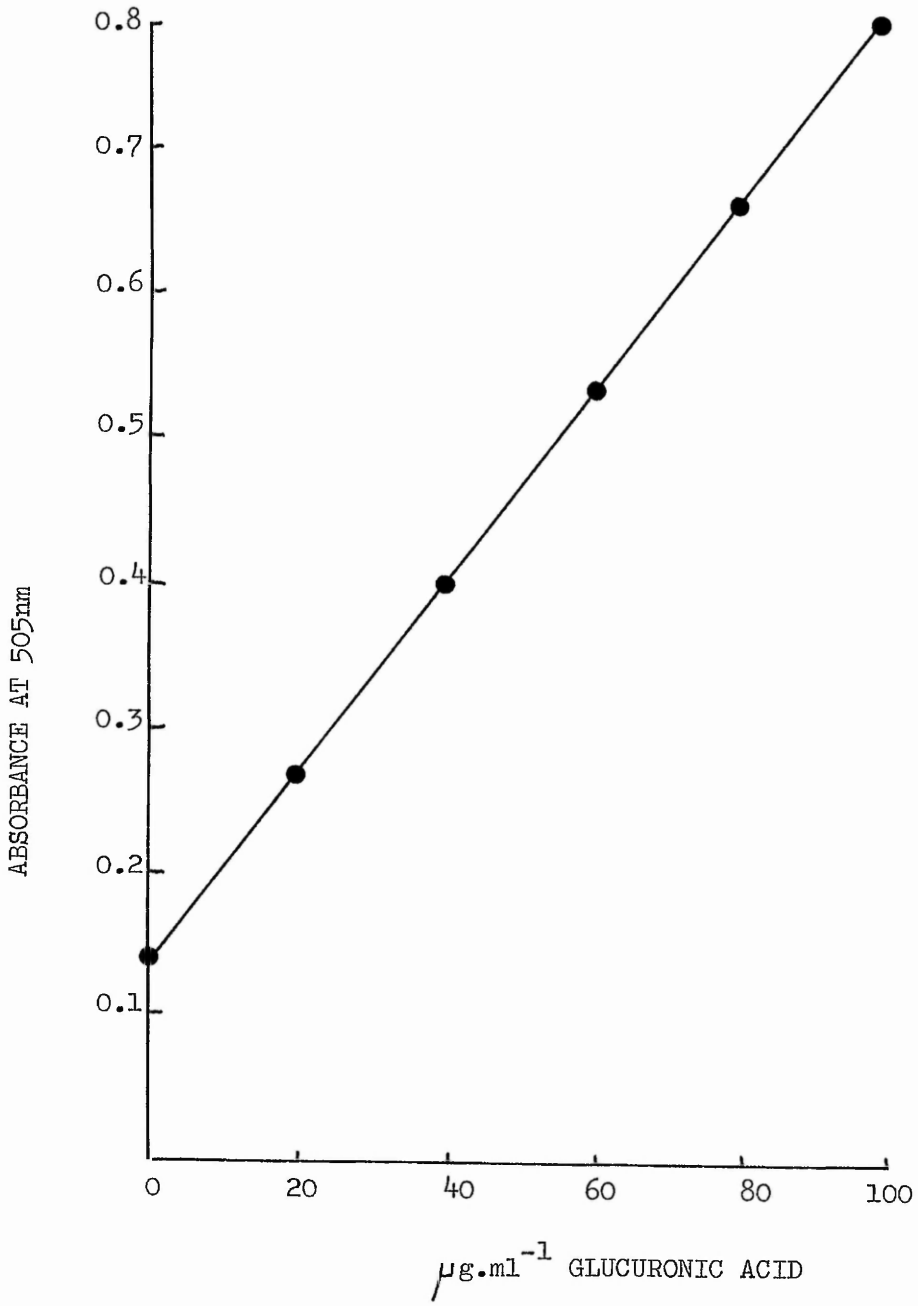

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 505nm
0
20
40
60
80
100
μg.ml-1 GLUCURONIC ACID

Fig. 7

Estimation of Intracellular Iodophilic Polysaccharides (DiPersio et al 1974)

Standard solutions of glycogen were prepared in duplicate over a range of 0-1000 $\mu g.ml^{-1}$ in 1ml.

To each solution was added 1ml of 1.0M potassium phosphate pH 7.0. After mixing 0.6ml of a freshly prepared 0.2% (w/v) iodine in 2.0% (w/v) potassium iodide solution was added with mixing.

Absorbance was read at 520nm.

Iodophilic polysaccharide estimation on cell extracts were performed in triplicate.

Fig. 7.

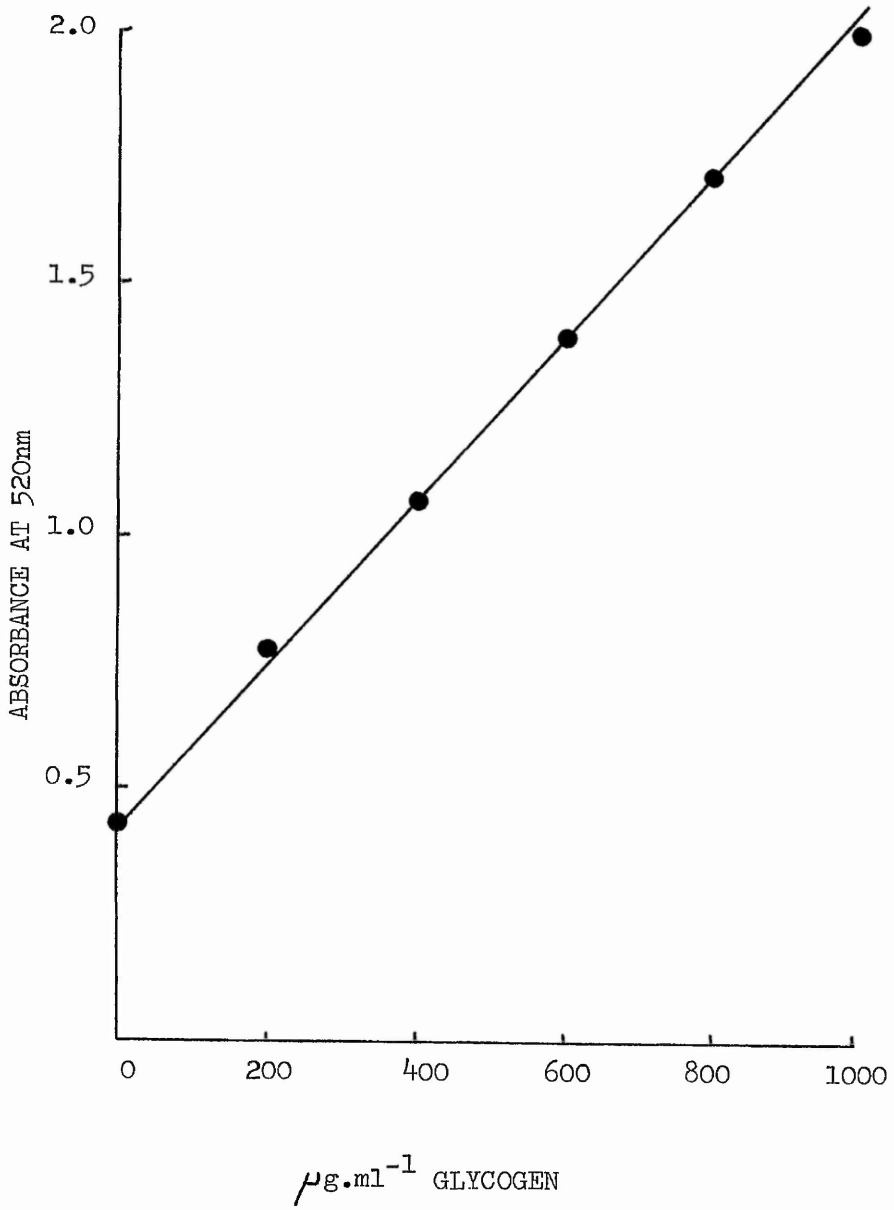

2.0
1.5
1.0
0.5
ABSORBANCE AT 520nm
0
200
400
600
800
1000
μg.ml-1 GLYCOGEN

Fig. 8

Estimation of Ribonucleic Acid by the Bial Method

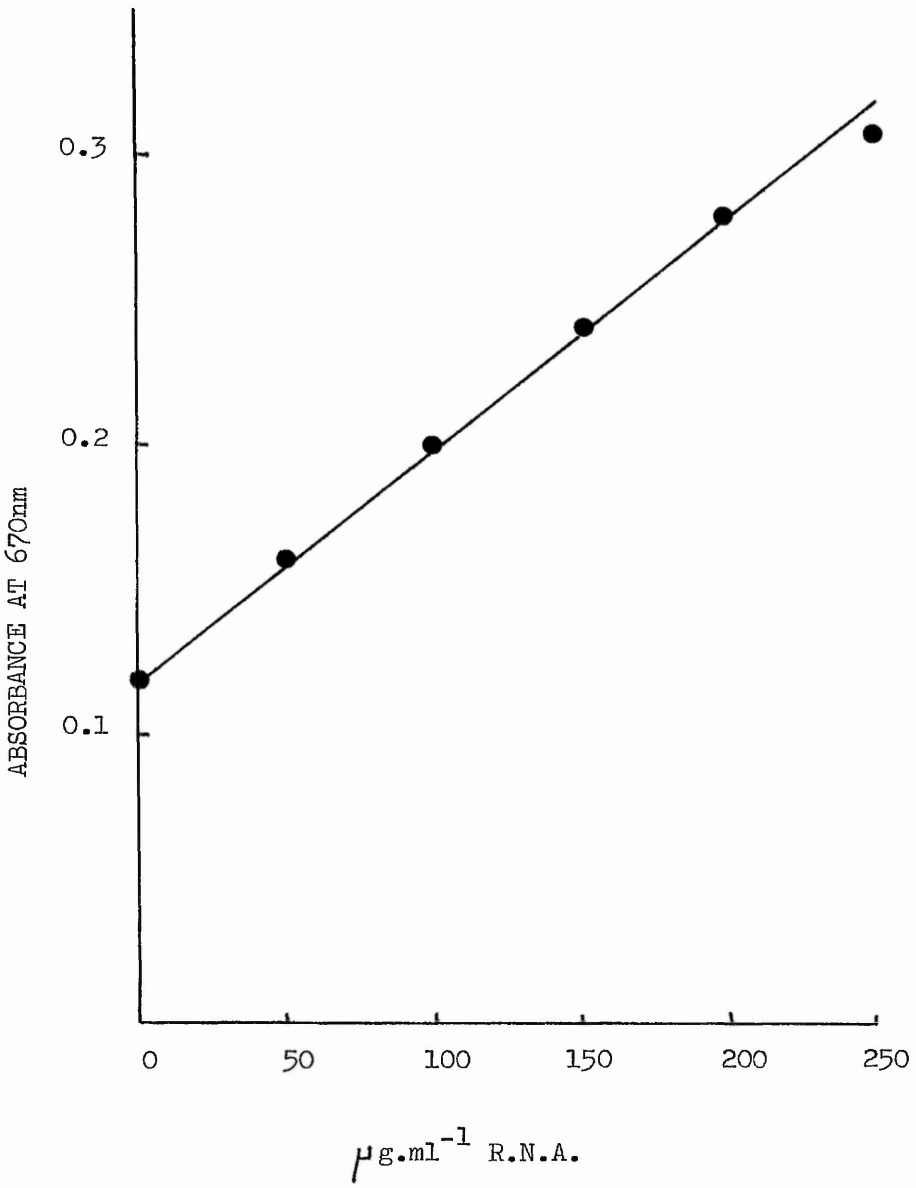
(Dische 1963)

Standard solutions of RNA were prepared in duplicate over a range of 0-250 $\mu g.ml^{-1}$ in 0.2ml.

To each solution was added 1ml of concentrated HCl containing ferric chloride (10% w/v in distilled water) at 0.5% (v/v), and 0.1ml orcinol (10% w/v in 90% ethanol) After mixing, the solution was heated at 80°C for 3 minutes, cooled to 4°C and the absorbance determined at 670nm against a distilled water blank.

RNA determinations on hydrolysed polysaccharide samples were prepared in triplicate.

Fig. 8.



2.4.8. Thin Layer Chromatography (t.l.c.)

Purified C.A.P. were subjected to acid hydrolysis in sealed glass tubes at 100°C in either (i) 0.1N HCl for one hour or (ii) NHCl for two hours. Two sets of hydrolysis conditions were used to detect possible degradation of acid labile constituents.

Hydrolysates were neutralised with NaOH and 10µl applied in small aliquots with drying between additions, to the origin of Kieselgel 60 t.l.c. plates (Merck). The plates were developed in butanol:methanol: glacial acetic acid (3:1:1) for 3 hours at room temperature. Plates were air dried, sprayed with concentrated H_2SO_4 and allowed to char at 150°C for 10 minutes.

A total of nine mono- and disaccharides were similarly chromatographed to provide standards. Results were expressed as Rf values.

2.4. 9. Silylation Analysis

Silylation analysis based on the original methods of Sweeley et al (1963) was used to investigate the constituent monosaccharides of the isolated streptococcal C.A.P's.

Lyophilised C.A.P. samples were subjected to partial hydrolysis in 1N HCl at 100°C in sealed glass tubes for 2 hours. Cooled hydrolysates were neutralised with NaOH and reduced under vacuum to dryness at 40°C. 10mg of sample were dissolved in 1ml of the commercially available silylating reagent Tri-Sil AQ

(Hexamethyldisilazane/Trimethylchlorosilane [21% in pyridine]). Silylation was complete on dissolution of the sample after 15 minutes at room temperature. Monosaccharides to serve as standards were silylated as above (10mg. ml^{-1}).

2.4.10. Gas Chromatography of Silylated C.A.P. Hydrolysates

0.5µl volumes of silylated C.A.P. hydrolysates and standards were injected onto a 180cm glass column packed with 3% OV-225 on Gas-Chrom W (mesh size 100/120). The chromatograph used was a Perkin Elmer Sigma 4B single channel isothermic instrument fitted with a flame-ionisation detector (F.I.D.). The carrier gas was Nitrogen at a flow rate of 20ml. min^{-1}. Injection port temperature was 200°C and the column temperature 165°C. Silylated C.A.P. monosaccharides were identified by reference to silylated standards, and the results presented as retention times. (minutes).

2.4.11. Methylation Analysis

Methylation analyses of the purified streptococcal C.A.P.s were performed by modifications to the methods of Lindberg (1972).

2.4.11.1. Methylation

10mg. of lyophilised C.A.P. were dissolved in 1ml benzyltrimethylammonium hydroxide (Triton B), by vigorous overnight stirring in a 5ml rubber-stoppered vial under Nitrogen. The resulting C.A.P. solution was stored on ice during the addition over a 12 hour period of 1ml of methyliodide (Sigma). The reaction vessel was

vented with a hypodermic needle and the reaction mixture agitated overnight at room temperature. Methylated polysaccharide was precipitated by the addition of 10ml distilled water, and the suspension dialysed for 48 hours against running tap water at $4^{o}C$. After dialysis the methylated polysaccharide was dried under vacuum in a rotary evaporator at $40^{o}C$.

Samples were methylated once, twice or three times to ensure permethylation.

2.4.11.2. Hydrolysis

The dried, methylated C.A.P. was dissolved in 3ml 90% formic acid (BDH) and heated to $100^{o}C$ for 2 hours in sealed glass tubes. The reaction mixture was cooled and reduced to dryness in a vacuum rotator at $40^{o}C$. The formolysed residue was taken up in 1ml 0.25M H_2SO_4 and hydrolysed at $100^{o}C$ for 12 hours in sealed glass tubes. When it was cooled and neutralised by the addition of barium carbonate. After removal of the sediment by centrifugation, the hydrolysed, methylated C.A.P. sample was reduced to dryness under vacuum at $40^{o}C$.

2.4.11.3. Acetylation

The methylated, hydrolysed C.A.P. was dissolved in 5ml distilled water and reduced by the addition of 10mg sodium borohydride (sigma). After 2 hours at room temperature, 1ml Dowex 50 H^{+} ion exchange resin was added and shaken occasionally over 5 minutes. The resin was removed by centrifugation and boric acid removed from the sample by co-distillation with methanol

(3 x 5ml) in a rotary evaporator at 40°C. The residue was treated with 2ml acetic anhydride (Aldrich): Pyridine (1:1) for 10 minutes at 100°C. Toluene (5ml) which gives an azeotrope with acetic anhydride was added and the mixture distilled as before, until the rate of distillation decreased, when a new aliquot of toluene (5ml) was added. The sample was concentrated to dryness and the residue taken up in 0.2ml acetone.

2,3,4,6, tetramethyl D glucose and 3-O methyl ∝ D glucose (sigma) were reduced and acetylated as above to provide standards.

2.4.11.4. Gas-Chromatography of Partially Methylated Sugars as their Alditol Acetates

1µl volume samples of derivatised C.A.P. were injected onto a 180cm glass column packed with 3% OV-225 on Gas-Chrom W. mesh size 100/120. The chromatograph was a Perkin Elmer sigma 4B single channel isothermic instrument fitted with an F.I.D. The carrier gas was Nitrogen at a flow rate of 20ml.min^{-1}.

Results were identified by interpolation of peak retention times between the two standards, and by reference to the published values of Lindberg (1972). Results were expressed as retention times, relative retention times and peak areas.

2.4.11.5. Relationship between relative retention times and Column Temperature

The values of relative retention times published by Lindberg (1972) for methylated sugars as their alditol

acetates were obtained at a column temperature of 160°C. However, running analyses at this temperature resulted in excessively long retention times e.g. 3-O methyl glucose had a retention time of 209 minutes. This relatively low column temperature also reduced the resolution of minor component peaks. To determine the relationship between column temperature and retention times of partially methylated alditol acetates, the retention times of 2,3,4,6, tetramethyl glucose, 3-O methyl glucose and two contaminating trace peaks in these standards were observed over a range of column temperatures.

Sample derivations and g.l.c. conditions were as described in section 2.4.10.

Mathematical investigation of the results obtained (section 3.2.4.1.) indicated that the relationship between column temperature and retention time of alditol acetates could be described mathematically and that using this relationship, the retention time of a given component could be predicted at any column temperature. This allowed identification of peaks by reference to Lindberg's published data, using a column temperature giving fast retention times and optimum peak resolution.

2.5. Immunological Investigation of C.A.P.

2.5.1. Preparation of Immunogens

Overnight cultures of S.mutans, S. sanguis and S. salivarius were grown at 37°C in 25ml phosphate buffered 2% proteose peptone broths. pH 7.6 which were

supplemented with either sucrose, glucose or maltose to a final concentration of 2.5% (w/v). Cells were harvested by centrifugation, washed three times in sterile physiological saline (0.85% w/v) and finally resuspended in this solution to 1ml. Cell suspensions were stored at 4°C prior to immunisation.

2.5.2. Experimental Animals and Inoculation Procedure

Adult, mixed sex, back-cross mice between 20g and 30g body weight were chosen for inoculation. In each series of experiments 10 mice were used for each immunogen and sera were pooled. Similarly, sera from 10 non-immunised mice were pooled to provide negative controls. 0.1ml of a whole cell suspension was administered subcutaneously on the abdomen, and the procedure repeated after 10 days. After a further 10 day period, mice were anaesthetised and exsanguinated by cardiac puncture.

The whole blood was cooled to 4°C, allowed to clot, and the serum removed by centrifugation at 4°C. 100µl volumes of serum were distributed to sterile, stoppered 1ml glass vials and stored at -20°C until required.

2.5.3. Absorption Techniques

Cross-absorption studies on sera were performed by incubation with washed cells of streptococci grown on various sugar substrates. Absorbing cells were prepared by harvesting cells by centrifugation of 20ml overnight cultures of streptococci grown at 37°C. The cells were washed three times in 10ml sterile distilled

water and finally resuspended in 1ml sterile physiological saline (pH 7.0). Neat sera (0.5ml) were absorbed with streptococcal cells (0.5ml) by incubation at 37°C for 2 hours.

Absorbing cells were removed by centrifugation at 10,000xg for 15 minutes at 5°C and subsequent millipore filtration (0.2μm diameter pore size) of sera.

2.5.4. Development of an Enzyme-Linked Immunosorbent Assay Technique for streptococcal C.A.P.

The method employed was a modification of that published by Gray (1979).

All assays were performed in 96-well, flat-bottomed polystyrene plates (Nunc). Before plates were used for assay their ability to accept polysaccharide antigens homogeneously was established by performing assays using standard antigen, antiserum and conjugate concentrations.

2.5.4.1. Antigen coating of plates

Polysaccharide molecules, which will not attach directly to the walls of plastic reaction vessels, were first covalently bound to the synthetic protein, poly-L-lysine. This was achieved using cyanuric chloride as the coupling agent. 5mg of purified, lyophilised C.A.P. was dissolved in 0.5ml 1N NaOH and then diluted with sterile distilled water to give a solution of 1000$\mu g.ml^{-1}$. 0.1ml of this antigen solution was added to a test-tube containing 0.5ml 0.01N NaOH + 0.001% phenolphthalein indicator and mixed. This solution was transferred to a second tube containing 0.5mg cyanuric chloride (Aldrich) crystals, and mixed vigorously for 10-15 seconds until the pink

colour of the indicator disappeared (pH 8.6). The solution was transferred to a third tube containing 0.1ml of 0.1% poly-L-lysine (Sigma) in distilled water. The contents of the tube were mixed and left at 4°C for 2 hours for coupling to occur.

The protein coupled C.A.P. was diluted in phosphate buffered saline pH 7.0 to an experimentally determined optimum. This was determined by assaying known + and - sera against a range of coating antigen dilutions, using standard conjugate and substrate concentrations. The optimal coating dilution of antigen was taken to be that which gave maximal separation between positive and negative test samples, but still gave low negative values. This technique indicated that the optimum antigen concentration was between 10μg and 50μg.ml^{-1}.

100μl of this coating solution were dispensed to each well of a microtitre plate which was covered and allowed to stand overnight at 25°C with constant gentle vibration.

Plates coated with antigen in this way were stored for up to one week at 4°C without discernible antigen deterioration.

2.5.4.2. Plate Washing Procedure

After coating, each well of the microtitre plate was filled with 300μl of a washing solution consisting of distilled water + 0.05% Brij-35 (Aldrich). The washing solution was left in the plates for 30 seconds before the plate was inverted and shaken dry. This

washing procedure was repeated three times.

2.5.4.3. Antiserum dilutions

Antisera were diluted in one of two ways, depending on the method of evaluation of results;-

(i) Antisera were double-diluted in the plates with phosphate-buffered saline + Brij-35 (0.05%) + bovine serum albumin (0.05%) to a final volume per well of 100µl. Plates were covered and incubated at 25°C for 3 hours, when they were washed as before.

(ii) Antisera were diluted to fall within a range of serum dilutions where it was known (from (i) above) that serum dilution was linearly related to Absorbance at 450nm.

2.5.4.4. Peroxidase-labelled Conjugates

The following enzyme-labelled conjugates were obtained from Flow Laboratories Limited -

(i) peroxidase labelled anti-rabbit IgG

(ii) peroxidase labelled anti-rabbit IgM

(iii) peroxidase labelled anti-mouse IgG

(iv) peroxidase labelled anti-mouse IgM

Lyophilised conjugates were reconstituted in sterile distilled water and dispensed to stoppered sterile 1ml glass vials in 50µl amounts, before storage at -20°C. Working dilutions of conjugates were determined experimentally by assaying various conjugate dilutions against known + and - sera using a standard antigen coating dilution, serum dilution and substrate

concentration.

The results (section 3.3.2.2.) indicated the optimal conjugate dilution; i.e. that giving maximal separation between + and - test samples, but giving low negative values. This dilution was normally 1:1000.

Conjugate dilutions were made up in phosphate buffered saline + Brij-35 (0.05%) + bovine serum albumin (0.05%). 100µl of freshly prepared conjugate at the appropriate dilution was added to each well of the microtitre plate, and the plate incubated at 25°C for 3 hours with constant gentle vibration. Plates were then emptied by inversion and washed as before.

2.5.4.5. Chromogenic substrate

0.01% (w/v) O-phenylene diamine (Sigma) + 0.005% (v/v) H_2O_2 in phosphate buffered saline pH 7.0 was used as the enzyme substrate. 150µl of freshly prepared substrate was added to each well of the microtitre plate and the reaction allowed to proceed in the dark, until a known positive control serum reached an absorbance of 1.0 unit at 450nm. This normally occurred after approximately 30 minutes incubation at room temperature. After colour development, the reaction was stopped by the addition of 0.025ml of 4N NaOH to each well of the microtitre plate.

2.5.4.6. Absorbance Measurements and Evaluation of Results

The absorbance of each well was determined at 450nm using a Titertek Multiscan vertical light-path

photometer (Flow Laboratories). Absorbance at 450nm was converted to ELISA units (Abs ^{450}/100 minutes) and the results expressed as either

(a) a titre i.e. the highest dilution of antiserum giving an arbitrary absorbance value e.g. 5 x the absorbance value of a negative control or

(b) as differences between absorbances given by the same dilution of different sera. Such an approach is valid when dilution of the sera can be shown to fall within a range of serum dilutions being linearly related to absorbance at 450nm.

2.5.4.7. Assay Controls

Inter-assay controls

Empty plates for use in assays were checked for optical homogeneity by taking Absorbance measurements of the wells at the wavelength used for the assay i.e. 450nm. Any plates showing unequal absorbances or distortion were discarded.

Peroxidase-labelled conjugates and antisera were dispensed to small volumes and frozen at -20°C immediately until required.

All the reagents used in the assay were freshly prepared before use from stock solutions.

All incubation steps were carried out under identical conditions at 25°C.

Automatic single and multi-channel pipettes were used with disposable tips to ensure uniformity of dilution.

Intra-assay controls

Rigorous intra-assay controls were necessary to allow comparison of results obtained over many months. Each plate assayed contained a known positive serum and a known negative serum.

The enzyme reaction was stopped when the known positive serum reached an absorbance of 1.0 at 450nm. Serum, conjugate and antigen controls were also incorporated into each plate assayed.

2.5.5. Agglutination Studies

Agglutination tests on sera were carried out in 96 well "u" bottomed microtitre plates (sterilin) Sera were double diluted to 1: 1024 in sterile 0.85% (w/v) saline pH 7.0, to a final volume of 100µl per well. Washed streptococcal cells from an overnight 20ml culture, supplemented with various sugar substrates, were harvested by centrifugation, washed three times in 10ml sterile distilled water and resuspended in 1ml sterile saline. 100µl of cell suspension were added to each serum dilution. Plates were shaken, incubated at 37°C for 2 hours and subsequently allowed to stand at 4°C overnight. Agglutinating titres were taken to be the highest dilutions of antisera showing streptococcal cell agglutination. Pooled sera from non-immunised animals were used as negative controls.

RESULTS

AND

DISCUSSION

3. Results and Discussion

3.1. Production and Quantitation of Cell-Associated Polysaccharides (C.A.P.)

3.1.1. Gross Cultural Characteristics

Growth of the five species of oral streptococci studied was homogeneous and disperse when sugars other than sucrose were used as the carbon source. The other sugar substrates investigated were glucose, fructose, lactose, maltose, trehalose, cellobiose and also an equimolar mixture of glucose and fructose. The growth of S. mutans and of S. sanguis in media containing 2.5% (w/v) sucrose was particulate and characterised by the formation of a cell-layer adherent to the walls of the glass culture vessel. Fig. (9) illustrates the dense, strongly adherent cell-layer formed by S. mutans and Fig. (10) the less adherent, gelatinous accumulation noted with S. sanguis. Growth of S. salivarius in sucrose-containing broth occurred as loosely aggregated floccules (Fig. 11). Such cultures also typically produced a cell-layer weakly adherent to the glass culture vessel. Cultures of S. mitior and of S. milleri showed no adherent properties and grew in the same homogeneous, disperse manner in sucrose as in the other sugars investigated. These results confirm the well documented phenomenon of sucrose-dependent in-vitro adherence observed, in certain members of the oral streptococci, by other workers (McCabe et al (1967; Gibbons and Van Houte 1975). Only strains of S. mutans, S. sanguis and S. salivarius are known to produce extra-cellular polysaccharides from sucrose, and the results

confirm the necessity of such a synthetic ability as a factor influencing this in-vitro adherence. Mukasa and Slade (1973) and Hamada(1977) have demonstrated the importance of active glucan synthesis for in vitro adherence.

The results also illustrate different degrees and types of adherence between the strains of S. mutans, S. sanguis and S. salivarius investigated. Such differences in adherence characteristics have been reported by other workers and have been shown to be mediated by the synthesis of quantitatively and qualitatively heterogeneous extracellular polysaccharides from sucrose (Kuramitsu and Ingersoll 1977). Detailed measurements of the quantity of extracellular polysaccharides produced by different species of oral streptococci have not been reported, and the initial stages of the present study were concerned with obtaining quantitative data on the cell-associated polysaccharides produced by five species of oral streptococci from various sugar substrates.

Fig. 9

In vitro Adherence to Glass of S. mutans cells grown in Broth containing 2.5% sucrose

Fig. 9

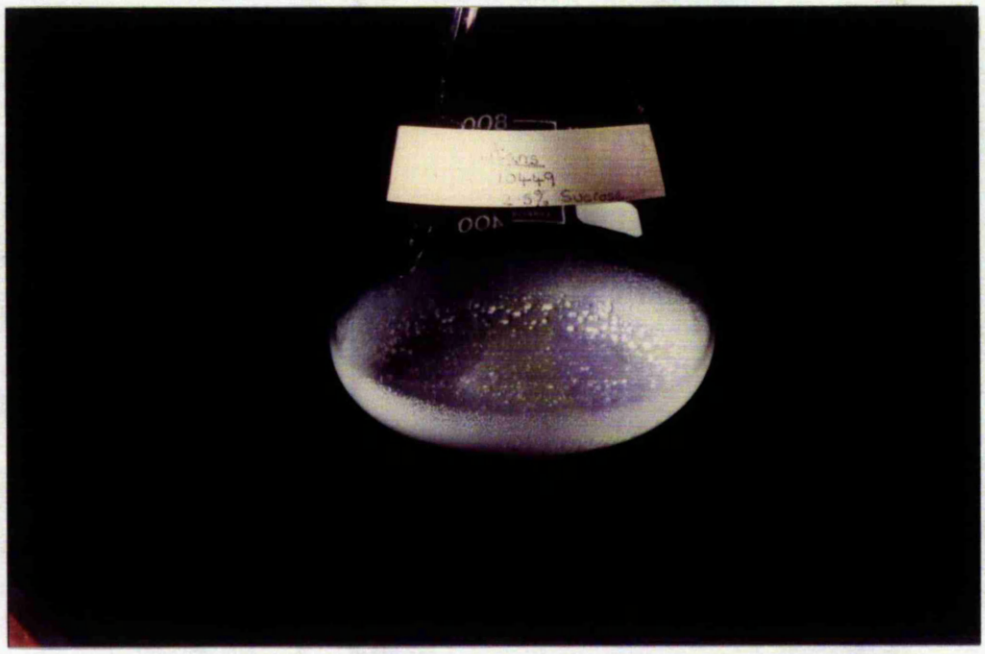


Fig. 10

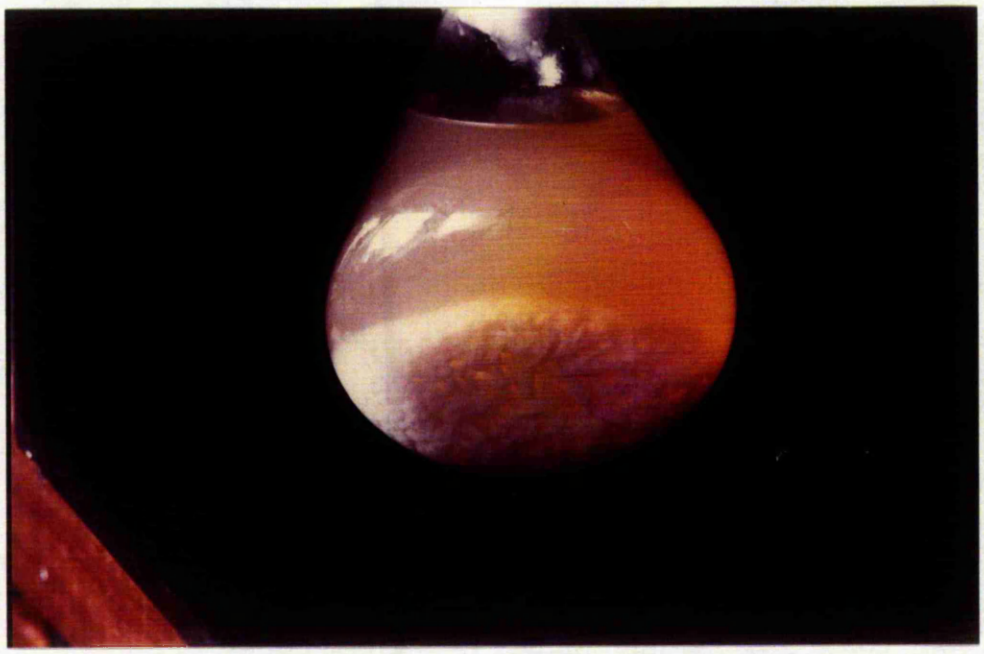
In vitro Adherence to Glass of S. sanguis cells grown in a Broth containing 2.5% sucrose

Fig. 10

Fig. 11

In vitro Abherence to Glass of S. salivarius cells Grown in Broth containing 2.5% sucrose

Fig. 11



3.1.2. Investigation of the Sensitivity and Specificity of the Assays used for C.A.P. Quantitation

Before using spectophotometric assays to quantitate the cell-associated polysaccharides produced by the oral streptococci, it was necessary to determine the specificity and sensitivity of these assays. This was achieved by producing, for each assay, absorption profiles for four classes of substance likely to be present in crude assay samples i.e. iodophilic polysaccharides from intracellular storage material, non-iodophilic polysaccharides, proteins and nucleic acids. These substances were represented by glycogen, dextran, bovine serum albumin and RNA respectively. Absorbance measurements of the various assays were made over the range 390nm to 750nm.

3.1.2.1. The Anthrone Assay for Total Carbohydrate

Fig. (12) illustrates the absorption profiles of dextran, glycogen, bovine serum albumin (BSA) and RNA when equal amounts of each were assayed by the Anthrone method. All four substances absorbed non-specifically over the range 390nm to 470nm. At the assay wavelength of 625nm only dextran and glycogen showed significant absorbance, although a minor peak at 625nm was observed with RNA. This was thought to be due to either the ribose moiety of the nucleic acid, or to contamination by hexoses of the yeast-derived RNA. BSA gave no appreciable activity at 625nm. Glycogen was shown to give lower absorbances at 625nm than dextran when equal amounts of each were assayed. Since all assay samples were lyophilised, this difference can only be due either to impurities in the

glycogen sample, or an intrinsic lack of sensitivity of the anthrone assay for glycogen. In general, the anthrone assay showed good specificity and sensitivity for carbohydrates in the presence of proteins and nucleic acids.

3.1.2.2. The Lowry Protein Assay

As Fig (13) clearly indicates, at the assay wavelength of 750nm, interference with the assay of proteins by carbohydrates or nucleic acids was minimal.

3.1.2.3. RNA Estimation by Bial's Method

At the assay wavelength of 670nm, RNA showed an absorbance peak whereas dextran, glycogen and BSA showed little absorbance at this wavelength (Fig 14).

Interference with the assay of RNA at 670nm by proteins or hexose contain polymers was therefore assumed to be minimal.

3.1.2.4. Iodophilic Polysaccharide Estimation

As Fig (15) indicates, the method of Dipersio et al (1974) showed little differentiation at the recommended wavelength of 520nm between glycogen and the other solutes tested. The specificity and sensitivity of the assay were low and consequently it was not used quantitatively in the present study. Assay of bacterial cell extracts was also hampered by the formation and precipitation of brown iodine/polysaccharide complexes, making absorbance measurements impossible.

The formation and comparative quantities of these iodophilic complexes were noted, however, and are recorded qualitatively in Section 3.2.1.

Fig.12

Specificity of the Anthrone Method for the Determination of Total Carbohydrate.

○	dextran	(100 $\mu g.ml^{-1}$)
●	Glycogen	(100 $\mu g.ml^{-1}$)
□	RNA	(100 $\mu g.ml^{-1}$)
■	bovine serum albumin	(100 $\mu g.ml^{-1}$)

sources

dextran - ex. L.mesenteroides (Sigma)
M.Wt. 60,000 - 90,000

glycogen - ex. Oyster (Sigma)

RNA - ex. Torula yeast (Sigma)

B.S.A. - (Sigma)

Fig. 12

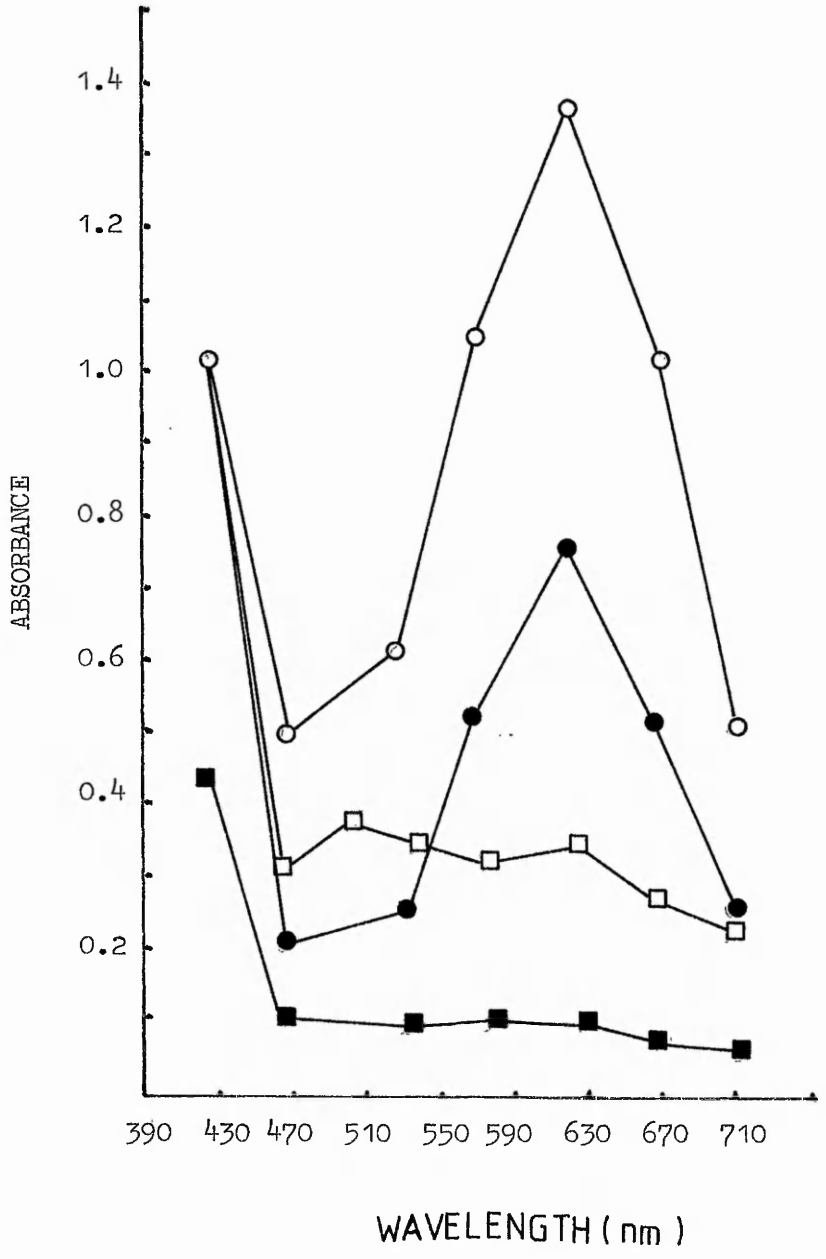

1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
390 430 470 510 550 590 630 670 710
WAVELENGTH (nm)

Fig. 13

Specificity of the Lowry Protein Assay

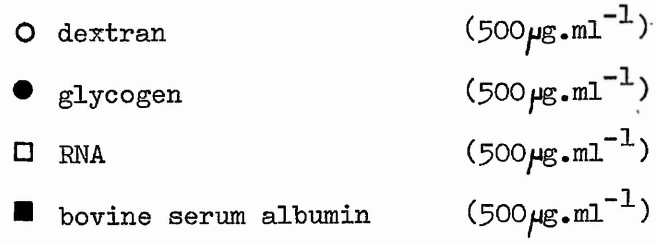


Fig. 13

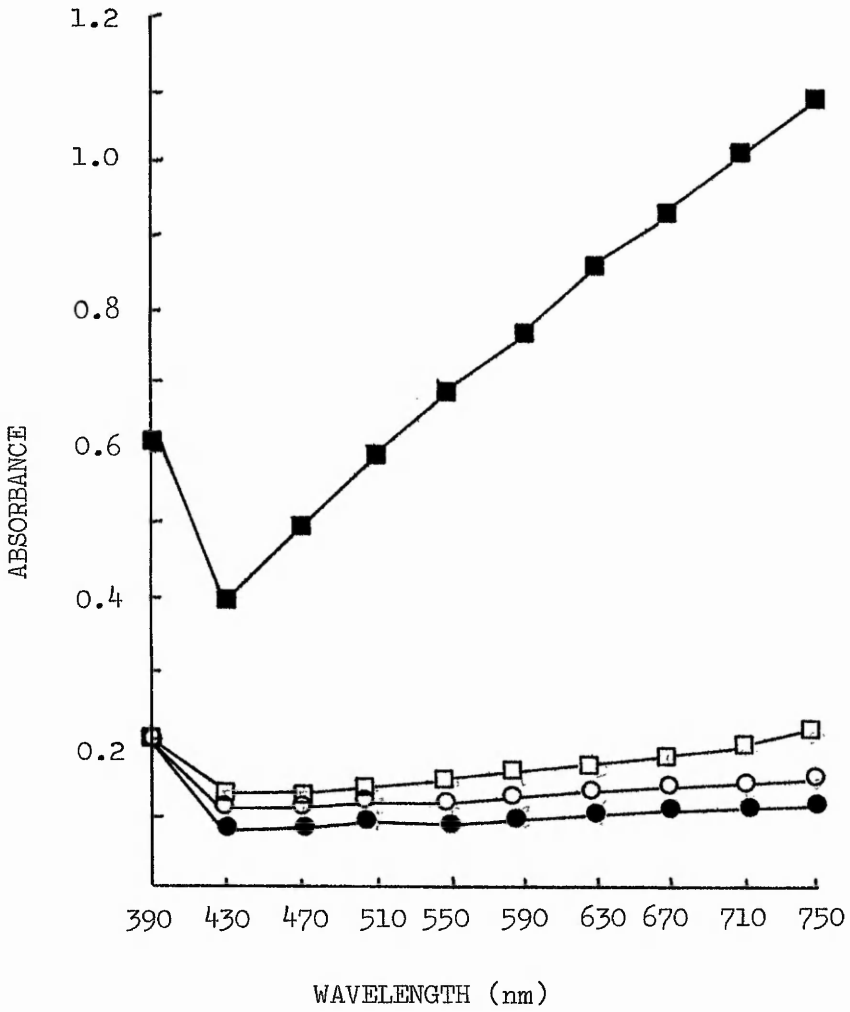

1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
390
430
470
510
550
590
630
670
710
750
WAVELENGTH (nm)

Fig. 14

Specificity of the Bial Method for RNA Determination

○	dextran	(500μg.ml^{-1})
●	glycogen	(500μg.ml^{-1})
□	RNA	(500μg.ml^{-1})
■	bovine serum albumin	(500μg.ml^{-1})

Fig. 14

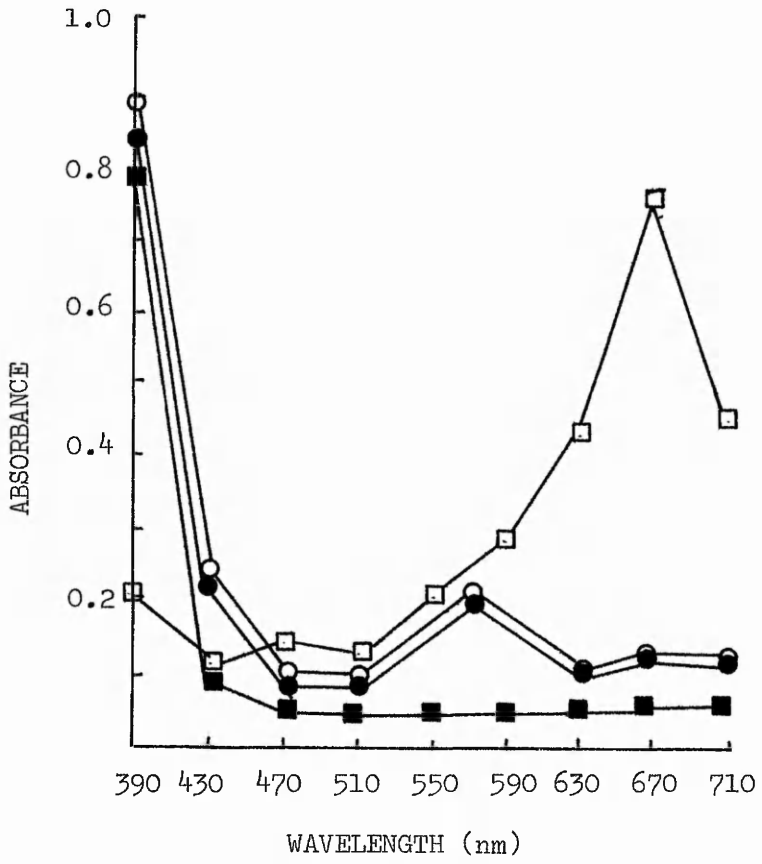
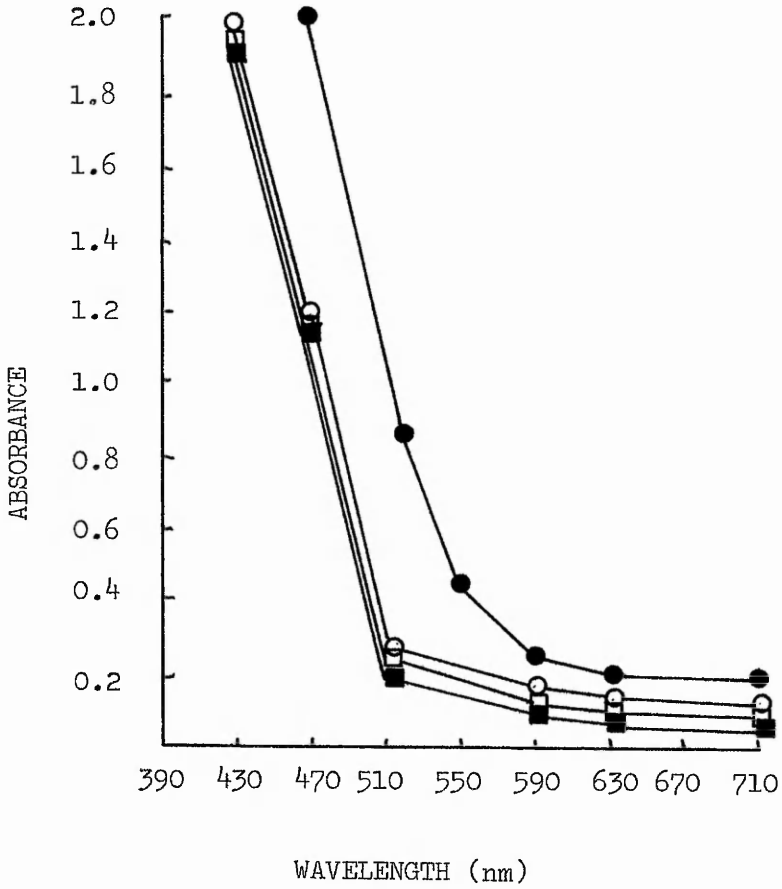

1.0
0.8
0.6
0.4
0.2
ABSORBANCE
390 430 470 510 550 590 630 670 710
WAVELENGTH (nm)

Fig. 15

Specificity of the Iodophilic Polysaccharide Assay of DiPersio

○	dextran	($500 \mu g.ml^{-1}$)
●	glycogen	($500 \mu g.ml^{-1}$)
□	RNA	($500 \mu g.ml^{-1}$)
■	bovine serum albumin	($500 \mu g.ml^{-1}$)

Fig. 15


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
390
430
470
510
550
590
630
670
710
WAVELENGTH (nm)

3.1.2.5. The Effect of the Concentration of NaOH in the Assay Samples on the Anthrone and Lowry Assays

Since extraction of cell-associated polysaccharides was achieved with aqueous sodium hydroxide solutions, it was important to determine the effect of using alkaline assay samples on the efficiency of both the anthrone carbohydrate assay, and on the assay of protein by the Lowry procedure. Fig (16) shows the effect of assaying 100μg.ml^{-1} solutions of glucose by the anthrone method, and BSA by the Lowry method, using sample NaOH normalities between 0 and 2.5N. It was determined that this range of sample concentrations had no effect on the efficienty of the carbohydrate assay, presumably because of the addition to the sample of two volumes of concentrated sulphuric acid during the assay. However, sample concentrations stronger than 0.5N NaOH resulted in a marked impairment of the Lowry assay for protein. For this reason, bacterial extracts in N NaOH were diluted at least by a factor of 2 and more usually by a factor of 5 before estimating their protein content using this assay.

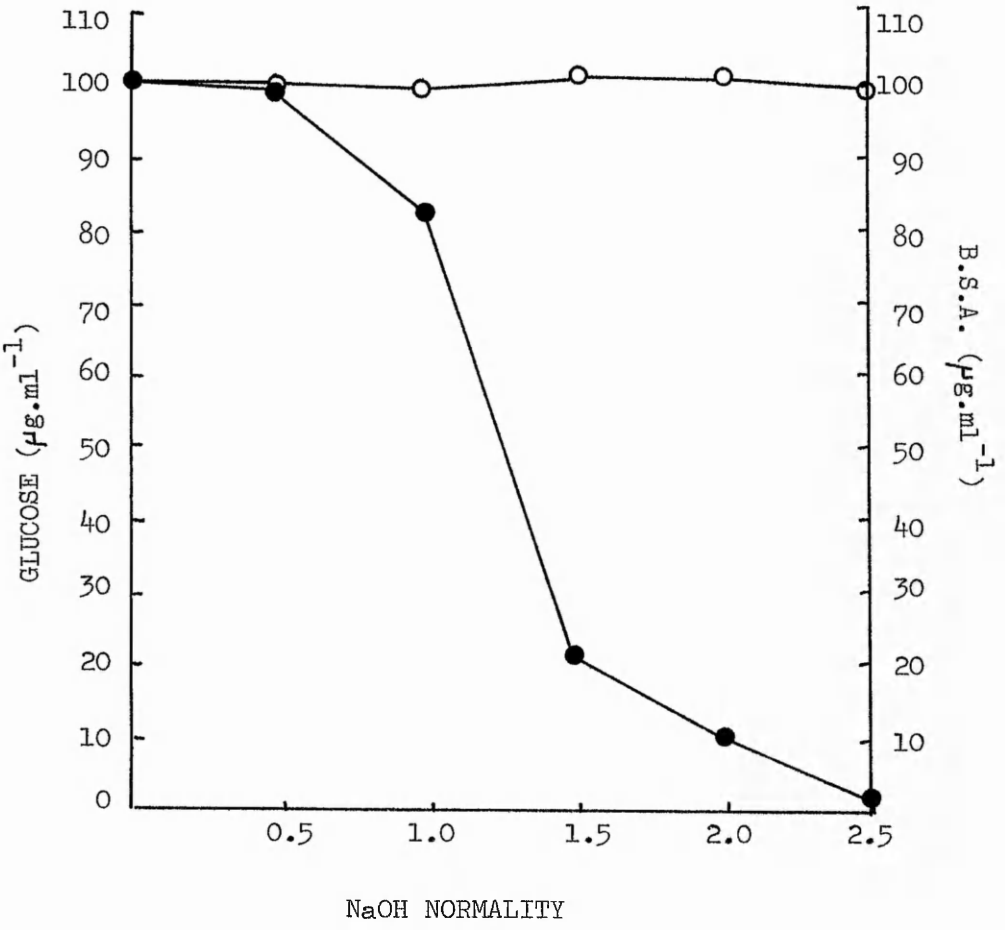
Fig. 16

Effect of NaOH Normality of Assay Samples on the Anthrone (Carbohydrate) and Lowry (Protein) Assays

o glucose ($100\ \mu g.ml^{-1}$)

● bovine serum albumin ($100\ \mu g.ml^{-1}$)

Fig. 16


110
100
90
80
70
60
50
40
30
20
10
0
GLUCOSE (μg.ml⁻¹)
B.S.A. (μg.ml⁻¹)
0.5
1.0
1.5
2.0
2.5
NaOH NORMALITY

3.1.3. The Extraction of Cell-Associated Polysaccharides from Streptococcal cells using Aqueous NaOH solutions

The aim of this series of experiments was to determine the optimum conditions for NaOH extraction of extracellular cell-associated glucans.

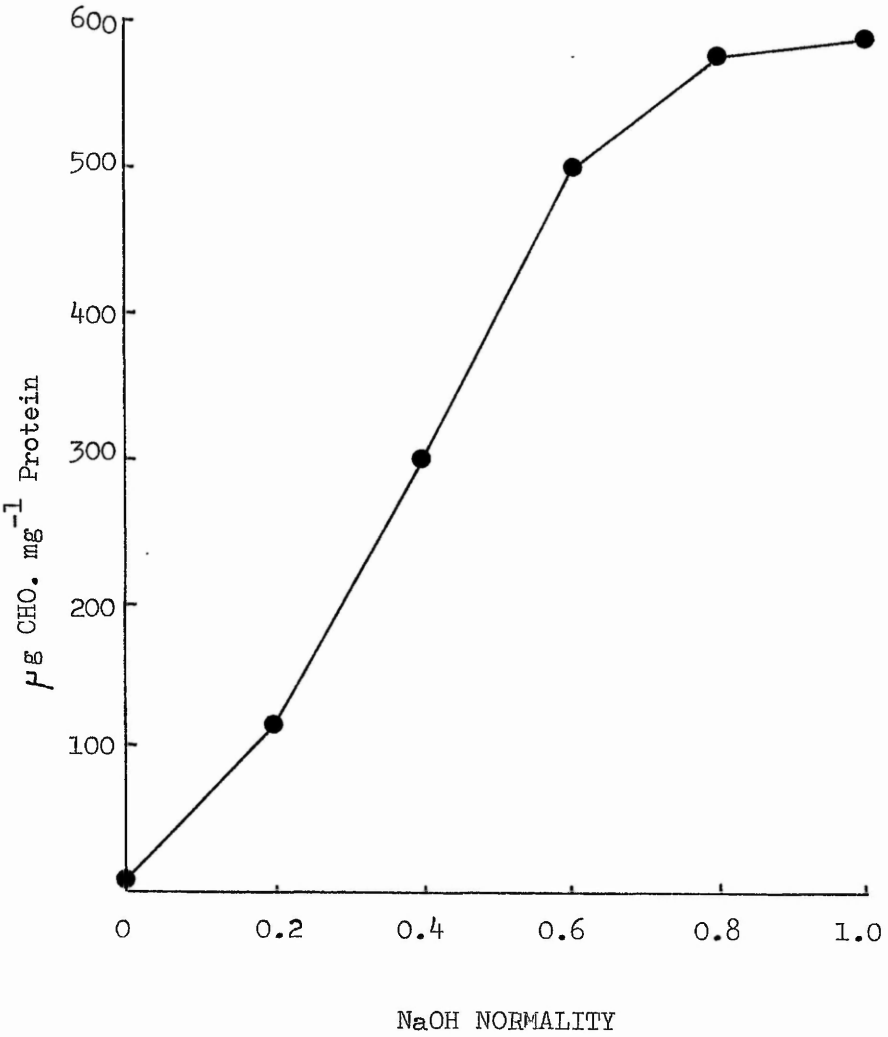
As Walker (1978) observes, alkaline extraction of glucans from cells which store glycogen is a risky procedure, but milder alkaline conditions are not effective in releasing water-insoluble glucans, releasing only the more water soluble "dextrans". Thus, the extraction conditions used, in terms of NaOH concentration and temperature, should produce the highest yield of C.A.P., without being severe enough to cause appreciable cell-lysis, resulting in measurement of intracellular storage polysaccharides. Several workers have used very high concentrations of alkaline extraction solutions, without apparently considering the possible contamination of their extracts with glycogen-like intracellular storage polysaccharides. Attempts were made later in the present study to obviate this possibility by incubating streptococcal cells overnight in neutral phosphate buffer so that much of the intracellular polysaccharide known to be stored by these organisms (DiPersio et al 1974; DiPersio 1978) could be metabolised prior to NaOH extraction of the extracellular glucans.

Fig (17) shows the relationship between the NaOH concentration of the extraction solution and the yield of C.A.P. per mg. of cell protein from washed cells of

Fig. 17

Effect of the Extraction Concentration of NaOH on the Yield of Cell-Associated Carbohydrates

Fig. 17


600
500
400
300
200
100
μg CHO. mg⁻¹ Protein
0
0.2
0.4
0.6
0.8
1.0
NaOH NORMALITY

S. mutans grown in 2.5% (w/v) sucrose broth. All extractions were performed at 25°C. It can be seen that increasing the NaOH concentration of the extraction solution increased the yield of C.A.P. up to a maximum at 1N NaOH. The extraction yield increased rapidly at NaOH concentrations up to 0.8N and then slowed at concentrations above this. This is presumably a reflection of the extraction of increasingly alkali insoluble material with increasing NaOH concentration, and the reduction in rate of extraction above 0.8N was taken to indicate removal of all alkali-soluble surface-associated glucan.

After 1N NaOH treatment, streptococcal cells retained their Gram positivity and structural integrity when stained and examined microscopically. It was assumed, therefore, that lysis of these cells during extraction at NaOH concentrations at 1N was minimal.

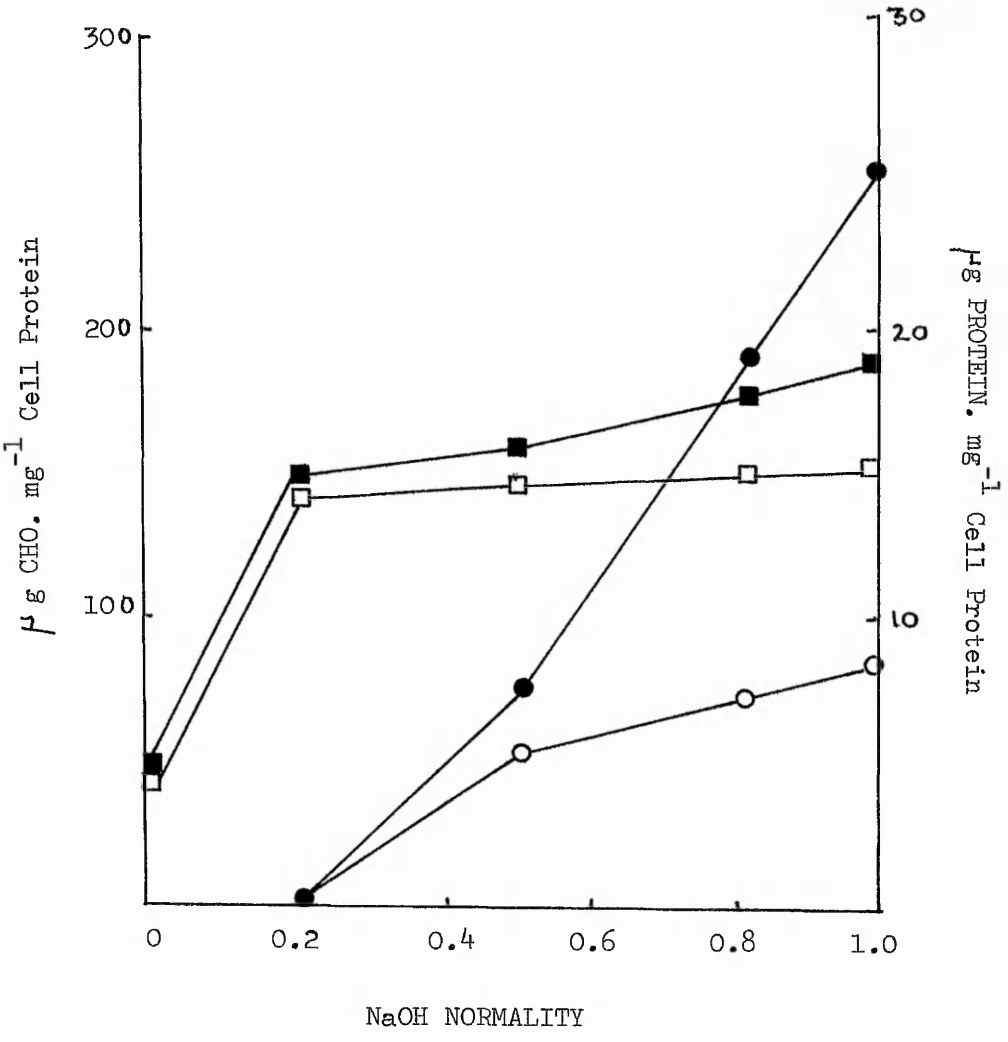
Fig (18) indicates the results of an investigation into the effect of extraction temperature and NaOH concentration on the protein and carbohydrate content of NaOH extracts of cells of S. mutans grown in 2.5% (w/v) sucrose broth. It can be seen that performing the polysaccharide extraction in N NaOH at a temperature of 25°C yielded 250% of the carbohydrate achieved at an extraction temperature of 0°C. This was presumably a reflection of the reduced alkaline solubility of carbohydrates at the lower temperature. Performing extractions at 25°C also resulted in higher concentrations of protein in the NaOH extracts, and was thought to be an indication of the measurement of proteins intimately associated with the extra-cellular

Fig. 18

Effect of Increasing NaOH Normality of Extraction, on Yields of Protein and Carbohydrate At Two Extraction Temperatures

- ● CHO Yield at 25°C.
- ○ CHO Yield at 0°C.
- ■ Protein Yield at 25°C.
- □ Protein Yield at 0°C.

Fig. 18


300
200
100
30
20
10
0
0.2
0.4
0.6
0.8
1.0
μg CHO. mg-1 Cell Protein
μg PROTEIN. mg-1 Cell Protein
NaOH NORMALITY

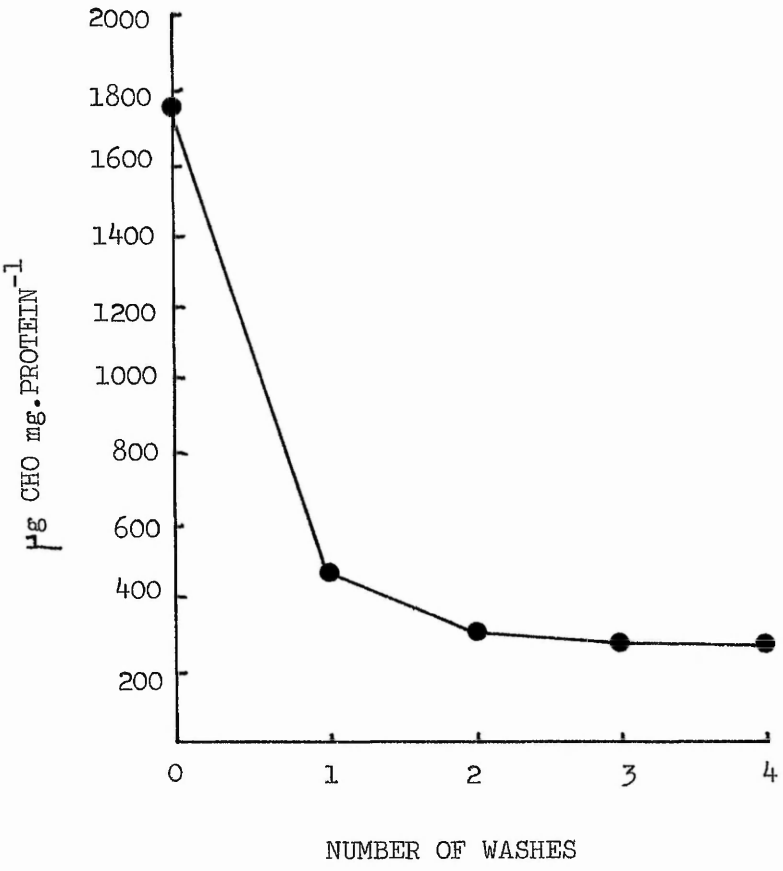
polysaccharides at the cell surface e.g. glycosyl and fructosyl transferases, rather than cell wall proteins. The increase in extracted protein at 25°C was small, however, and since cells retained their integrity and Gram positivity after treatment at 25°C, this temperature was chosen for subsequent extractions, using an NaOH concentration of 1N. The conditions used for extraction in the present study differ markedly from those used by other workers. Genco et al (1976) extracted their "Cell Associated Glucan" with 20% Potassium hydroxide at 100°C for 60 minutes. Such treatment almost certainly caused the lysis of a proportion of their cells, and it seems likely that much of the polysaccharide recovered by them from ethanolic precipitation of their neutralised extract was derived from intracellular locations.

The present study was concerned with the extracellular, cell-associated glucans of the oral streptococci, and not with the more water-soluble glucans released into the culture medium, or loosely attached at the cell surface. For this reason, and also to ensure that carbohydrates derived from the growth medium, adherent to streptococcal cells were not measured as extracellular polysaccharides, cells were washed in distilled water prior to NaOH extraction. Fig (19) shows that three such cell washing procedures were sufficient to remove both nutrient carbohydrates and also the more water-soluble, loosely associated extracellular polysaccharides. Further washes failed to remove significant quantities of what was assumed to be polysaccharide intimately associated with the cell surface.

Fig. 19

Effect of Cell Washing on Yield of Carbohydrate

Fig. 19


2000
1800
1600
1400
1200
1000
800
600
400
200
µg CHO mg.PROTEIN^{-1}
0
1
2
3
4
NUMBER OF WASHES

3.1.4. Measurement of C.A.P. Produced by Cultures of S. mutans Grown on Four Sugar Substrates

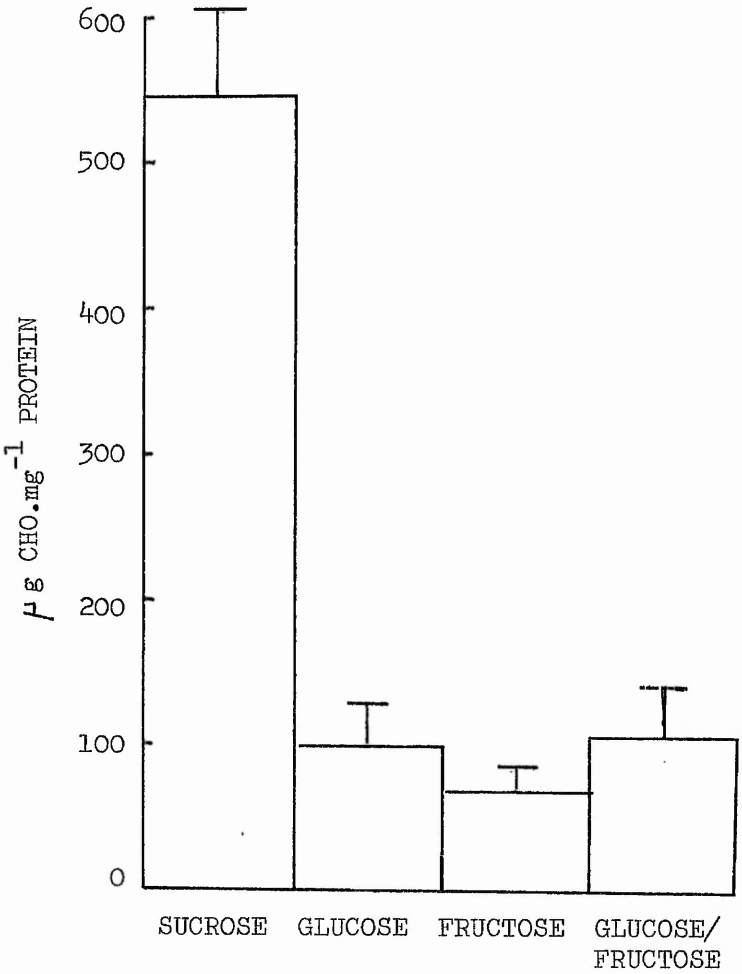
Preliminary experiments on the measurement of cell-associated polysaccharides from the oral streptococci were performed on cultures of S. mutans. Measurements of cell-associated polysaccharides produced from sucrose, glucose, fructose and from an equimolar mixture of glucose and fructose were obtained and summarised in Fig (20). All cultures were harvested after 24 hours of incubation at $37^{\circ}C$, and it was found that growth of the organism on sucrose resulted in an average C.A.P. yield of 552 ± 49.8 $\mu g.mg^{-1}$ Cell Protein. Growth of S. mutans on glucose produced a C.A.P. yield of 98 ± 26 $\mu g.mg^{-1}$ Cell Protein which was very similar to that produced by growth on an equimolar mixture of glucose and fructose. When fructose was used as a substrate for the growth of S. mutans the yields of C.A.P. produced were always lower than those produced from glucose, but not significantly so at the 95% confidence level when analysed statistically. The figures represent the averages of five triplicate experiments.

The aims of these experiments were to establish quantitative data on the amount of C.A.P. produced from different sugar substrates, to determine the accuracy of the measurements possible, and also to establish whether S. mutans was capable of synthesising glucans from an equimolar mixture of glucose and fructose, rather than from sucrose. The method was shown to be very reproducible between replicates

Fig. 20

Yields of Cell-Associated Polysaccharide from S. mutans grown on sucrose, glucose, fructose and an Equimolar Mixture of Glucose and Fructose

Fig. 20


600
500
400
300
200
100
0
µg CHO.mg-1 PROTEIN
SUCROSE
GLUCOSE
FRUCTOSE
GLUCOSE/
FRUCTOSE

(S.E < 2%) but that larger variations in measurements were observed between experiments. This indicates that the method itself appears reasonably reproducible, but that the production of C.A.P. by streptococci is quantitatively quite variable, even under tightly controlled cultural conditions. The results obtained indicate that growth of S. mutans on sucrose produced approximately five times the anthrone reacting substances at the cell surface of cells grown on the other three substrates. An equimolar mixture of glucose and fructose appears to be incapable of promoting synthesis of C.A.P. This excludes the possibility that such a mixture, shown to be as cariogenic as sucrose when fed to monkeys (Colman et al 1977) is subject to polymerisation by S. mutans into glucans or fructans. It should be noted that while glucose, fructose and the mixture of glucose and fructose produced much lower levels of cell-surface carbohydrate than sucrose, the amounts produced from these sugar substrates was higher than might be expected, since only sucrose is reported to mediate C.A.P. synthesis in the oral streptococci. The nature of these anthrone-reacting substances present at the cell surface was later investigated and is discussed in Section 3.2.

3.1.5. Measurement of C.A.P. Produced by Five Species of Oral Streptococci Grown on Seven Sugar Substrates

The aim of this section of the work was to compare the maximum C.A.P. yields of S. mutans, S. sanguis, S. salivarius, S. mitior and S. milleri grown on several sugars. To ensure that the maximum yields were compared. it was essential to determine for each organism and each sugar substrate, when this maximum value was produced. Therefore, cell protein measurements and measurements of C.A.P. yield were taken for each organism, grown on each substrate at 12 hour intervals over a total incubation time of 60 hours at 37°C.

The results obtained are illustrated in Figs (21 to 25) and represent averages of triplicate experiments.

3.1.5.1. S. mutans

Fig (21a) illustrates the growth (as cell protein) of S. mutans in broth culture containing 2.5% (w/v) sucrose, glucose, fructose, maltose, lactose, cellobiose and trehalose. Growth of S. mutans occurred on all of these sugar substrates but it was noted that growth on cellobiose was preceeded by an extended lag phase, and was presumably due to enzyme induction or to a preliminary extracellular enzyme modification of this substrate before assimilation. Growth on six of the seven substrates entered the stationary phase after 24 hours of incubation, but cells grown on maltose were limited more quickly and growth reached a maximum after 12 hours of incubation. The final cell yields of S. mutans grown on maltose, fructose,

lactose and trehalose were similar at approx. 0.2 mg. Protein. ml^{-1}. However, the final cell yields of cells grown on sucrose and glucose were similar to each other at approx. 0.3 mg. Protein. ml^{-1}, but were consistently higher than those achieved on the other sugars. Cellobiose commonly produced low final cell yields (0.1mg Protein. ml^{-1}).

Why growth of S. mutans on sucrose and glucose should provide higher terminal cell yields than growth on the other sugars tested is not clear, but it may be that these substrates can be utilised at a lower energy expenditure than the other substrates investigated because the enzymes necessary for their assimilation and metabolism are constitutive in this organism. This could explain the finding that cellobiose, which required a significant lag period before utilisation, also produced the lowest terminal cell protein yield of the substrates investigated. If the data from these growth curves are related to those obtained for S. mutans C.A.P. production outlined in Fig (21b), it can be seen that only growth on sucrose or, surprisingly, on maltose stimulated the production of more than 100μg. Carbohydrate (CHO). mg^{-1} cell protein. The maximum yield of C.A.P. from organisms grown on sucrose was achieved at 24 hours, and production appeared to occur during the logarithmic phase of cell growth. The yield of C.A.P. began to decrease markedly as cells entered the stationary phase. It is likely that this decrease in C.A.P. yield was mediated by the activity of glucan hydrolysing enzymes.

Several workers have reported the constitutive production by S. mutans of an α1-6 glucanase. An endoglucanase specific for the α 1-6 linkage has been purified from the culture supernatant of S. mutans by Guggenheim and Burckhardt (1974), and it may be that the structural heterogeneity of the S. mutans glucans may be a result of the combined enzymatic actions of glucosyltransferases and glucanases. During the stationary phase, the net effect would be an enzyme degradation of cell-associated glucans. There is little doubt that α 1-6 glucans of streptococci act as reserve carbohydrates and contribute to the growth of cariogenic organisms. (Wood 1967; 1969). Rapid degradation by plaque dextranases explains why the proportion of α 1-6 glucan found among the carbohydrates in dental plaque by Hotz et al (1972) was low, whereas the level of glucose and oligosaccharides was high.

Cells grown on maltose showed a quite different pattern of C.A.P. synthesis. The maximum yield of C.A.P. from cells of S. mutans grown on maltose occurred some 12 hours after the cells had entered the stationary phase of growth, but, after achieving this level, C.A.P. decreased markedly with prolonged incubation, eventually after 60 hours approaching the levels of C.A.P. produced by sugars other than sucrose. Investigations into the nature of the C.A.P. produced by maltose-grown cells of oral streptococci were undertaken and are discussed in Sections 3.1.6. and 3.2.

Fig. 21a

The Growth of S. mutans on Seven Sugar Substrates

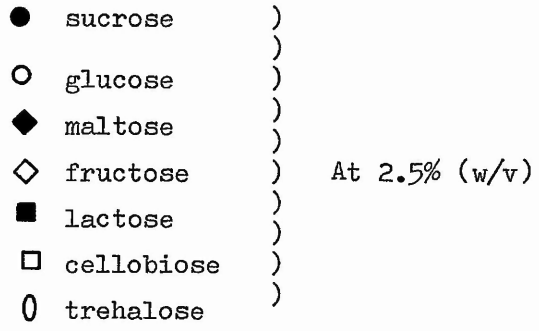


Fig. 21b

Production of Cell-Associated Polysaccharides by S. mutans Grown on Seven Sugar Substrates

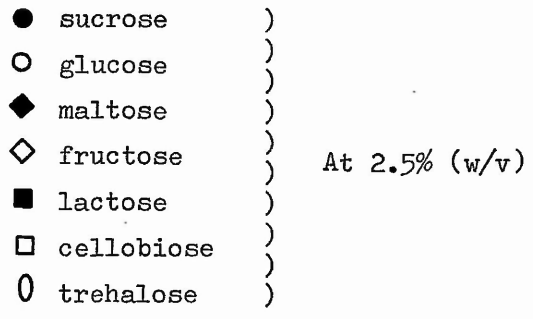


Fig. 21a

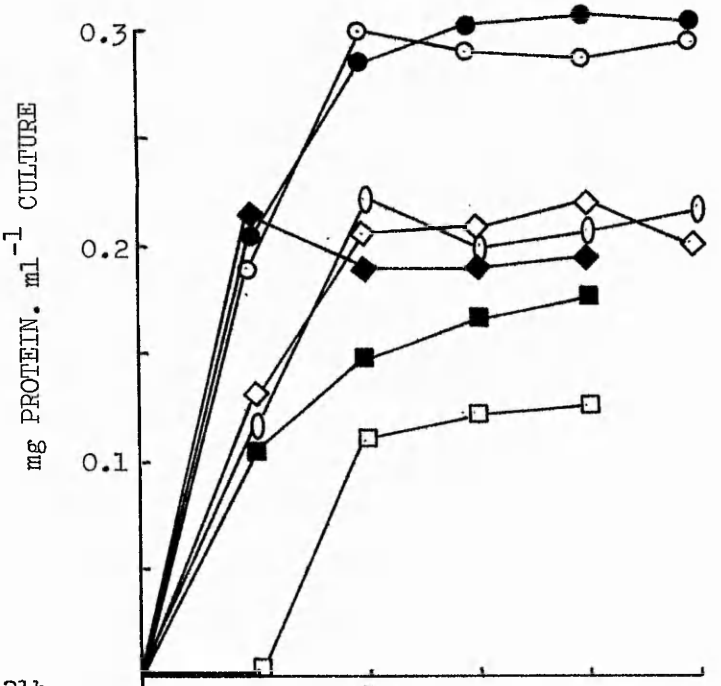
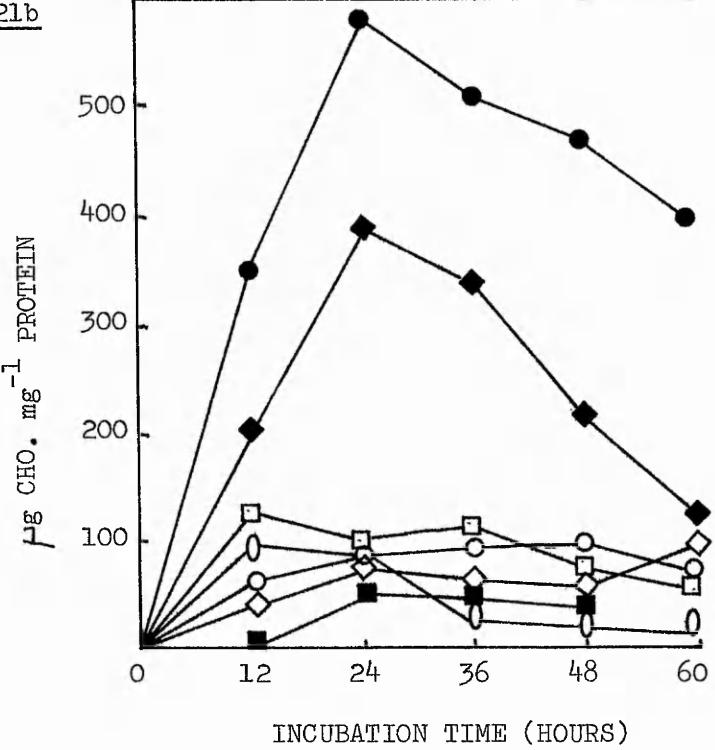


Fig. 21b



3.1.5.2. S. sanguis

Fig (22a) illustrates the growth of S. sanguis in broth culture, containing 2.5% (w/v) sucrose, glucose, fructose, maltose, lactose, cellobiose and trehalose.

Cellobiose and trehalose were found not to support the growth of the S. sanguis strain used in the investigations. The utilisation of these two sugars has been reported as a variable characteristic in this species (Cowan 1974).

As with S. mutans it was noted that growth by S. sanguis on sucrose and glucose produced similar levels of cell protein, and that this level was significantly higher than terminal cell protein yields achieved by cultures growing on maltose, lactose or fructose. This may be for the same energy and enzymic induction considerations postulated for S. mutans in Section 3.1.5.1, since growth of S. sanguis on lactose and fructose was characterised by an extended lag phase, with no cell growth measurable after 12 hours of incubation. S. sanguis grown on maltose exhibited the same early limitation at 12 hours seen with S. mutans grown on the same substrate.

Results obtained for the production of C.A.P. by S. sanguis (Fig 22b) indicated that, as with S. mutans only sucrose and maltose grown cells produced more than 100μg.CHO. mg^{-1} Protein. The nature of the C.A.P. produced from these two sugars will be discussed in detail in a later section. The maximum C.A.P. production by S. sanguis grown on sucrose occurred later than that of S. mutans and amounted to only 50% of the quantity produced by S. mutans. This related well to the gross appearances of cultures described in

Section 3.1.1. where it was shown that the adherent cell-layer produced in vitro by S. mutans from sucrose was thicker and more adherent than that produced by S. sanguis under similar conditions.

Quantitative data obtained in this section indicated a relationship between the amount of C.A.P. produced and in vitro adherence characteristics, although the nature of the synthesised glucans is also important in this respect. Fig (22b) shows that, while yields of C.A.P. did decline after the organism had entered the stationary phase, the rate of this decline was less apparent in S. sanguis than in S. mutans. This may in part be due to the reported lack of a constitutive α1-6 glucanase in S. sanguis (Dewar and Walker 1975).

C.A.P. production by S. sanguis grown on maltose showed a similar pattern to that of S. mutans but again, produced much lower maximum levels (190μg.mg^{-1} as opposed to 390μg.mg^{-1} for S. mutans).

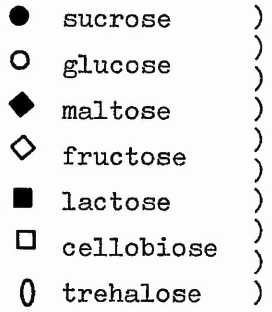
3.1.5.3. S. salivarius

Fig (23a) illustrates the growth of S. salivarius in broth culture containing 2.5% (w/v) sucrose, glucose, fructose, maltose, lactose, cellobiose and trehalose.

Cellobiose was found not to support the growth of the strain of S. salivarius used in the study. The six sugar substrates which did support the growth of this organism were rapidly assimilated and in no case was growth subject to any extended lag phase. S. salivarius appeared to have the constitutive ability to utilise all the sugar substrates equally well and consequently the growth of

Fig. 22a

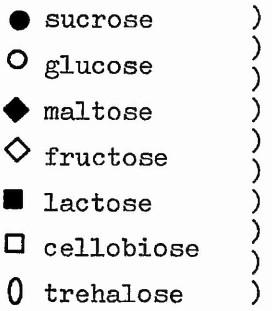
The Growth of S. sanguis on Seven Sugar Substrates



At 2.5% (w/v)

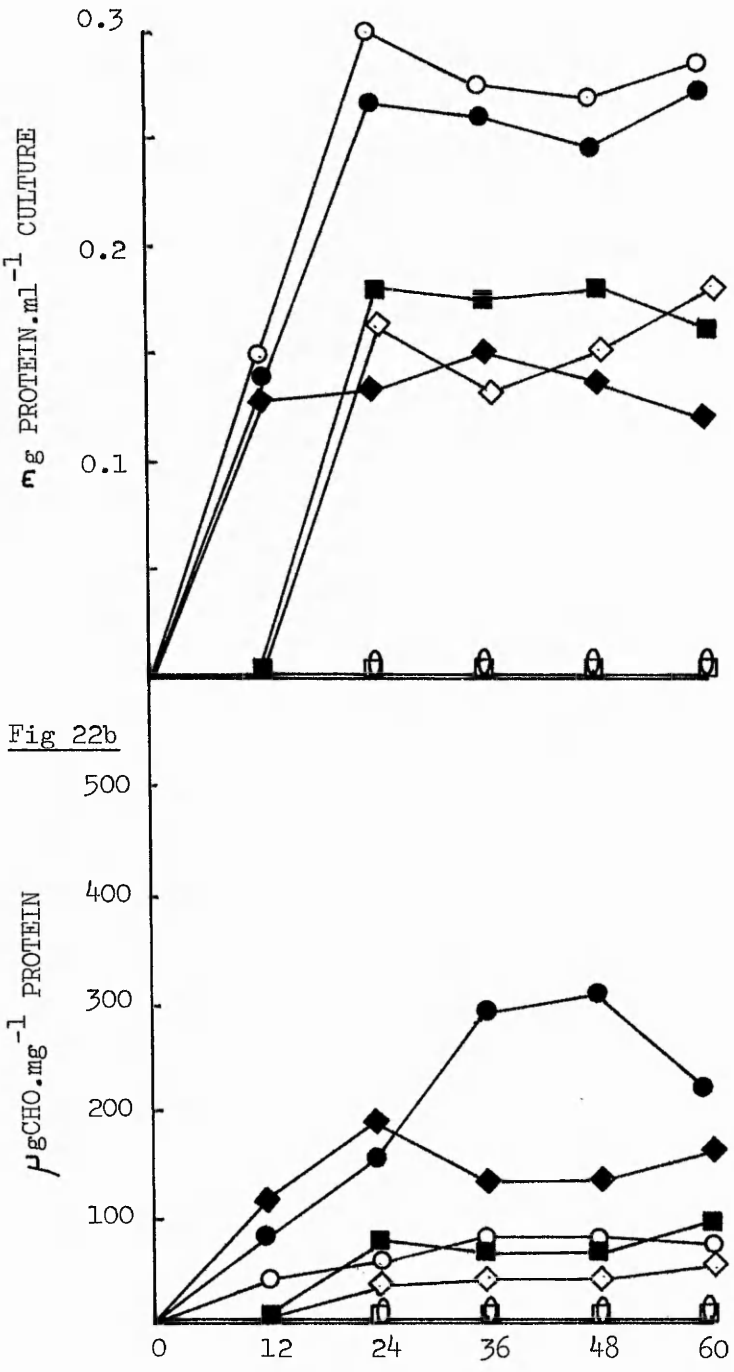
Fig. 22b

Production of Cell-Associated Polysaccharides by S. sanguis Grown on Seven Sugar Substrates



At 2.5% (w/v)

Fig. 22a


0.3
0.2
0.1
mg PROTEIN.ml-1 CULTURE
Fig 22b
500
400
300
200
100
µgCHO.mg-1 PROTEIN
0
12
24
36
48
60
INCUBATION TIME (HOURS)

this organism was more rapid than that of either S. mutans of S. sanguis. Growth limitation, in the case of each sugar occurred at 12 hours after incubation and terminal cell protein yields fell within the range 0.14mg.ml^{-1} to 0.22mg.ml^{-1}. There was no evidence of higher terminal cell protein yields of cultures grown on sucrose and glucose as was the case with S. mutans (Fig. 21a) and with S. sanguis (Fig. 22a), indicating that these sugar substrates were more available, in terms of net energy expenditure to S. salivarius than to the other two species.

Results obtained for the production of C.A.P. by S. salivarius grown on the seven sugar substrates (Fig. 23b) indicated almost impossibly high quantities of C.A.P. produced by growth on sucrose (14 mg.CHO.mg^{-1} Protein). It is likely that this figure is an overestimate because of certain practical difficulties encountered in the measurement of S. salivarius C.A.P. grown on this sugar. The extraction of C.A.P. from sucrose grown cells of S. salivarius into NaOH resulted in a dramatic increase in the viscosity of the extraction solution, and it was found that volumes of NaOH less than 200ml. were solidified by the C.A.P. extracted from cells of S. salivarius derived from 1l of culture. This extract was diluted with fresh NaOH to facilitate the removal, by centrifugation of bacterial cells.

However, the viscosity exhibited by even dilute extracts of S. salivarius C.A.P. made complete removal of these cells very difficult. For this reason, it is likely that the cell protein yields for S. salivarius grown on sucrose are

Fig. 23a

Growth of S. salivarius on Seven Sugar Substrates

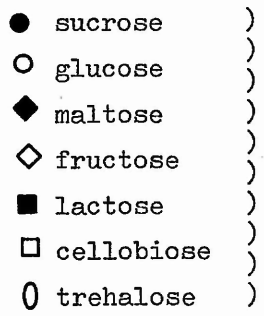


Fig. 23b

Production of Cell-Associated Polysaccharides by S. salivarius Grown on Seven Sugar Substrates

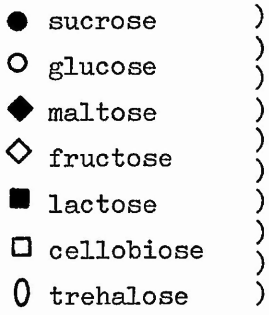


Fig. 23a

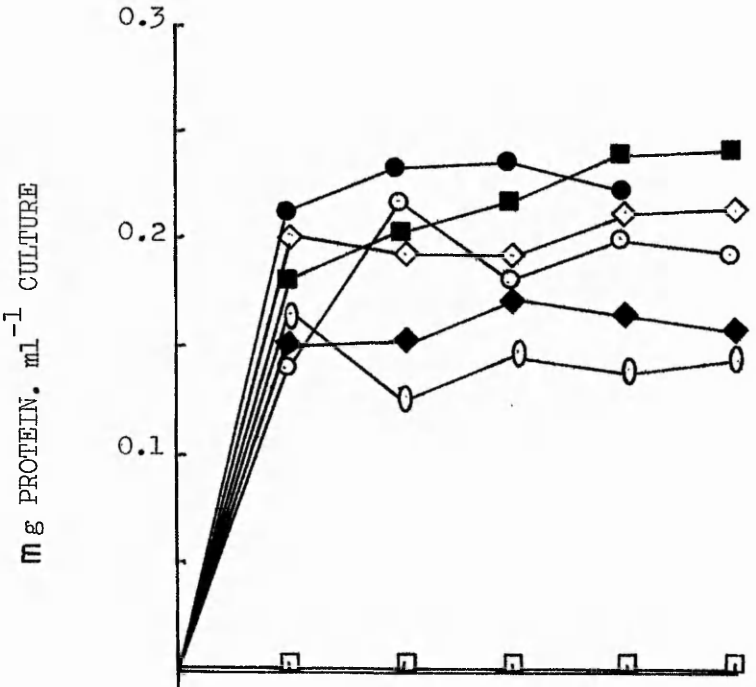
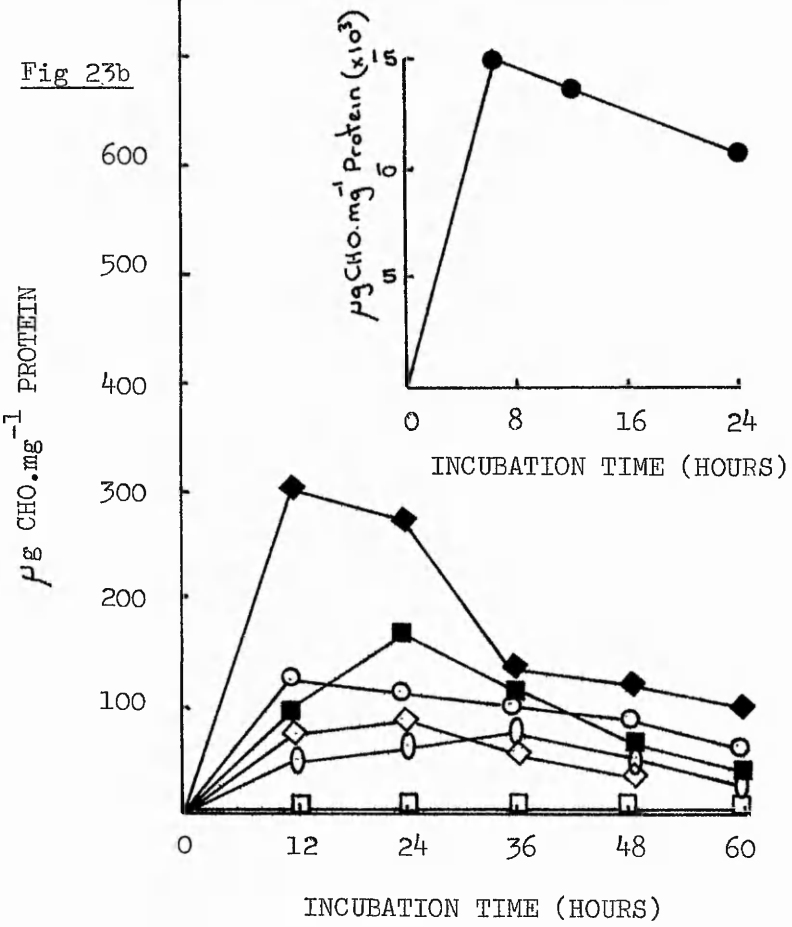


Fig 23b



underestimates, and for the same reason, that the C.A.P. yields are overestimates. It should be noted, however, that the cell protein yields for this organism grown on sucrose are not lower than expected and so error due to this difficulty may not have been large.

The maximum C.A.P. yield obtained by growth on sucrose was obtained after only 6 hours of incubation at 37°C, and rapidly decreased thereafter to approximately 70% of this value after 24 hours of incubation, probably as the result of enzymic degradation.

As with S. mutans and S. sanguis the only other sugar from which S. salivarius would produce substantial amounts of C.A.P. was maltose. This production was highest after 12 hours of incubation and decreased after 24 hours.

S. salivarius grown on sugars other than sucrose and maltose did not produce C.A.P. levels significantly higher than $100\mu g.mg^{-1}$. This significance of the C.A.P. levels derived from maltose will be discussed in detail in Sections 3.1.6. and 3.2.

3.1.5.4. S. mitior

Fig (24a) illustrates the growth of S. mitior in broth cultures containing 2.5% (w/v) sucrose, glucose, fructose, maltose, lactose, cellobiose and trehalose.

Neither cellobiose nor trehalose would support the strain of S. mitior used in the study. Growth patterns for the other five sugar substrates differed considerably. Lactose would support growth only after a 12 hour lag phase, and 24 hours of incubation were required before growth on fructose could be detected. Maltose and sucrose-grown cultures

Fig. 24a

Growth of S. mitior on Seven Sugar Substrates

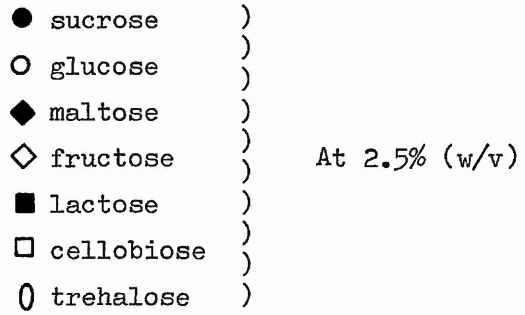


Fig. 24b

Production of Cell-Associated Polysaccharides by S. mitior Grown on Seven Sugar Substrates

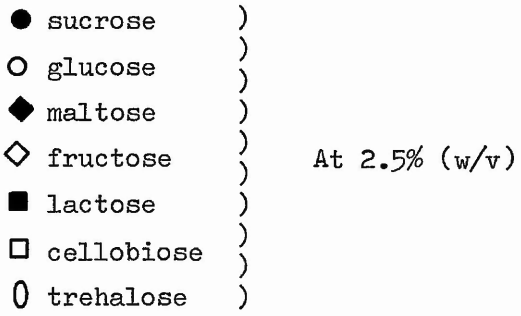


Fig. 24a

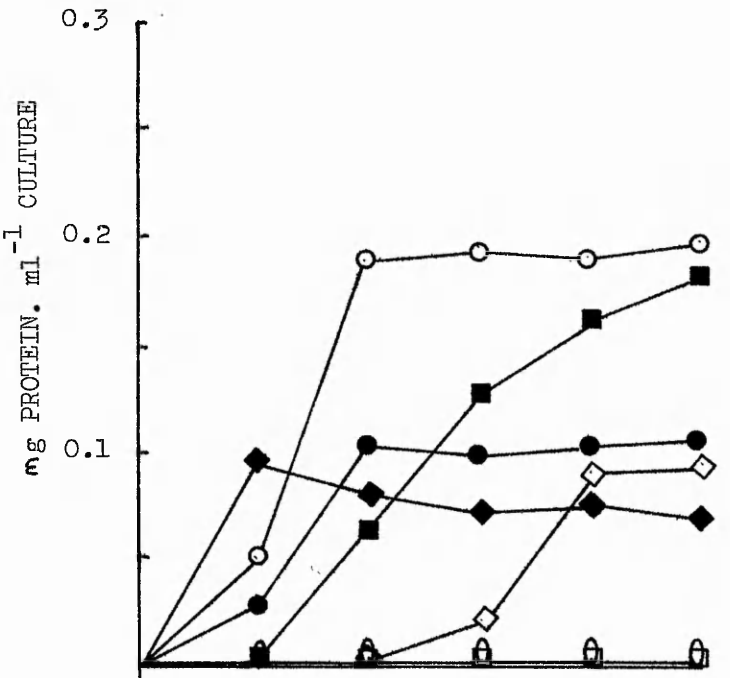
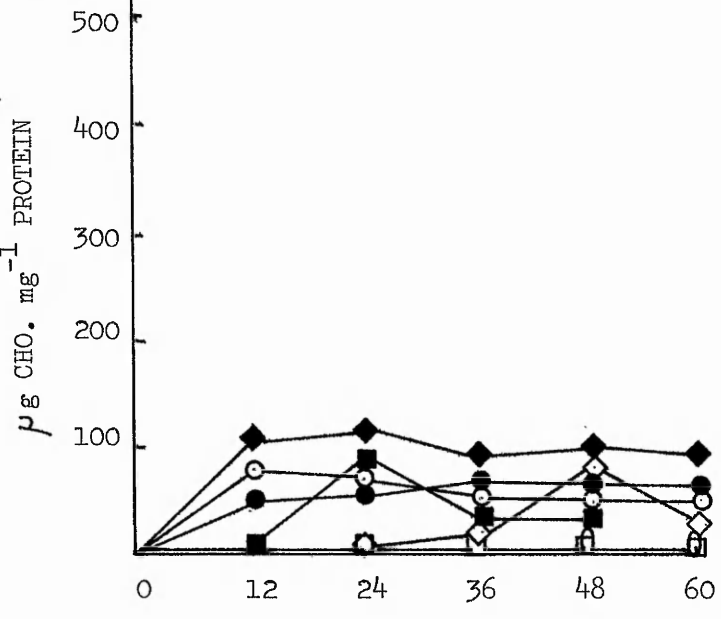


Fig 24b



achieved similar terminal cell protein yields at approx. 0.1mg.ml^{-1}, but this yield was achieved after 12 hours incubation on maltose and 24 hours incubation on sucrose. Growth on glucose produced the highest cell yield of S. mitior after incubation for 24 hours at 37°C. Fig (24a) indicates also that the terminal cell yields achieved by S. mitior on glucose and lactose were similar and were significantly higher than those achieved by growth on sucrose, fructose or maltose.

The results obtained for the production of C.A.P. by cells of S. mitior (Fig 24b) show that the levels produced by growth on all the sugar substrates tested are low and do not substantially exceed 100μg.mg^{-1}. It was concluded that the strain of S. mitior used in the present study was incapable of the synthesis of significant levels of C.A.P. from any of the sugar substrates investigated.

3.1.5.5. S. milleri

Fig (25a) illustrates the growth of S. milleri in broth cultures containing 2.5% (w/v) sucrose, glucose, fructose, maltose, lactose, cellobiose and trehalose. Fructose, cellobiose and trehalose did not support the growth of the strain of S. milleri used in the study, although most strains of S. milleri are reported to ferment trehalose (Cowan 1974). The growth of this organism on sucrose, glucose and lactose was characterised by an extended lag phase and subsequently by slow growth rates. S. milleri grew more rapidly on maltose than on the other substrates, with cultures normally reaching stationary phase after

Fig. 25a

Growth of S. milleri on Seven Sugar Substrates

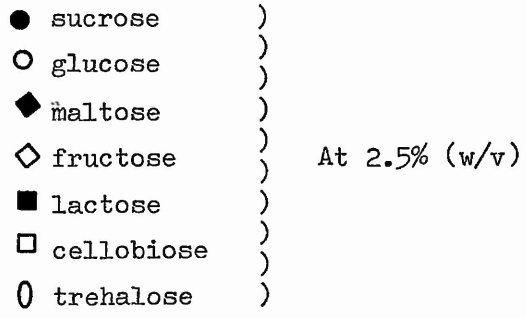


Fig. 25b

Production of Cell-Associated Polysaccharides by S. milleri Grown on Seven Sugar Substrates

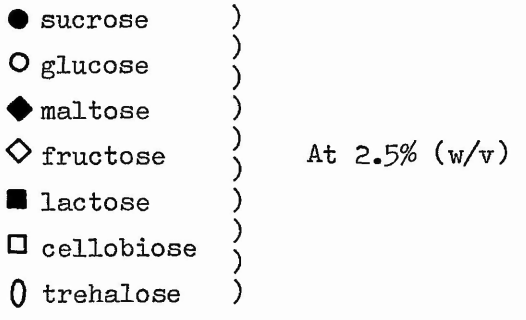


Fig. 25a

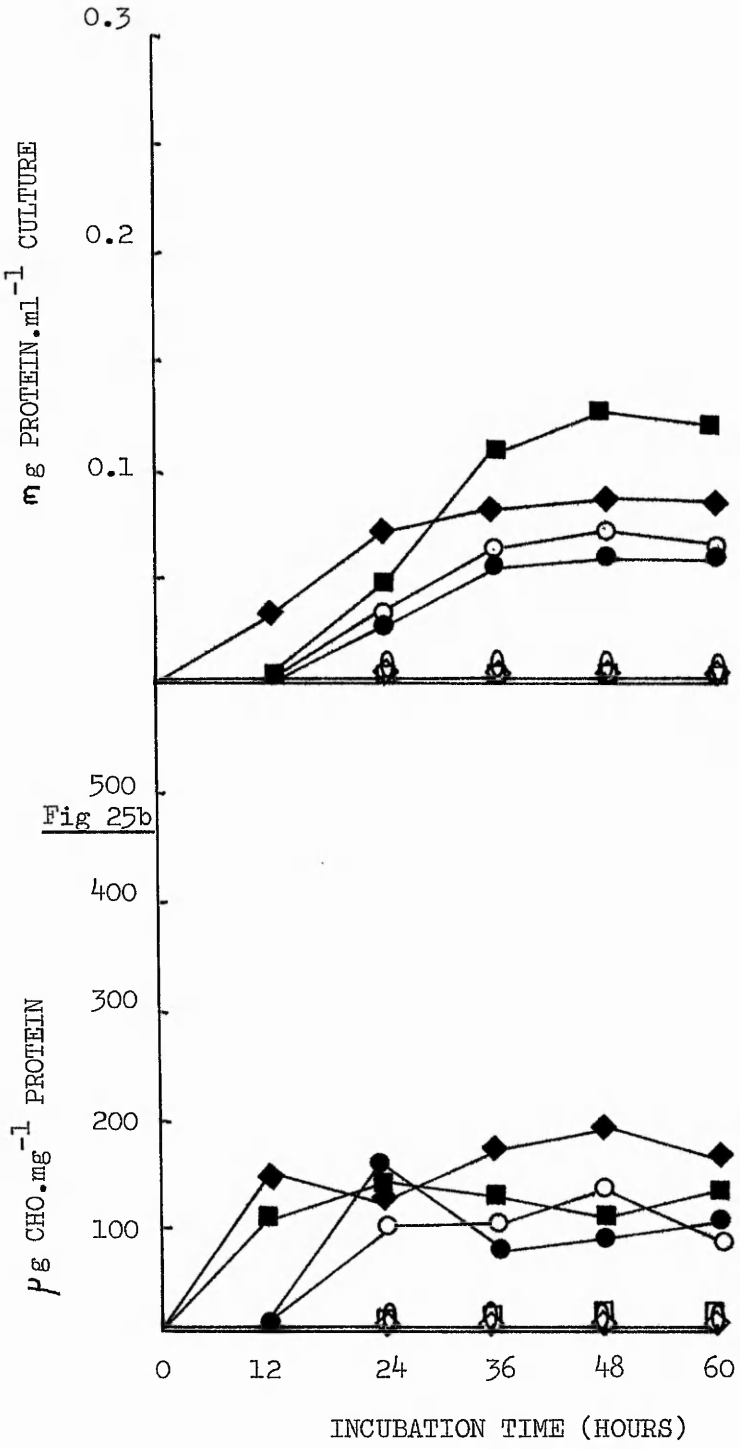


Fig 25b

24 hours of incubation, but produced the highest terminal cell yield when grown on lactose.

The growth of this organism on the seven sugar substrates did not produce high levels of C.A.P. from any of them although it was noted that growth on maltose resulted in slightly elevated C.A.P. levels occurring during the stationary phase of growth. It was also apparent that the "basal" level of C.A.P. production was higher for this organism than for the other four species, with the range of maximum yields being between 120μg.mg^{-1} and 200μg.mg^{-1} (Fig 25b).

Summarising the results of growing the five species of streptococci on seven sugar substrates, several points can be made. Cultures varied in their abilities to assimilate the metabolise the substrates, and this was thought to be due to the differing constitutive or inducible natures of the enzyme systems responsible for sugar metabolism in the five species. Cultures of S. mutans, S. sanguis and S. salivarius produced high levels of cell-associated anthrone reacting substances when grown on sucrose and these were assumed to be extracellular glucans and fructans synthesised from this sugar. The growth of these three species on maltose also produced elevated levels of surface polysaccharides. The relevance of these results was investigated further, (Section 3.1.6.).

When these three species were grown on substrates other than sucrose or maltose, the amount of cell-associated anthrone-reacting substances measured was low and fairly constant at a level of approx. 100μg.mg^{-1}. Similarly, cultures of

S. mitior and S. milleri produced a low level of such substances when grown on all the substrates investigated, including sucrose and maltose. The identity of these sucrose-independent carbohydrates was investigated in a later section (3.2.).

Quantitative studies of this kind on the comparative production of extracellular polysaccharides by the five species of oral streptococci, grown on a range of sugar substrates has not previously been reported. Trautner et al (1981) attempted such a study of the five serotypes of one organism, S. mutans. These workers established differences in amount and structure of the glucans elaborated by the five serotypes, but also expressed concern over the validity of their data because of the high number of bacteria found to be present in their glucan fractions.

In the present study, to avoid the bacterial contamination encountered by these workers, the alkaline extracts of bacterial cells were centrifuged at high speeds and subjected to millipore filtration prior to neutralisation, ethanol precipitation and lyophilisation.

3.1.6. Investigation of the Significance of Intracellular Polysaccharides in C.A.P. Measurement

The measurement of high levels of C.A.P. produced by cultures of S. mutans, S. sanguis and S. salivarius from maltose was unexpected; since maltose has not been reported to mediate the synthesis of extracellular polysaccharides in these organisms.

To ensure that these high levels were not the result of

sucrose contamination of the maltose used in the investigations, the AnalaR grade maltose was hydrolysed and subjected to both thin-layer chromatography and to silylation analysis as described in sections 2.4.8. and 2.4.9. No detectable sucrose, or fructose was detected in these analyses, allowing the exclusion of sucrose contamination.

Experiments were next undertaken to investigate the possibility that intracellular polysaccharides, liberated during the NaOH extraction of extracellular glucans by cell lysis or leakage, were causing measurement of elevated values for C.A.P. production. In the absence of a satisfactory assay for iodophilic polysaccharides in the presence of other polysaccharides, it was decided to monitor any reduction in C.A.P. yield produced by cells incubated in a neutral phosphate buffer at 37°C over a 12 hour period. It was assumed that any reduction in C.A.P. levels during this treatment would be a reflection of the catabolic breakdown of intracellular storage products.

Fig (26) indicates the results of C.A.P. determinations obtained when cells of S. mutans, grown on four different sugar substrates were first incubated, for various times in neutral phosphate buffer at 37°C.

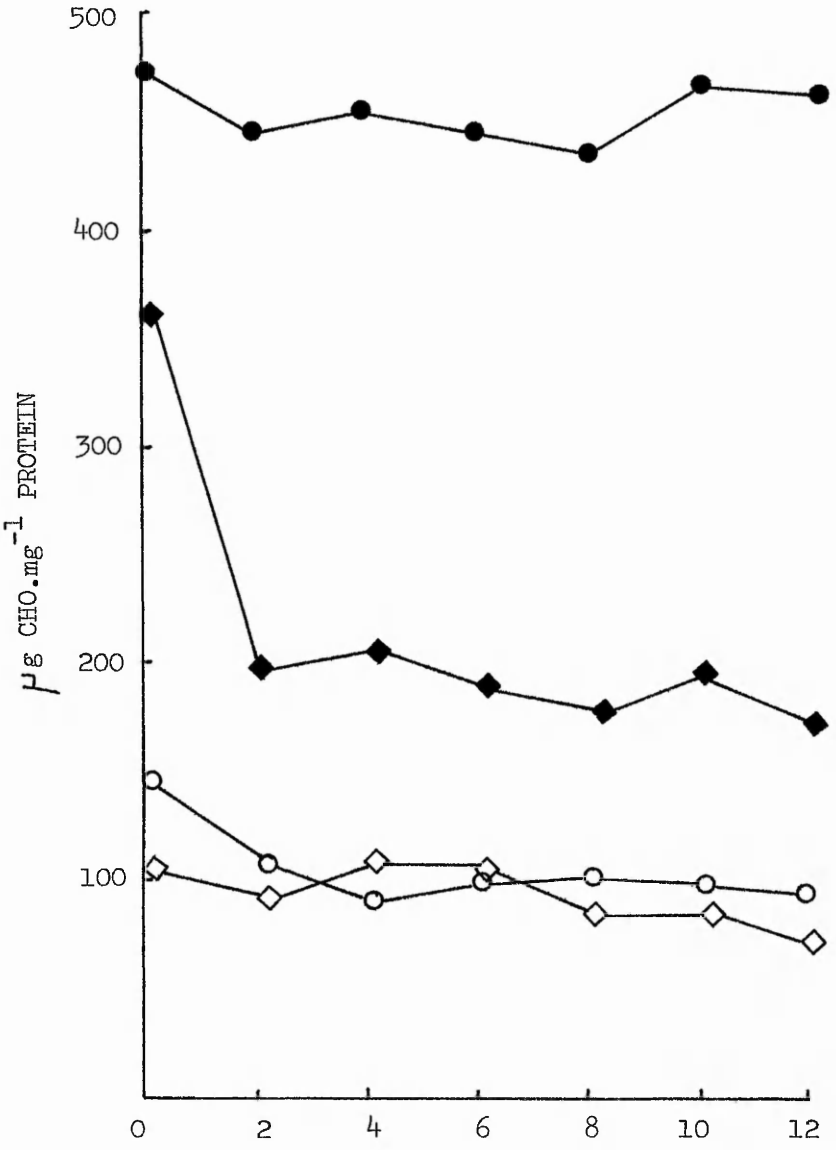
Little change was observed in the levels of C.A.P. measured on cells grown in sucrose and after 12 hours incubation in buffer the results revealed only a 1.7% drop in C.A.P. measurement. Under the same conditions, after 12 hours of incubation glucose grown cells lost 29% of their C.A.P. and

Fig. 26

Effect of Incubation in Neutral Buffer at 37°C on Yield of Cell-Associated Polysaccharides

- ● *S. mutans* cells grown in 2.5% sucrose
- ◆ *S. mutans* cells grown in 2.5% maltose
- ○ *S. mutans* cells grown in 2.5% glucose
- ◇ *S. mutans* cells grown in 2.5% fructose

Fig. 26


500
400
300
200
100
μg CHO.mg⁻¹ PROTEIN
0
2
4
6
8
10
12
INCUBATION TIME (HOURS)
(IN NEUTRAL BUFFER)

fructose-grown cells lost 22% on average. The largest drop in measurable C.A.P. occurred when maltose-grown cells were subjected to this buffer treatment. 47% of the anthrone reactive carbohydrates were lost after 2 hours of incubation and 51% were lost after 12 hours. The data represent the averages of triplicate experiments.

It has been reported that glycogen-like polyglucose polymers occur as storage products intracellularly and these have been studied in S. salivarius (Hamilton 1968), S. mutans (Freedman and Coykendall 1975), S. mitior (Gibbons and Socransky 1962), S. sanguis (Eisenberg et al 1974) and S. milleri (DeStoppelaar and Van Damme 1978). This polyglucose storage has been shown to be a variable characteristic in all but S. mutans (De Stoppelaar and Van Damme 1978). Interest focussed on these substances because of the acidogenesis resulting from their breakdown upon exhaustion of exogenous carbon sources, possibly implicating them in the aetiology of caries. Intracellular polysaccharides (IPS) accumulate under conditions of carbohydrate excess, and their metabolism is controlled by changes in the external culture pH (De Stoppelaar and Van Damme 1978), with the optimum pH for glycogen degradation being 7.0, and with degradation being depressed at successively lower pH values. It is likely that the results illustrated in Fig (26) reflect this pH dependent IPS degradation. It is proposed that the large decrease in anthrone-reactive substances occurring between 0-2 hours of incubation was the result of IPS degradation, and that this degradation became less rapid after 2 hours, when the buffer

pH fell below a level conducive to IPS metabolism.
A similar effect occurred to a lesser degree, with both fructose and glucose-grown cells. No such fall was observed to occur in cells grown on sucrose.
It seems likely that the decrease in measurable carbohydrates noted with maltose, glucose and fructose grown cells was due to the intracellular metabolism of intracellular storage polysaccharides. This indicates that a proportion of the anthrone-reactive substances measured as being extracellular in early experiments was due to either the lysis of some cells, liberating IPS, or to a leakage of these components from cells treated with NaOH. However, cells grown on sucrose did not lose appreciable quantities of measurable carbohydrates, indicating that IPS measurement was not a problem with cells grown on this substrate. It may be that such cells were protected from NaOH lysis by the polysaccharides associated with their cell surfaces. For these reasons, after initial investigations on the nature of the anthrone-reactive substances measured in section 3.1.5. subsequent extractions were performed on cells which had been pre-incubated for 2 hours at 37°C in neutral phosphate buffer, to minimise the possibility of IPS contamination.
Two other points arising from the experiments summarised in Fig (26) should be considered. It was noted that cells of maltose-grown S. mutans contained much higher levels of presumptive IPS than did cells grown on glucose, which in turn had a higher IPS content than did fructose-grown cells. Recently McFarland et al (1984) investigated the accumulation

of IPS by cells of S. pyogenes grown on various sugar substrates. Qualitative tests performed on 207 cultures of group A streptococci showed that only maltose or maltose-yielding saccharides were suitable substrates for IPS storage in these organisms. They found that glucose, fructose or lactose were not suitable IPS precursors regardless of medium sugar concentration. They suggested that the principal precursor to IPS synthesis was α 1-4 D-glucoside because in preliminary experiments isomaltose was shown to be inactive as an IPS precursor. It is suggested from experimental data obtained in the present work that α 1-4 D glucoside is the precursor for IPS storage in S. mutans, since growth of this organism on glucose and fructose results in much lower levels of IPS accumulation.

It was also noted from the data illustrated in Fig (26) that the buffer treatment employed did not reduce the measurable carbohydrate levels below approx. $100\mu g.mg^{-1}$ cell protein. This was the value achieved during earlier experiments with organisms not known to produce extracellular polysaccharides Figs 21-25).

It was concluded that the measurement of approximately $100\mu g.mg^{-1}$ of cell-associated polysaccharide in our experiments reflected the measurement of stable cell wall carbohydrate components. The nature of these components was investigated more fully in the next section.

3.2. Biochemical Characterisation of the Cell-Associated Polysaccharides Produced from Various Sugars by Five Species of Oral Streptococci

3.2.1. Composition of Crude C.A.P. Preparations.

Before purifying stages, or steps to minimise I.P.S. contamination of extracts were made, it was decided to investigate the nature of the anthrone-reactive substances measured in Section 3.1.5. by analysing crude preparations of C.A.P. obtained by the lyophilisation of ethanol precipitates of neutralised NaOH cell extracts. Table (1) illustrates the results obtained by assaying the C.A.P. produced by S. mutans cells grown on sucrose, glucose, fructose and maltose. The figures presented are average μg(anthrone positive material).mg^{-1} of the lyophilised crude preparation.

Glucose oxidase assays were performed on C.A.P. samples hydrolysed in NHCl for 2 hours at 100^{o}C.

Assays for total carbohydrate, R.N.A. and iodophilic polysaccharides were carried out on untreated C.A.P. samples.

The C.A.P. samples derived from cells grown on sucrose had a much lower R.N.A. content than did those derived from cells grown on the other sugars, with the highest R.N.A. content occurring in samples from cells grown on maltose. These results indicate a greater degree of cell lysis, during extraction, in cells grown on glucose, fructose and maltose, than those grown on sucrose. It was assumed that

this was due to a protective effect exerted by the extracellular polysaccharides on the cell during NaOH extraction, and was also noted in the results outlined in Fig. (26)

C.A.P. samples obtained from cells grown on sucrose and maltose had a high content of anthrone-reacting carbohydrates compared to the low levels measured in samples from cells grown on glucose and fructose. Assay of the hydrolysed samples for glucose by the glucose oxidase technique revealed that a large proportion of the anthrone-reacting carbohydrates present in C.A.P. samples derived from sucrose and maltose-grown cells were glucans. The differences in values obtained for total carbohydrate, and glucose content was assumed to be a reflection of the incomplete hydrolysis of the C.A.P. samples. C.A.P. samples derived from glucose and fructose grown cells contained only traces of glucose (0.056% and 0.044% respectively).

Qualitative assay of crude C.A.P. samples for iodophilic polysaccharides was based on the production of iodine-polysaccharide complexes as brown precipitates by neutral solutions of C.A.P. samples on the addition of a few drops of a 0.2% (w/v) iodine in 2.0% (w/v) potassium iodide solution. The quantity of precipitation is indicated in Table (1) as - being no precipitation and ~~+++~~ as heavy precipitation. C.A.P. samples from maltose grown cells precipitated strongly in this test, with glucose and fructose C.A.P. samples producing only weak precipitates and C.A.P. from sucrose-grown cells showing no precipitation.

TABLE 1

Preliminary Biochemical Characterisation of Crude C.A.P. from S. mutans Grown on Four sugar Substrates

TABLE 1

		C.A.P. from cells grown on:-			
ASSAY	FOR	SUCROSE	GLUCOSE	FRUCTOSE	MALTOSE
BIAL'S	RNA	30.0	200.0	250.0	320.0
ANTHRONE	CARBOHYDRATE	460.0	32.0	35.0	410.0
GLUCOSE OXIDASE	GLUCOSE	400.0	5.6	4.4	360.0
IODINE	INTRACELLULAR POLYSACCHARIDE	-	+	±	+++

Figures represent $\mu g.mg^{-1}$ C.A.P. sample

It was concluded from this series of experiments that the high levels of C.A.P. measured in Section 3.1.5. from maltose grown cells was the result of lysis of a proportion of these cells during NaOH extraction, resulting in the measurement of intracellular iodophilic polysaccharides, which maltose-grown cells were shown to synthesise in abundance.

The low levels of R.N.A. and iodophilic polysaccharides measured in C.A.P. samples derived from sucrose-grown cells indicated that lysis of these cells during NaOH extraction was minimal, and that this may be due to a protective effect exerted by extracellular polysaccharides on these cells.

Both glucose and fructose grown cells appeared to suffer some cell lysis during extraction, but their iodophilic polysaccharide content was considerably lower than those of maltose-grown cells.

3.2.2. Thin-Layer Chromatographic Analysis of Crude C.A.P. Samples

Analysis of the monosaccharide composition of the cell-associated polysaccharides prepared from cells of *S. mutans*, *S. sanguis* and *S. salivarius* were initially undertaken by hydrolysis and neutralisation of C.A.P. samples followed by thin-layer chromatography. Identification of component monosaccharides was made by reference to nine similarly chromatographed monosaccharides. Fig (27) shows a diagramatic representation of the separations achieved, and Table (2) gives the Rf values of polysaccharide components, compared to those of the nine standard sugars.

Fig. 27

Diagramatic Representation of Monosaccharide Separation Using Thin-Layer Chromatography

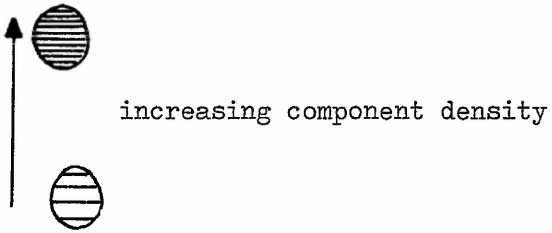


Fig. 27

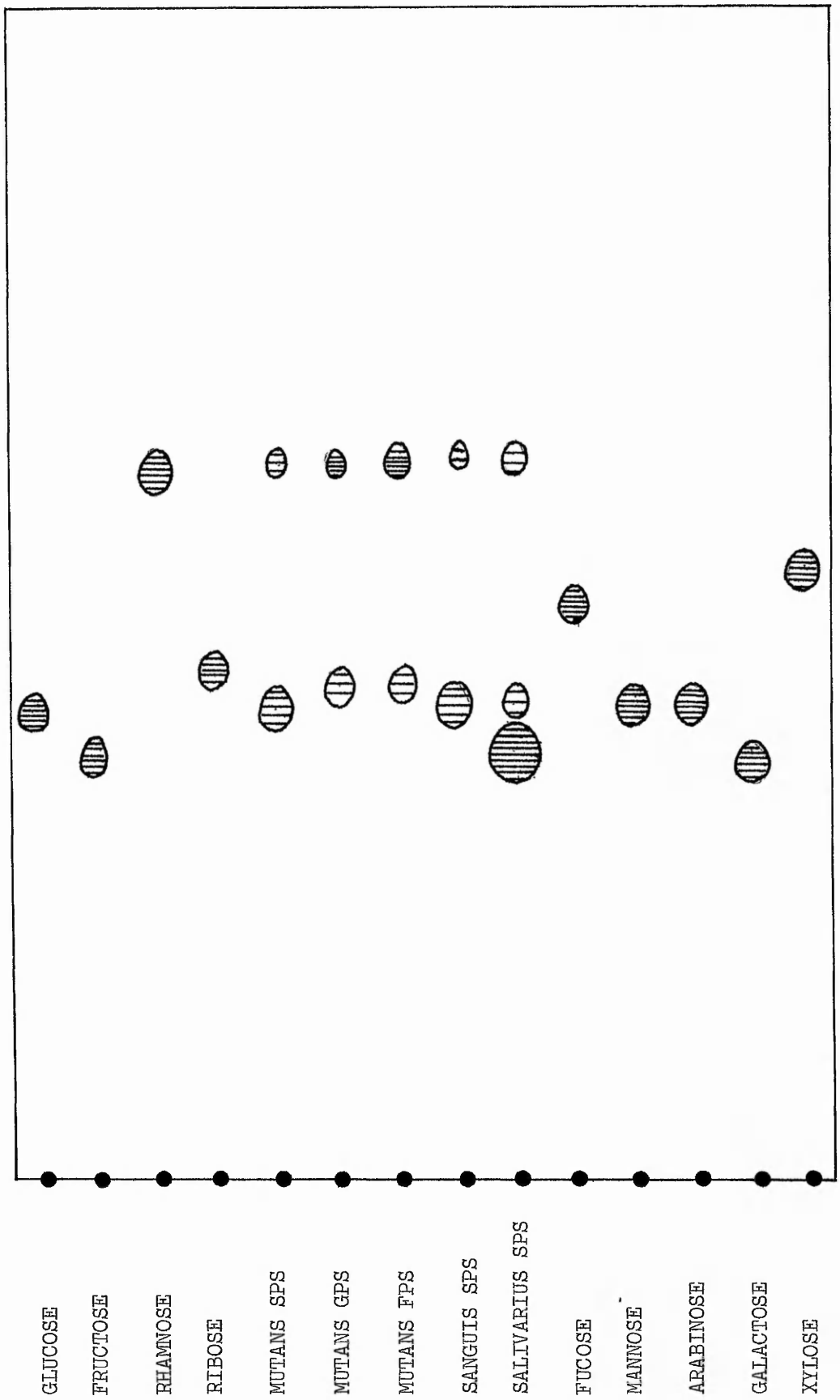

GLUCOSE
FRUCTOSE
RHAMNOSE
RIBOSE
MUTANS SPS
MUTANS GPS
MUTANS FPS
SANGUIS SPS
SALIVARIUS SPS
FUCOSE
MANNOSE
ARABINOSE
GALACTOSE
XYLOSE

TABLE 2

Thin-Layer Chromatographic Analysis of Cell-Associated Polysaccharides from S. mutans, S. sanguis and S. salivarius Grown on sucrose (SPS); glucose (GPS); and fructose (FPS).

TABLE 2

SAMPLES	Rf
GLUCOSE	.40
FRUCTOSE	.37
RHAMNOSE	.60
RIBOSE	.44
FUCOSE	.48
MANNOSE	.42
ARABINOSE	.42
GALACTOSE	.35
XYLOSE	.52
S. MUTANS SPS	.37 .40 .60
S. MUTANS GPS	.43 .60
S. MUTANS FPS	.43 .60
S. SANGUIS SPS	.40 .60
S. SALIVARIUS SPS	.37 .40 .60

Chromatography of the C.A.P. produced by S. mutans from sucrose yielded three components with Rf values corresponding to glucose, fructose and traces of rhamnose. C.A.P. from cells from S. salivarius grown on sucrose contained large amounts of fructose, with a smaller glucose component and traces of rhamnose. C.A.P. from cells of S. sanguis grown on sucrose yielded a component of glucose together with small traces of rhamnose.

When C.A.P.'s derived from S. mutans grown on glucose and fructose were chromatographed, the major component of both samples corresponded to rhamnose, with traces of a component not readily identifiable in the system used. This may have been a non-sugar component, or alternatively, a sugar breakdown product of acid hydrolysis. Rhamnose was characterised in the chromatographic system used not only by its Rf value, but also by a distinctive yellow/orange colouration.

These results were largely as expected, since it has long been known that strains of S. mutans, and S. salivarius can elaborate glucans and fructans from growth on sucrose, and that S. sanguis strains synthesise a glucan from this sugar. (Colman and Williams 1972). The traces of rhamnose detected in all the samples chromatographed were thought to have been derived from the cell walls of these bacteria during NaOH extraction. It is known that the polysaccharide antigens of S. mutans which provide the basis for the serological typing of these bacteria are glucose-rhamnose heteropolymers (Linzer et al 1982), and this may be the origin of the

rhamnose detected in our studies, and also the low levels of anthrone-reactive substances measured on cells grown on sugars other than sucrose.

3.2.3. Silylation Analysis of C.A.P. Samples

The present study was predominantly concerned with the glucans produced from sucrose by the species of oral streptococci shown to be capable of their synthesis. No detailed study of the fructans elaborated by these species was made.

In order to investigate more accurately and sensitively the composition of de-proteinised, purified glucan samples from sucrose-grown cells of S. mutans, S. sanguis and S. salivarius, silylation analysis was employed. This was achieved as described in Section (2.4.9). Individual components of the glucans, revealed by gas-liquid chromatography (g.l.c.) of hydrolysed, silylated samples, were identified by reference to similarly treated monosaccharide standards. Fig (28a) shows the typical trace obtained by G.L.C. analysis of five silylated monosaccharides at $10mg.ml^{-1}$. Figs (28b) (28c) and (28d) indicate typical traces obtained for the analysis of ($10mg.ml^{-1}$) glucan samples from S. mutans, S. sanguis and S. salivarius respectively, grown on sucrose. No sugar components were detected by the analysis of C.A.P. samples derived from cells grown on glucose or fructose.

Glucan samples derived from cells of S. mutans, S. sanguis and S. salivarius grown on sucrose contained only two detectable monosaccharides. These were identified as glucose and rhamnose (Table 3).

Fig. 28

Gas-Chromatographic Separation of Silylated Monosaccharides from Hydrolysis of Cell-Associated Polysaccharides

a) Silylated Monosaccharide Standards

b) Hydrolysed, Silylated C.A.P. from *S. mutans* grown on sucrose

c) Hydrolysed, Silylated C.A.P. from *S. sanguis* grown on sucrose

d) Hydrolysed, Silylated C.A.P. from *S. salivarius* grown on sucrose

Conditions

Sample C.A.P. from *S. mutans*, *S. sanguis*, *S. salivarius*

Injection volume 0.5 μl

Injection temperature 200^{o}C.

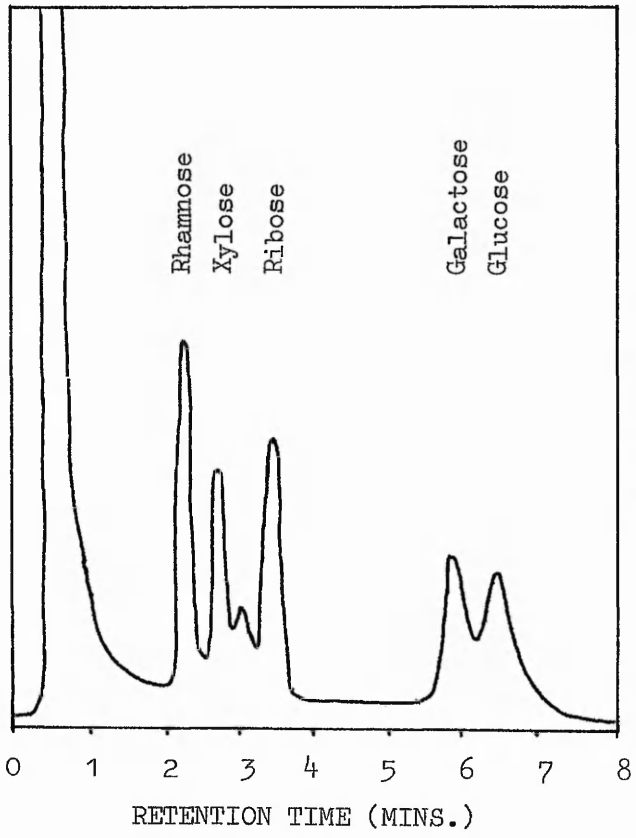
Column Temperature 165^{o}C.

Flow rate 20ml.min^{-1}.

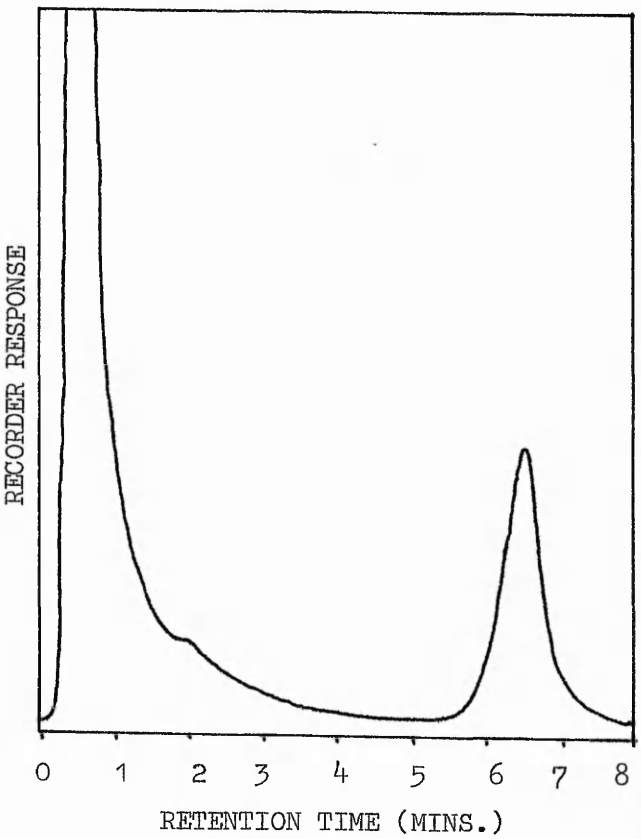
Chart Speed 10mm. min^{-1}.

Fig 28

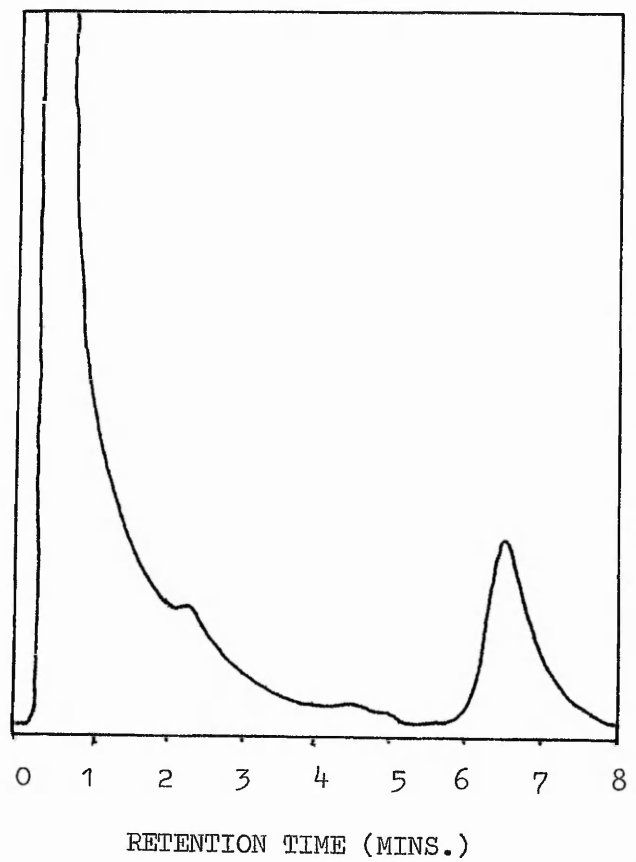
(a) STANDARDS



(b) S. MUTANS SPS



(c) S. SANGUIS SPS



(d) S. SALIVARIUS SPS

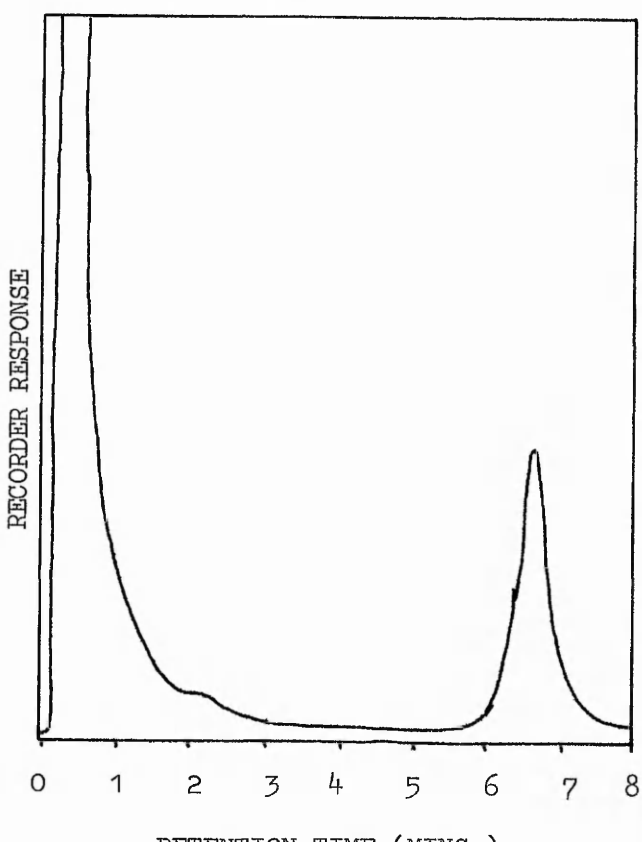


Table 3

Peak Retention Times of Monosaccharide Components of Cell-Associated Polysaccharides from S. mutans, S. sanguis and S. salivarius Grown on sucrose.

TABLE 3

SUGAR	RETENTION TIME (MINUTES)				
Rhamnose	2.2				
Xylose		2.7			
Ribose			3.4		
Galactose				5.8	
Glucose					6.5
Mutans SPS	2.2				6.5
Sanguis SPS	2.2				6.5
Salivarius SPS	2.2				6.5

The compositions (as % peak area) were determined and were as follows;- S. mutans (0.7% rhamnose, 99.3% glucose); S. sanguis (2% rhamnose, 98% glucose); S. salivarius (0.8% rhamnose, 99.2% glucose).

The relatively high proportion of rhamnose observed in these investigations for glucans derived from sucrose-grown cells of S. sanguis may be related to the quantity and nature of the extracellular glucose-homopolymers produced by this organism. Experiments in Section 3.1.5. indicated that S. sanguis produced much lower levels of extracellular polysaccharide than either S. mutans or S. salivarius from sucrose, and it may be that these lower levels of surface associated glucan rendered the cell surface type polysaccharide more accessible to NaOH extraction. Such type-specific polysaccharides have been shown to contain rhamnose (Linzer et al 1982).

It was concluded that the glucans elaborated by S. mutans, S. sanguis and S. salivarius were glucose homopolymers, contaminated with traces of rhamnose-containing type-specific polysaccharides derived from the cell surface.

3.2.4. Methylation Analysis of Cell-Associated Polysaccharide Linkages

3.2.4.1. Investigation Into the Relationship Between Column Temperature and Retention Times

As described in Section (2.4.1.5.) it was decided to perform methylation analyses at GLC column temperatures higher than those recommended by Lindberg (1972). However, so that it was still possible to use the published figures to identify

the position of $-OCH_3$ groups and hence determine the structure of polysaccharide samples, it was necessary to investigate the relationship between column temperature and peak retention times.

Fig. 29 illustrates this relationship for 2 known and 2 unknown methylated sugars over a temperature range of $160^{\circ}C$ - $210^{\circ}C$. In order to predict the retention time of a methylated sugar at any column temperature it was necessary to define the relationship shown in Fig (29) mathematically. It was found that plotting the natural logarithm of column temperature ($^{\circ}C$) against the natural logarithm of retention time (mins) gave a linear relationship with a very high correlation coefficient in linear regression analysis, but that the slope for each component was different (Fig. 30). From the equation for a straight line:-

(1) $y = \alpha + \beta x$

where y = vertical axis

x = horizontal axis

α = intercept on y

β = slope

Using data in Fig. (30), where r = retention time (mins), and t = column temperature ($^{\circ}C$):-

(2) $$\ln r = \alpha + \beta \cdot \ln t$$

Note that α and β are different for each sugar. Mathematical investigation of the data showed that

(3) $$\alpha = \frac{\ln r + (-0.6558 \times \ln t)}{1 - (-0.1645 \times \ln t)}$$

(4) $$\beta = (-0.6558 - (0.1645 \times \alpha))$$

From published data (Lindberg 1972), for any sugar the values of t and r are known. These values can, by

Fig. 29

Relationship Between Retention Times of Methyl Glycosides as Their Alditol Acetates and G.L.C. Column Temperature

A) 2,3,4,6 Tetramethyl Glucose

B) Unidentified, minor peaks,

C) contaminating A + D

D) 3 - O Methyl Glucose

Fig. 29

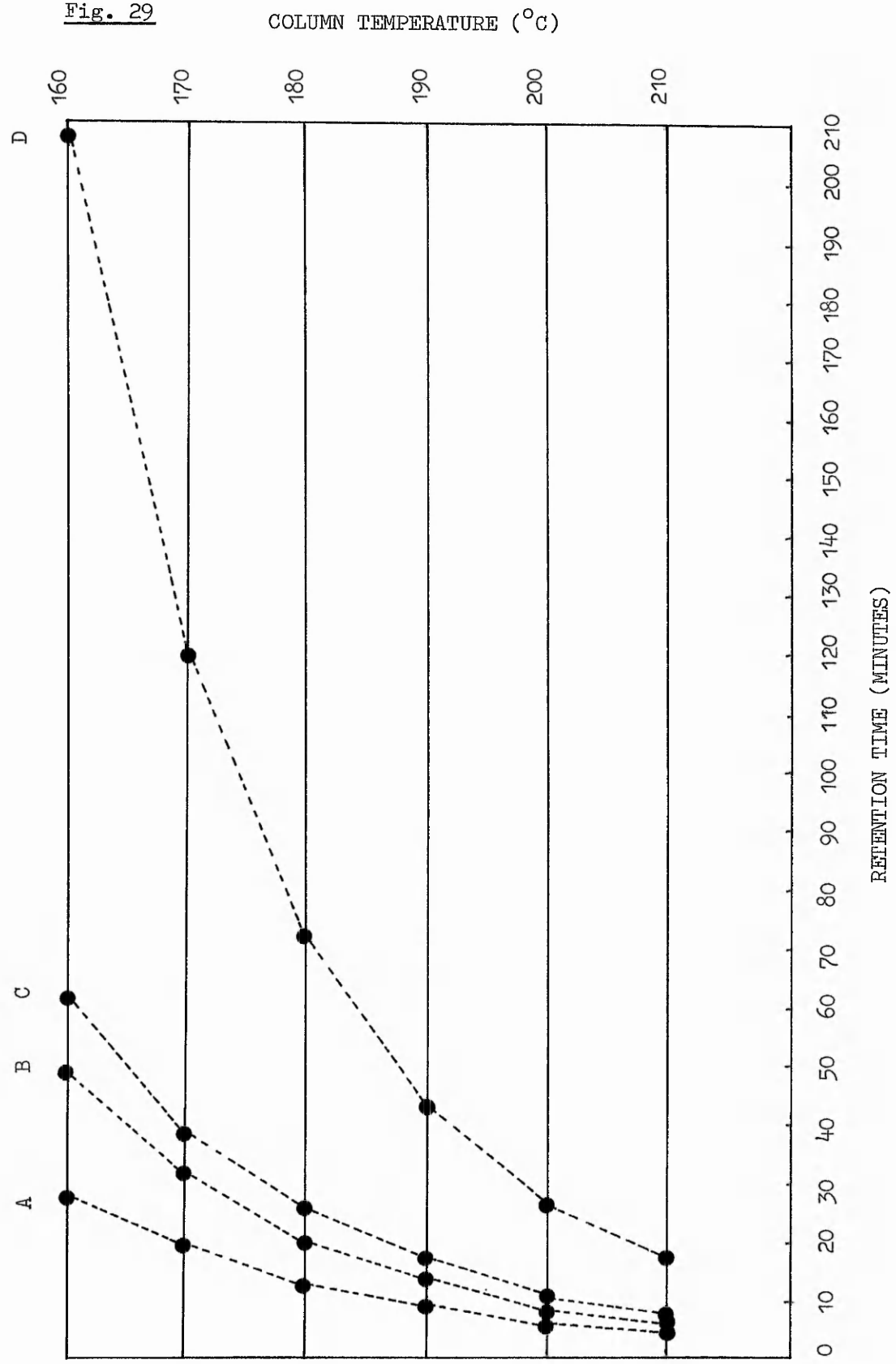

COLUMN TEMPERATURE (°C)
160
170
180
190
200
210
D
C
B
A
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210
RETENTION TIME (MINUTES)

Fig. 30

Relationship Between ln Retention Time of Methyl Glycosides and ln G.L.C. Column Temperature

A	2,3,4,6 - Tetramethyl Glucose	(1.0000)
B	Unidentified minor Peak	(1.0000)
C	Unidentified Minor Peak	(1.0000)
D	3 - O Methyl Glucose	(0.9998)

Figures in parenthesis indicate the correlation co-efficient for each sugar from linear regression.

Fig. 30

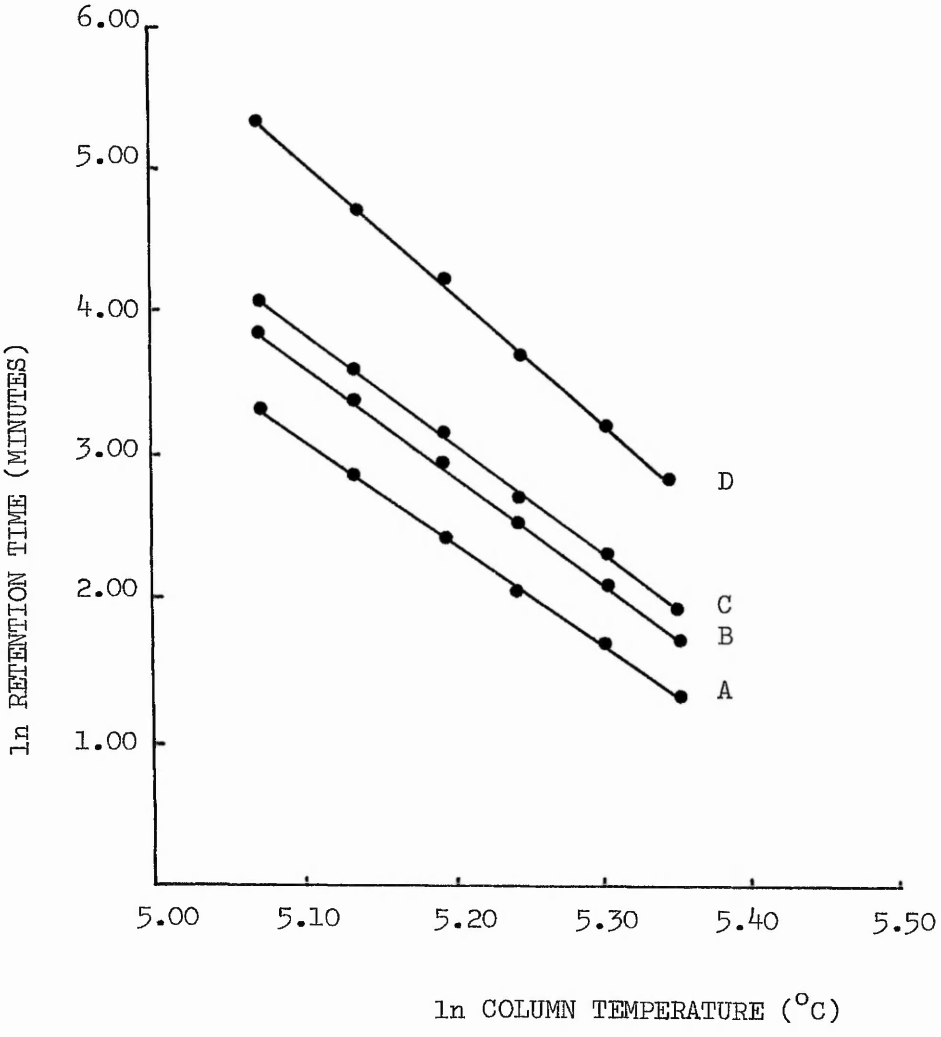

6.00
5.00
4.00
3.00
2.00
1.00
ln RETENTION TIME (MINUTES)
D
C
B
A
5.00
5.10
5.20
5.30
5.40
5.50
ln COLUMN TEMPERATURE (°C)

Fig. 31

3.2.4.2.1. Gas-Chromatographic Separation of 2,3,4,6 and 3-O Methyl Glycosides as Their Alditol Acetates

Conditions

(sample)	2,3,4,6 and 3-O methyl glucose
Injection volume	1 µl
Injection temperature	210°C
Column Temperature	190°C
Flow rate	200 ml.min^{-1} (N_2)
Chart Speed	5mm.min^{-1}.

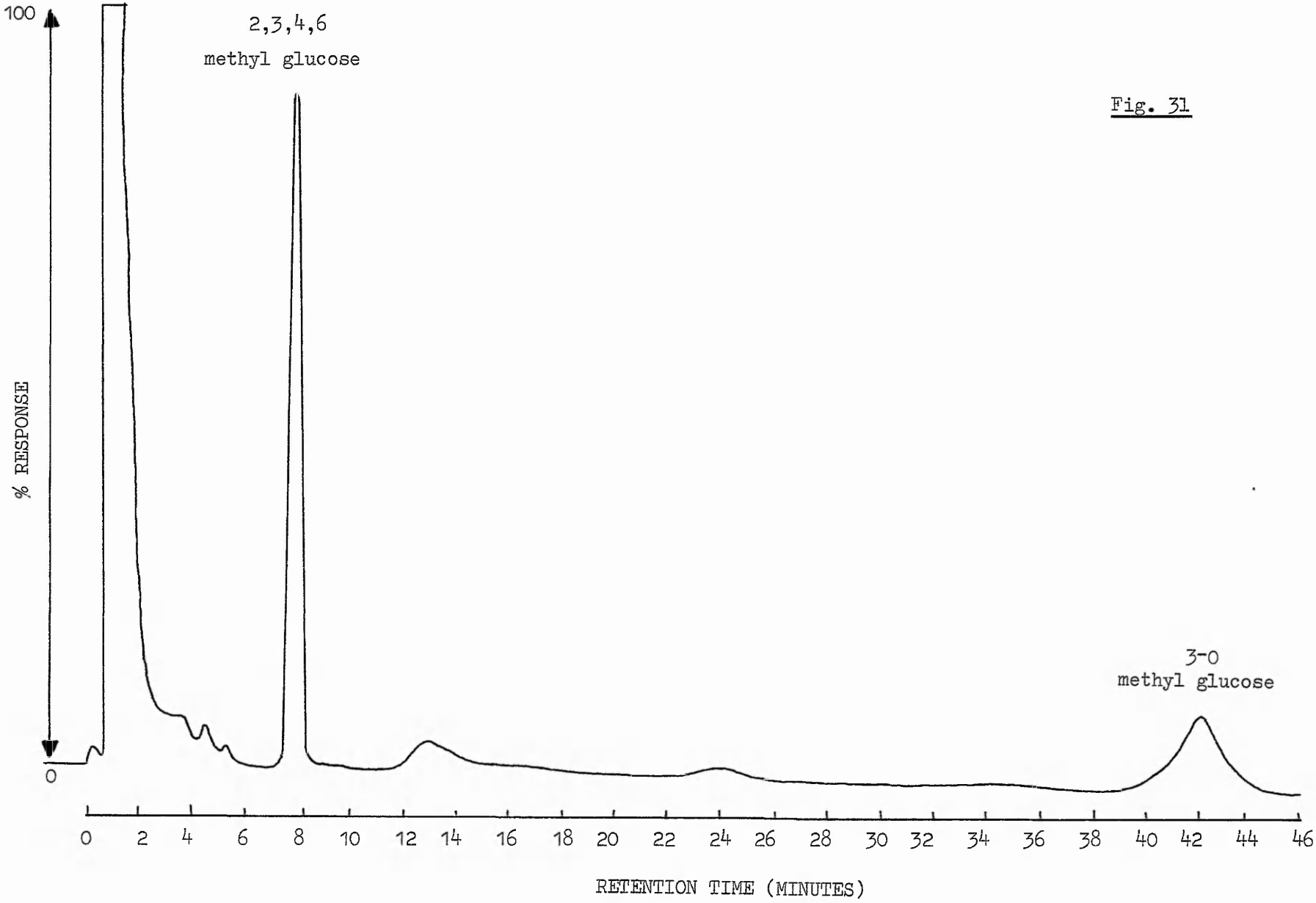

100
% RESPONSE
0
2,3,4,6
methyl glucose
3-O
methyl glucose
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
RETENTION TIME (MINUTES)

Fig. 31

substitution in equation (2) yield values for α and β for that sugar.

Using this information, the retention time of a particular sugar can be derived for any value of column temperature.

3.2.4.2.1. Linkage Analysis

The degree and position of sugar methylation was determined for methylated, hydrolysed and acetylated glucans by interpolation of retention times between two similarly derivatised standards i.e. 2,3,4,6 tetramethyl glucose and 3-O methyl glucose. Fig. (31) shows a typical trace obtained by g.l.c. analysis of these two standards at a column temperature of 190°C. Note that under these conditions the retention time of 3-O methyl glucose relative to that of 2,3,4,6 tetramethyl glucose is 5.38. However, by converting these retention times to those expected at 160°C using the conversion described in the previous section, the relative retention time of 3-O methyl glucose relative to 2,3,4,6 tetramethyl glucose becomes 7.60. This is the relative retention time published by Lindberg (1972) and used subsequently for the identification of methylated components of our streptococcal glucans.

3.2.4.2.2. Linkage Analysis of C.A.P. from sucrose grown S. mutans, S. sanguis and S. salivarius

In order to determine the linkages involved in the polysaccharides derived from sucrose-grown cells of S. mutans, S. sanguis and S. salivarius, methylation analysis, as described in Section (2.4.11) was performed on lyophilised C.A.P. samples.

Figs. (32,33, and 34) illustrate typical GLC traces obtained by triple methylation of C.A.P. samples from S. mutans, S. sanguis and S. salivarius respectively. It was noted in each case that several major peaks could not be identified, and these were assumed to be non-sugar components derived from bacterial cells since similar analysis of dextran gave only methylated glucose residues.

No attempt was made to identify components whose peak areas decreased on successive methylation of C.A.P. samples.

The results of these analyses and subsequent identification of major components are summarised in Table (4). Since both thin-layer chromatography and silylation analysis of the C.A.P. samples revealed only the presence of glucose and rhamnose, peaks were identified by reference to relative retention times published by Lindberg (1972) for these two sugars.

It can be seen from Table (4) that samples of C.A.P. from S. sanguis contained only five methylated residues i.e. 2,3,4,6-; 2,4,6- and 2,3,4 methyl glucose, and 3,4- and 2,3,4- methyl rhamnose. These residues suggest the glucan to be α1-6, and α1-3 linked, with a large proportion of terminal glucose groups. The same linkages, present in different amounts are present in both S. mutans and S. salivarius samples, but in these samples there was also a significant peak corresponding to 2,4- methyl glucose. These latter two samples also contained mono substituted derivatives, assumed to be the products of undermethylation i.e. 6-O methyl glucose and 4-O methyl rhamnose. The presence of a

Fig. 32

3.2.4.2.2. Typical Trace Obtained by Methylation Analysis of C.A.P. from S. mutans Grown on Sucrose.

Conditions

As for standards.

Peak Identities

1	2,3,4 methyl rhamnose
2	3,4 methyl rhamnose
3	2,3,4,6 methyl glucose
4	4 methyl rhamnose
5	2,4,6 methyl glucose
6	2,3,4 methyl glucose
7	3,6 methyl glucose
8	2,4 methyl glucose
9	6 methyl glucose

Fig. 32

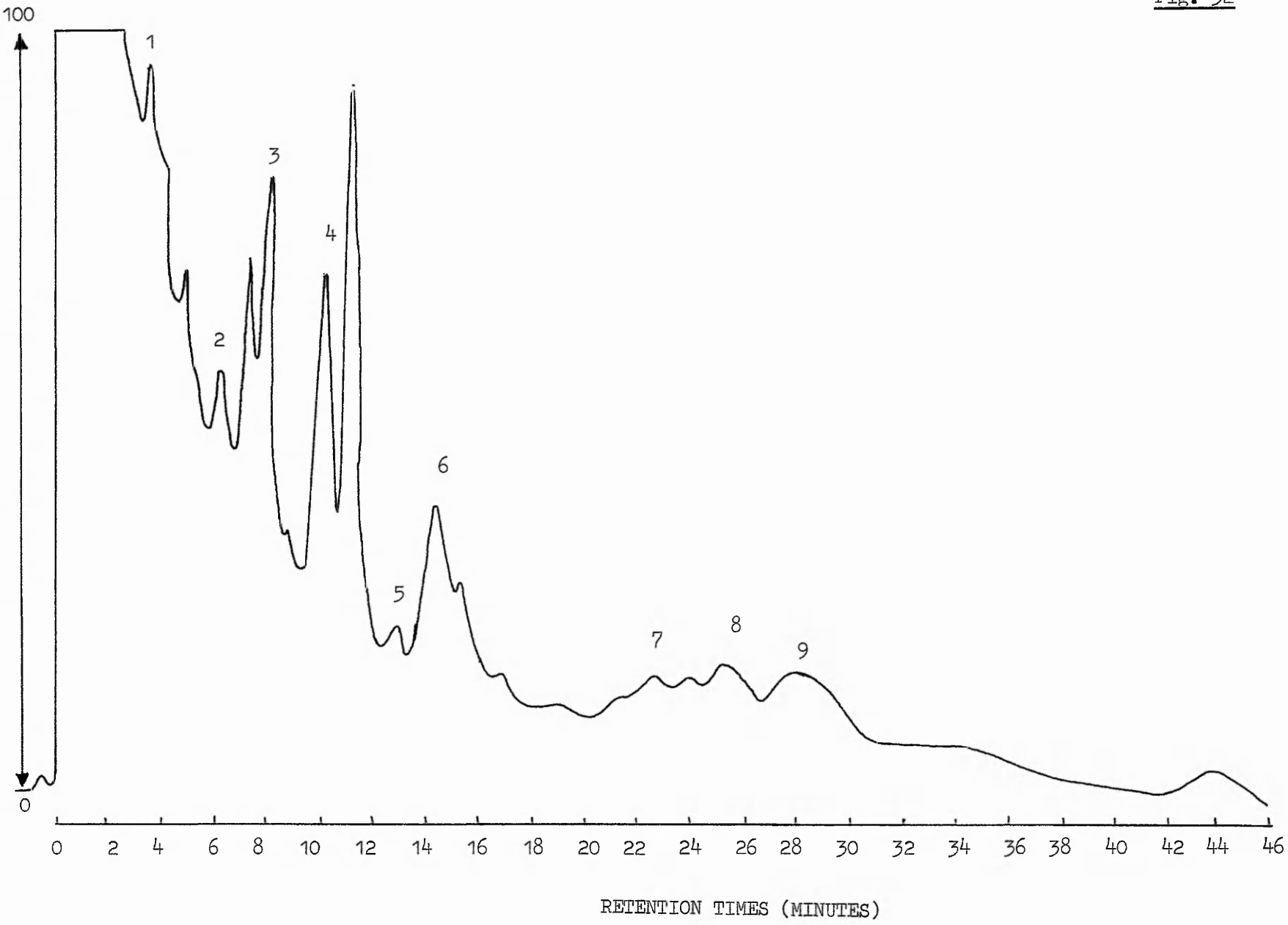

100
0
% RESPONSE
1
2
3
4
5
6
7
8
9
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
RETENTION TIMES (MINUTES)

Fig. 33

Typical G.L.C. Trace Obtained by Methylation Analysis of C.A.P. from S. sanguis Grown on Sucrose

Peak Identities

1	2,3,4 methyl rhamnose
2	3,4 methyl rhamnose
3	2,3,4,6 methyl glucose
4	2,4,6 methyl glucose
5	2,3,4 methyl glucose

Fig. 33

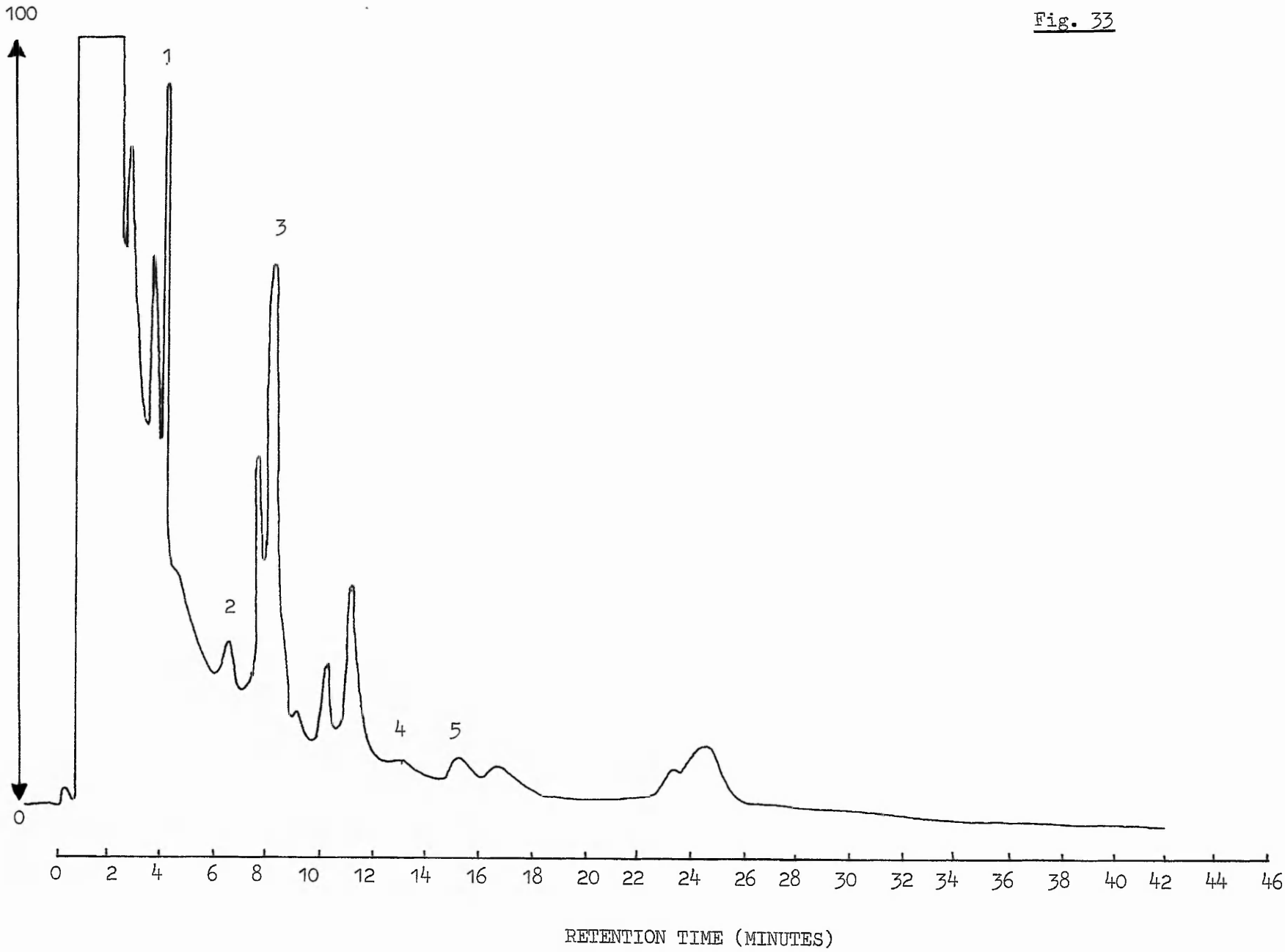

100
0
% RESPONSE
1
2
3
4
5
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
RETENTION TIME (MINUTES)

Fig. 34.

Typical G.L.C. Trace Obtained by Methylation Analysis of C.A.P. from S. salivarius Grown on Sucrose

Peak Identities

1	2,3,4 methyl rhamnose
2	3,4 methyl rhamnose
3	2,3,4,6 methyl glucose
4	4 methyl rhamnose
5	2,4,6 methyl glucose
6	2,3,4 methyl glucose
7	3,6 methyl glucose
8	2,4 methyl glucose
9	6 methyl glucose

Figg. 34

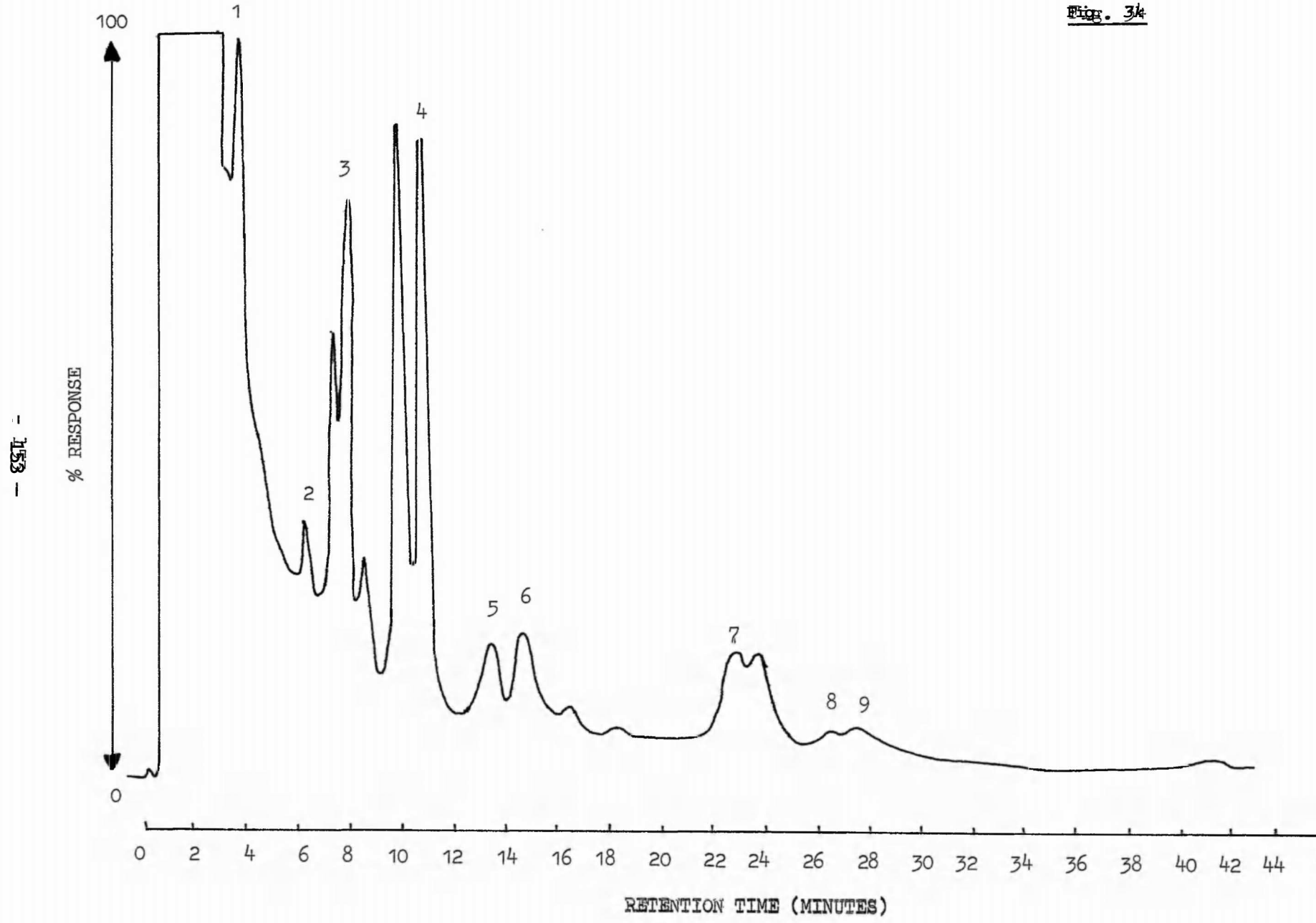

100
0
% RESPONSE
1
2
3
4
5
6
7
8
9
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
RETENTION TIME (MINUTES)

TABLE 4

Comparison of the Relative Retention Times of Partially Methylated Sugars as their Alditol Acetates (ex Lindberg 1972), with those obtained by Methylation Analysis of C.A.P. Produced by S. mutans, S. sanguis and S. salivarius from sucrose

Table 4

Position of OCH_3 Group	Relative Retention Time (ex Lindberg 1972) Parent Sugar		Relative Retention Times of Partially Methylated Sugars from C.A.P. of:-		
	GLUCOSE	RHAMNOSE	S. MUTANS	S. SANGUIS	S. SALIVARIUS
2	6.6	1.37	-	-	-
3	7.60	1.67	-	-	-
4	-	1.57	1.55	-	1.55
6	5.00	-	4.98	-	4.98
2,3	4.50	0.92	-	-	-
2,4	4.21	0.94	4.20	-	4.20
2,6	3.38	-	-	-	-
3,4	4.26	0.87	0.89	0.89	0.89
3,6	3.73	-	3.75	-	3.75
4,6	3,49	-	-	-	-
2,3,4	2.22	0.35	2.22,0.35	2.22, 0.35	2.22, 0.35
2,3,6	2.32	-	-	-	-
2,4,6	1.82	-	1.82	1.82	1.82
3,4,6	1.83	-	-	-	-
2,3,4,6	1.00	-	1.00	1.00	1.00

Table 5

Predominant Glucosidic linkages of the C.A.P.'s Produced by S. mutans, S. sanguis and S. salivarius from sucrose.

Figures represent the proportion of each linkage type as a percentage of the total peak areas identified as methylated glucose.

Table 5

	Terminal 2,3,4,6	Branch 2,4	(α1-6) 2,3,4	(α1-3) 2,4,6	Non-glucose
S. mutans S.P.S.	24.0	12.0	26.0	3.0	35.0
S. sanguis S.P.S.	84.0	0	11.5	4.5	0
S. salivarius S.P.S.	42.0	3.0	18.0	13.0	24.0

component corresponding to 2,3,4-rhamnose suggests the presence of an α1-6 linked rhamnose polymer in all three C.A.P. samples.

The relative proportions of the major glucose components are shown in Table (5) and represent the proportion of each linkage type as a percentage of the total peak areas identified as methylated glucose.

The results indicated that S. sanguis glucan had a very large proportion of terminal glucose groups, and was predominantly α1-6 linked with a smaller component of α1-3 linkages. The absence of detectable quantities of 2,4-methyl glucose, suggested that the polymers from S. sanguis were not branched to any degree.

The glucan obtained from S. mutans, however, was relatively rich in 2,4- methyl glucose indicating a branched structure of glucose residues predominantly α1-6 linked, with some α1-3 linkages. S. salivarius glucan had a higher proportion of α1-3 linkages than did glucans from the other species. The predominant linkage was α1-6 however, and the glucan appeared to exhibit only moderate (3%) branching.

Overall, it appeared that the glucans from S. mutans and S. salivarius were more closely related to each other than they were to the glucan produced by S. sanguis.

Early investigations of the linkages involved in the glucans synthesised from sucrose by members of the oral streptococci used the techniques of periodate oxidation and Smith degradation and established the predominant linkages to be of the α1-6 and α1-3 glucosidic type. (Guggenheim and Schroeder 1967; Guggenheim 1970). The latter author, using

cell-free synthesis of glucans by crude enzyme preparations found differences in structure between the glucans synthesised by S. mutans and S. sanguis enzyme systems. It was reported that the insoluble glucan elaborated by S. mutans GTases was a predominantly α1-3 linked glucan, whereas that produced by S. sanguis GTases was composed of almost equal amounts of α1-3 and α1-6 linked residues. It was also noted that the S. sanguis glucan had a more "gelatinous" consistency than that produced from S. mutans. This difference in consistency was also noted in the present study.

The present study used glucans derived from whole bacterial cells since it has been reported that the composition of glucans prepared in cell-free systems is dependent on the purity of the enzyme preparation (Ceska et al 1972). It was therefore felt to be more relevant to investigate the glucans produced in vivo. This approach was previously taken by Nisizawa et al (1976) using periodate oxidation techniques on the extracted polysaccharides and it was demonstrated that a water-soluble fraction contained higher levels of α1-6 linked residues than α1-3 linked residues (56% α1-6; 37% α1-3), but that a water-insoluble fraction contained more α1-3 linkages (57%) than α1-6 linked residues (38%). These authors found that both an insoluble and a soluble fraction were produced by S. mutans samples, but that glucans extracted from S. sanguis cells contained only an essentially water soluble fraction. Because of the improved sensitivity offered by methylation analysis over periodate oxidation reported by Lindberg (1972), subsequent

investigators adopted this technique. Nisizawa et al (1977) repeated their earlier investigations (Nisizawa et al (1976) using methylation analysis and found results largely equivalent to their earlier findings. However, these authors also pointed out the difficulties involved in the methylation of glucans extracted from cells, rather than synthesised by GTases in cell-free systems. This was reported to be due to their more complex structure and larger molecular weights. Results obtained in the present study confirm the difficulties involved in the methylation of glucans obtained in this way, as shown by the presence of undermethylated residues, even after triple methylation. The complex structure and high molecular weights of these glucans rendered them insoluble in most solvents commonly used for methylation analysis e.g. dimethylsulphoxide, and for these reasons the methylations were carried out in benzyltrimethylammonium hydroxide whose use has not previously been reported for these substances. Another difficulty encountered in the present study was the presence of a large number of unidentifiable component peaks. The presence of these peaks is thought to be a reflection of the complex nature of glucans derived from whole cells rather than from cell-free systems. These difficulties were pointed out by Lindberg (1972) in his definitive report on the methylation analysis of polysaccharides. He pointed out that it is not always possible even in the methylation of a homoglucan to identify all methylated sugars from the retention times of their alditol acetates. Moreover, as his report points out, non-sugar components from impurities in the original

sample or in solvents and reagents can sometimes give strong peaks which may cause confusion. The presence of contaminants in the solvents used in the present study was eliminated by the running of reagent "blanks", which did not give any peaks corresponding to the unidentifiable peaks found in our analysis.

It was concluded that glucan samples investigated in the present study may not have been permethylated, and that non-sugar components in these glucans were responsible for the peaks not readily identifiable.

In spite of these limitations, several interesting points were raised by the analysis of these glucans.

The glucan derived from S. mutans had only a small α 1-3 linked component, but the highest α 1-6 linked component of the three glucans investigated. This was unexpected since it was assumed that the glucans extracted from the cell surface were essentially water-insoluble and rich in α 1-3 glucosidic linkages. There may be two reasons why the α 1-3 linked component was present at only low levels in the extracted glucan. In a comparative study of the composition of glucans extracted from the five serotypes of S. mutans (Trautner et al 1981) it was found that there were only small differences in the proportions of soluble glucan produced by the five serotypes, but that the percentage of insoluble glucan was lowest amongst members of the serotypes a and c. Furthermore it was shown that the percentage of α 1-3 linked glucose units was highest in the insoluble glucan from strains of type d and e, and was lowest in strains of type c. The S. mutans strain used in the present study was

NCTC 10449 (type c).

A second possible reason for the unexpectedly low proportion of ∝1-3 linkages in glucans extracted from S. mutans was emphasised by Trautner and Felgenhauer (1979). They suggested that the ∝1-6 linked soluble glucans may be surrounded by insoluble polysaccharides at the cell surface, or might be linked to cell-associated GTase, thus becoming part of an insoluble complex. In this way, during our extraction procedure, the more soluble ∝1-6 linked glucan components may not have been removed by the extensive washing procedures adopted before extraction. This would have had the effect of decreasing the proportion of ∝1-3 linked residues measured relative to the ∝1-6 linked residues. The results obtained for the linkage analysis of S. sanguis glucan can only satisfactorily be explained by hypothesising a "dextrin" like structure consisting of large numbers of short, linear ∝1-6 linked and ∝1-3 linked chains. This is thought to be the only explanation for the very high proportion of terminal residues detected in these samples (84%).

The linkage analysis of S. salivarius glucan indicated a relatively high ∝1-3 linked component, being almost equivalent to the ∝1-6 component, and it has been reported that the water-insoluble, ∝-D-glucan obtained from S. salivarius consists of up to 80% ∝1-3 linked residues making up the backbone with smaller proportions of ∝1-6 and ∝1-4 residues (Tsumuraya and Misaki 1979).

It is interesting to note from Table (5) that those glucans showing the presence of 1,3,6 substituted branch residues are also those having the highest unidentifiable peaks i.e.

S. mutans glucan and *S. salivarius* glucan. The more complex structure of these glucans, as demonstrated by their content of branched residues, may have been related to their difficulty of methylation.

3.3. Immunological Characterisation of Cell-Associated Polysaccharides from S. mutans, S. sanguis and S. salivarius

3.3.1. Agglutination Studies

The results of agglutination tests on mouse antisera to sucrose and glucose-grown cells of S. mutans, S. sanguis and S. salivarius are shown in Table (6). For each antiserum the indicator cells used were glucose-grown cells of the same species used to produce that antiserum.

Antisera to glucose-grown cells of S. mutans consistently agglutinated glucose-grown S. mutans cells to a higher titre than did antisera raised against sucrose-grown cells. A similar, though less marked effect was noted with antisera to S. sanguis grown on sucrose and glucose. There was no such difference in agglutinating activity between sucrose and glucose-grown S. salivarius antisera. It was noted, however, that these antisera had only poor agglutinating activity against homologous bacterial cells. Pre-immune mouse sera showed the ability to agglutinate cells of S. mutans weakly, whereas no such activity was apparent against cells of S. sanguis and S. salivarius.

It is proposed that the lower glucose-cell agglutinating activity of sera raised against sucrose-grown cells of S. mutans and S. sanguis may have implications in the aetiology of bacterial endocarditis. The pathogenesis of bacterial endocarditis has been shown to be a complex process involving the interaction of several independent factors (Hayward 1973), and several in vivo studies have suggested that bacterial adherence could be one of these factors (Ramirez-Ronda 1978;

Table 6

Agglutinating Titres of Mouse Antisera to Sucrose and Glucose grown cells of S. mutans, S. sanguis and S. salivarius

The results represent the reciprocal of the agglutinating titre. Each serum was a pooled preparation from 10 mice, and agglutinating titres are the averages of five separate groups of sera.

Table 6

Indicator cells	Serum	Reciprocal of Agglutinating Titre
Glucose grown *S. mutans*	Anti-sucrose grown *S. mutans*	320
	Anti-glucose grown *S. mutans*	1280
Glucose grown *S. sanguis*	Anti-sucrose grown *S. sanguis*	640
	Anti-glucose grown *S. sanguis*	1280
Glucose grown *S. salivarius*	Anti-sucrose grown *S. salivarius*	80
	Anti-glucose grown *S. salivarius*	80
Glucose grown:-		
S. mutans	Pre-Immune Serum	20
S. sanguis	"	10
S. salivarius	"	10

Pelletier *et al* 1978). Although these findings on the increased pathogenicity of dextran producing rather than non-dextran producing strains of oral streptococci suggested an important role of bacterial adherence in the pathogenesis of endocarditis, it is likely that other mechanisms of increased infectivity related to the production of extracellular polysaccharides exist.

Recently Meddens *et al* (1984) investigated the role of granulocytes in the induction of endocarditis with a dextran-producing *S. sanguis* and a dextran negative mutant of this strain. These authors noted that dextran-negative strains were more readily cleared from the circulation than were dextran-positive organisms, although it was noted that the intracellular killing of streptococci was not influenced by dextran production. Their study suggested that an impaired phagocytic removal of attached bacteria from the vegetational surface can be a factor promoting the induction of endocarditis by dextran-producing streptococci.

Results obtained in the present study suggest that extracellular glucan production may enhance the pathogenicity of these organisms by "masking" their cell-surface antigenic determinants and rendering them less "visible" to the immune system. There appeared to be no such effect with *S. salivarius*, possibly because of the more soluble nature of the polysaccharides associated with these organisms. The apparently low agglutinating antibody response of the mice used in the present study to *S. salivarius* was unexpected and is difficult to explain except in terms of the genetic responsiveness of the mouse strains used.

3.3.2. Development of an Enzyme-linked Immunosorbent Assay for Streptococcal C.A.P.

3.3.2.1. Antigen Coating of Assay Plates

Modifications of the method published by Gray (1979) were used to facilitate the detection of anti-glucan antibodies in mouse sera.

Preliminary investigations showed that the polystyrene assay plates used could not be coated directly with streptococcal C.A.P. However, the plates could be coated evenly and consistently with preparations of poly-L-lysine-coupled C.A.P. samples. The concentration of poly-L-lysine used for coupling was found to be critical. Fig (35a and b) show the results of using poly-L-lysine concentrations, during antigen coupling of 500, 100, 20 and 10 $\mu g.ml^{-1}$. The antigen used was lyophilised C.A.P. from sucrose-grown S. mutans at a concentration of 1000 $\mu g.ml^{-1}$. Fig (35a) illustrates the assay of a pre-immune mouse serum, and Fig (35b) the assay of an antisucrose-grown S. mutans serum. In the assay of both sera, the use of 500 $\mu g.ml^{-1}$ of poly-L-lysine during antigen coupling led to non-specific absorbance increases as the sera were diluted above 1:32. This effect did not occur with the lower poly-L-lysine concentrations. A coupling concentration of 100 $\mu g.ml^{-1}$ appeared optimal for the concentration of antigen used since this concentration gave low absorbance values with the pre-immune sera, showed no non-specific absorbance at high serum dilutions, and resulted in a direct relationship between antiserum dilution and absorbance at 450nm in the post-immune serum.

Fig. 35

Effect of Poly-L-lysine Concentration on the Assay of Antisera to Streptococcal Glucans

a) Assay of Pre-Immune Serum.

b) Assay of Anti Sucrose-grown S. mutans Serum.

○ 500 μg poly-L-lysine. ml^{-1} coating solution

● 100 μg poly-L-lysine. ml^{-1} coating solution

□ 20 μg poly-L-lysine. ml^{-1} coating solution

■ 10 μg poly-L-lysine. ml^{-1} coating solution

Fig 35a

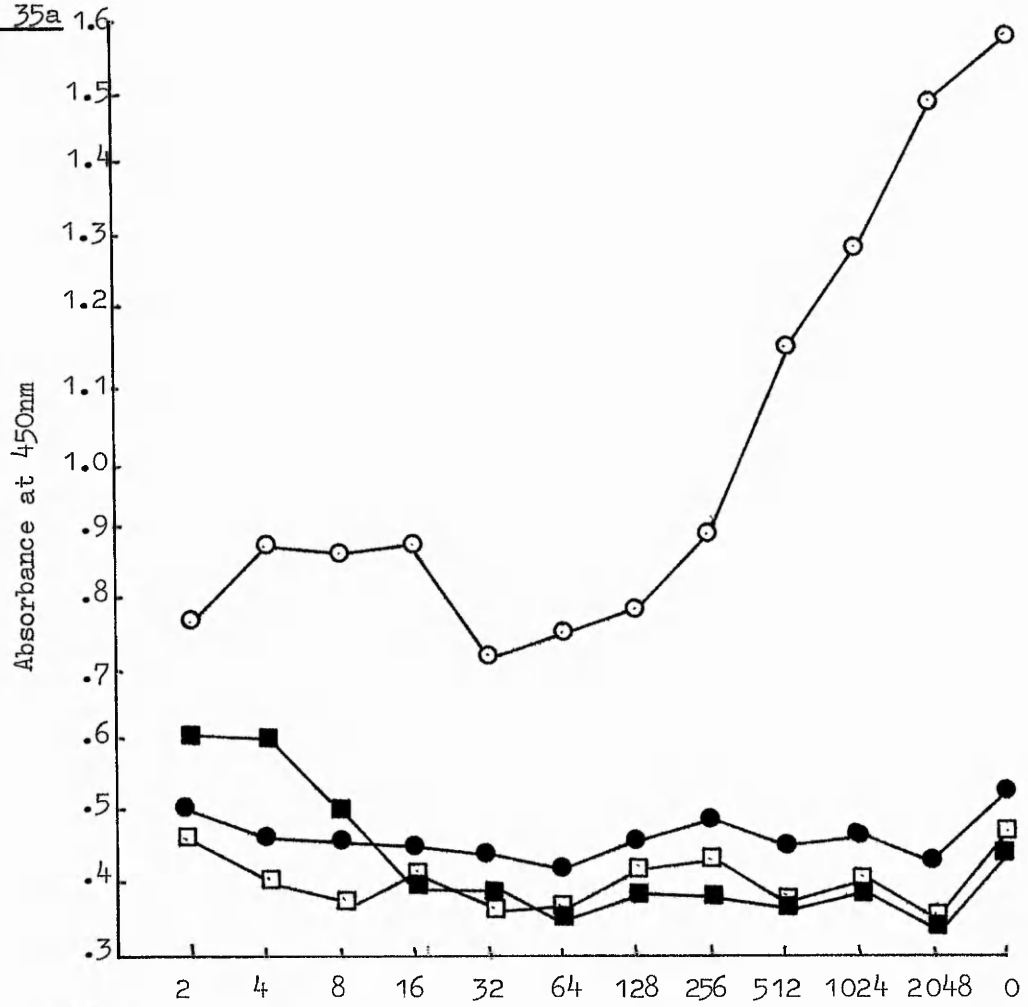
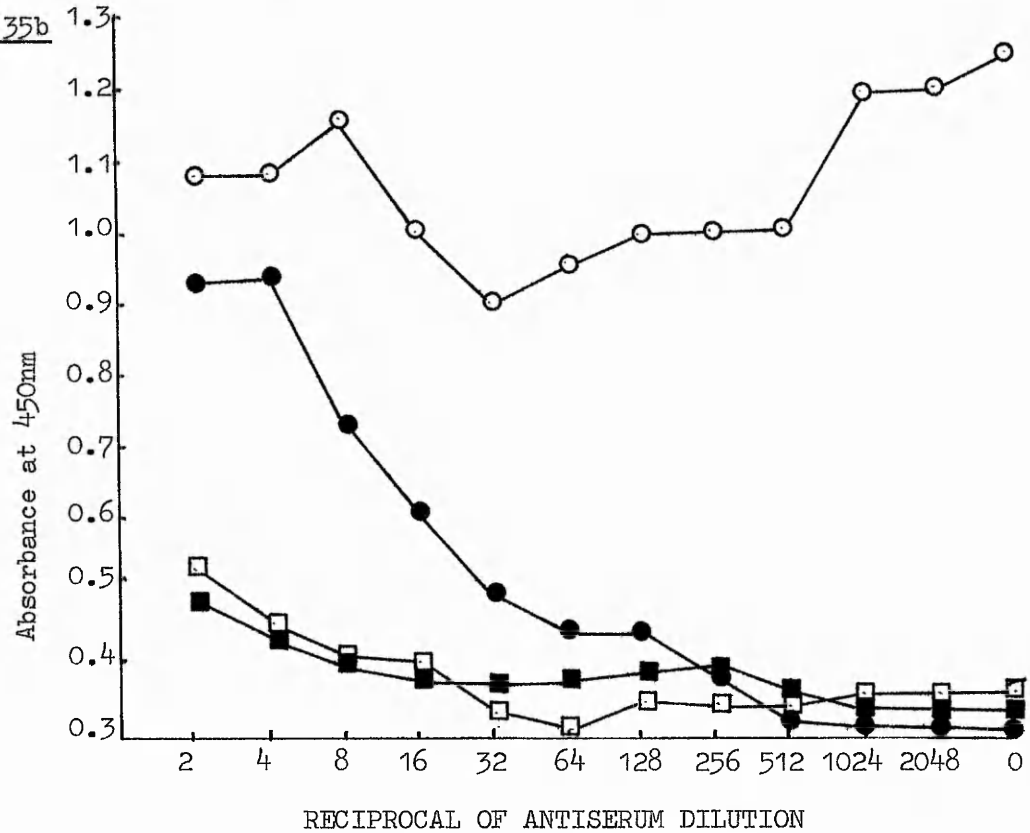


Fig 35b



RECIPROCAL OF ANTISERUM DILUTION

The non-specific absorbances noted at high antiserum dilutions, using high concentrations of poly-L-lysine during antigen coupling is thought to involve two effects. During antigen coating of plates there may be a competition effect between free and antigen bound poly-L-lysine, leading to a relative reduction of the amount of antigen attached to the plate. Secondly, it would appear from the data shown in Fig. (35 a and b) that the peroxidase-labelled antimouse lgG used during the assay binds non-specifically to poly-L-lysine. This leads to competition between the antibody-antigen complex and poly-L-lysine for the antimouse IgG, and explains the increase in lysine mediated, non-specific absorbance observed with increasing antiserum dilution.

This effect could be almost totally overcome by the use of a lower poly-L-lysine concentration during antigen coupling e.g. 100 $\mu g.ml^{-1}$. It was found that small non-specific absorbances noted, using lower poly-L-lysine concentrations could further be reduced by the inclusion of bovine serum albumin (0.05% w/v) in the antiserum dilution buffer and in the peroxidase labelled anti-mouse conjugates. The effectiveness of this treatment, due to masking of non-specific antimouse conjugate binding sites on the plate can be seen in the data presented in Table (7). The coating antigen used in this experiment was C.A.P. from sucrose-grown S. mutans, and the antiserum was post-immune serum to sucrose-grown S. mutans cells at a dilution of 1:1000.

TABLE 7

Effect of Pre-Treating Antigen Coated Plates with Bovine Serum Albumin on Non-Specific Absorbance

Figures represent Absorbance at 450nm

Table 7

lysine coupling conc^{n} ($\mu g.ml^{-1}$)	Anti IgG		Anti IgM	
	-BSA	+BSA	-BSA	+BSA
100	0.34	0.096	0.454	0.12
20	0.29	0.078	0.38	0.09
10	0.29	0.082	0.39	0.11

3.3.2.2. Peroxidase-Labelled Anti-Mouse Conjugate Concentration

To assess the optimum conjugate concentration for use in anti-glucan antibody detection, assays of post-immune sera to sucrose-grown *S. mutans* cells were performed, with *S. mutans* C.A.P. as the coating antigen, using four concentrations of antimouse Ig conjugate. The results (Fig 36) indicate that the use of higher concentrations of conjugate result in the earlier saturation of the substrate conversion reaction. From the data shown, using a substrate incubation time of 30 minutes, a conjugate concentration of 1:500 appeared optimal. This conjugate titre, after 30 minutes produced a high absorbance, but without substrate reaction saturation.

However, experiments performed to determine the effect of conjugate concentration on non-specific "background" absorbance, using pre-immune serum and *S. mutans* C.A.P. as the coating antigen indicated that non-specific absorbance increased dramatically at conjugate concentrations higher than 1:700 (Fig 37). For this reason, both anti IgG and anti IgM (which behaved similarly) were used in assays at a titre of 1:1000.

The observed increase in non-specific absorbance at conjugate concentrations higher than 1:700 was thought to be due to the inefficiency of our washing procedure in completely removing such high concentrations of conjugate.

Fig. 36

Relationship Between Anti Mouse IgG Conjugate Concentration, Substrate Incubation Time and Absorbance at 450nm.

Fig 36

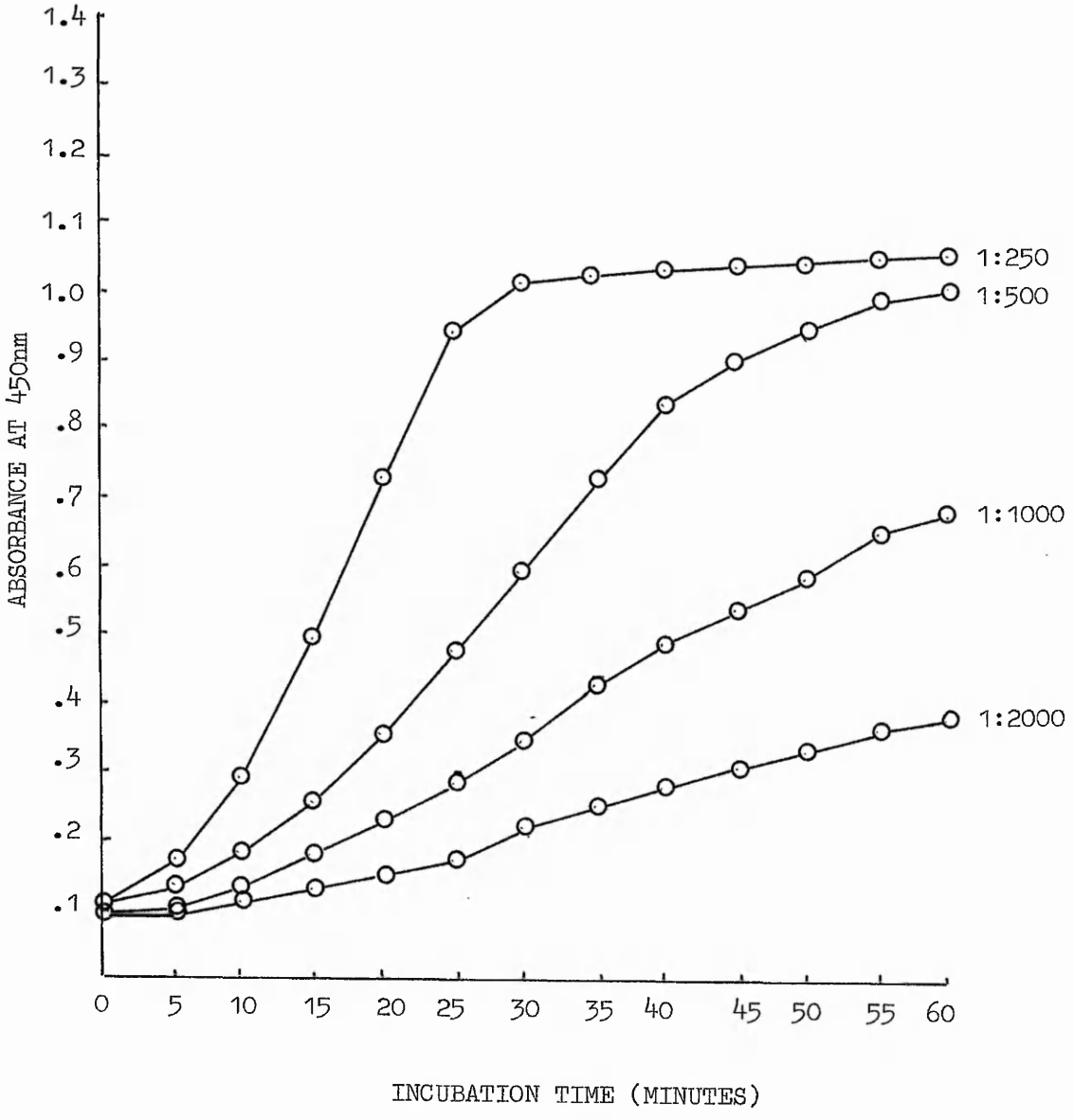
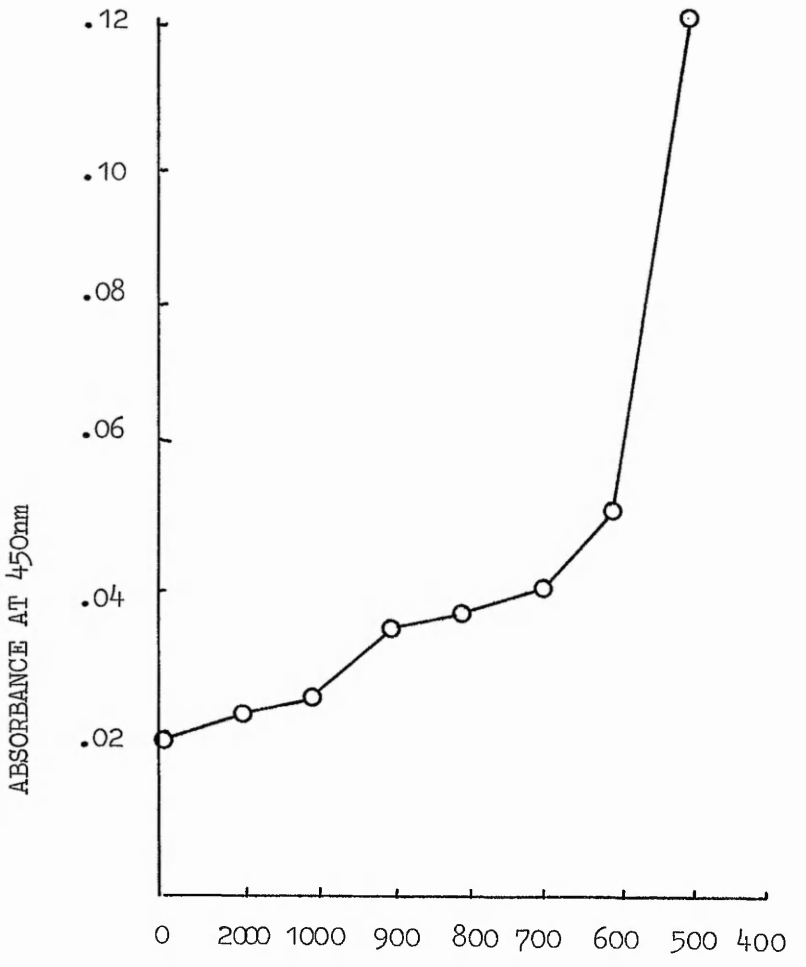

1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCE AT 450nm
1:250
1:500
1:1000
1:2000
0
5
10
15
20
25
30
35
40
45
50
55
60
INCUBATION TIME (MINUTES)

Fig 37

Increase in Non-Specific Background Absorbance With Increasing Conjugate Concentration

Fig 37


.12
.10
.08
.06
.04
.02
ABSORBANCE AT 450nm
0
2000
1000
900
800
700
600
500
400
Reciprocal of Conjugate dilution

3.3.2.3. Antiserum Titrations

To determine the dilutions of antisera at which antibody concentration was directly related to Absorbance at 450nm, it was necessary to assay dilutions of both positive and negative sera. This was achieved using pre-immune mouse serum as the presumptive negative control, and post immune anti sucrose-grown S. mutans serum as the presumptive positive control. The plate coating antigen used was C.A.P. from sucrose-grown cells of S. mutans, and both anti IgG and anti IgM conjugates were used at a titre of 1:1000.

Fig. (38) shows the typical results of such titrations for S. mutans. For both IgG and IgM assays of positive sera, antiserum dilutions of greater than 1:1000 were necessary to ensure that the Absorbance measured was directly related to antibody concentration, and in subsequent assays sera were routinely diluted 1:1000.

Pre-immune sera gave higher absorbances when assayed for IgG anti S. mutans antibodies than when assayed for IgM antibodies and it may be that this is a reflection of the presence of native IgG antibodies directed against S. mutans determinants in these sera.

3.3.3. Investigations of the IgG Response of Mice to Streptococcal C.A.P.

To quantify and determine the specificity of the IgG antibody response of mice to challenge with sucrose-and glucose-grown cells of S. mutans, S. sanguis and S. salivarius, sera were assayed for activity against dextran, glycogen, levan and C.A.P. from sucrose-grown cells of S. mutans, S. sanguis and

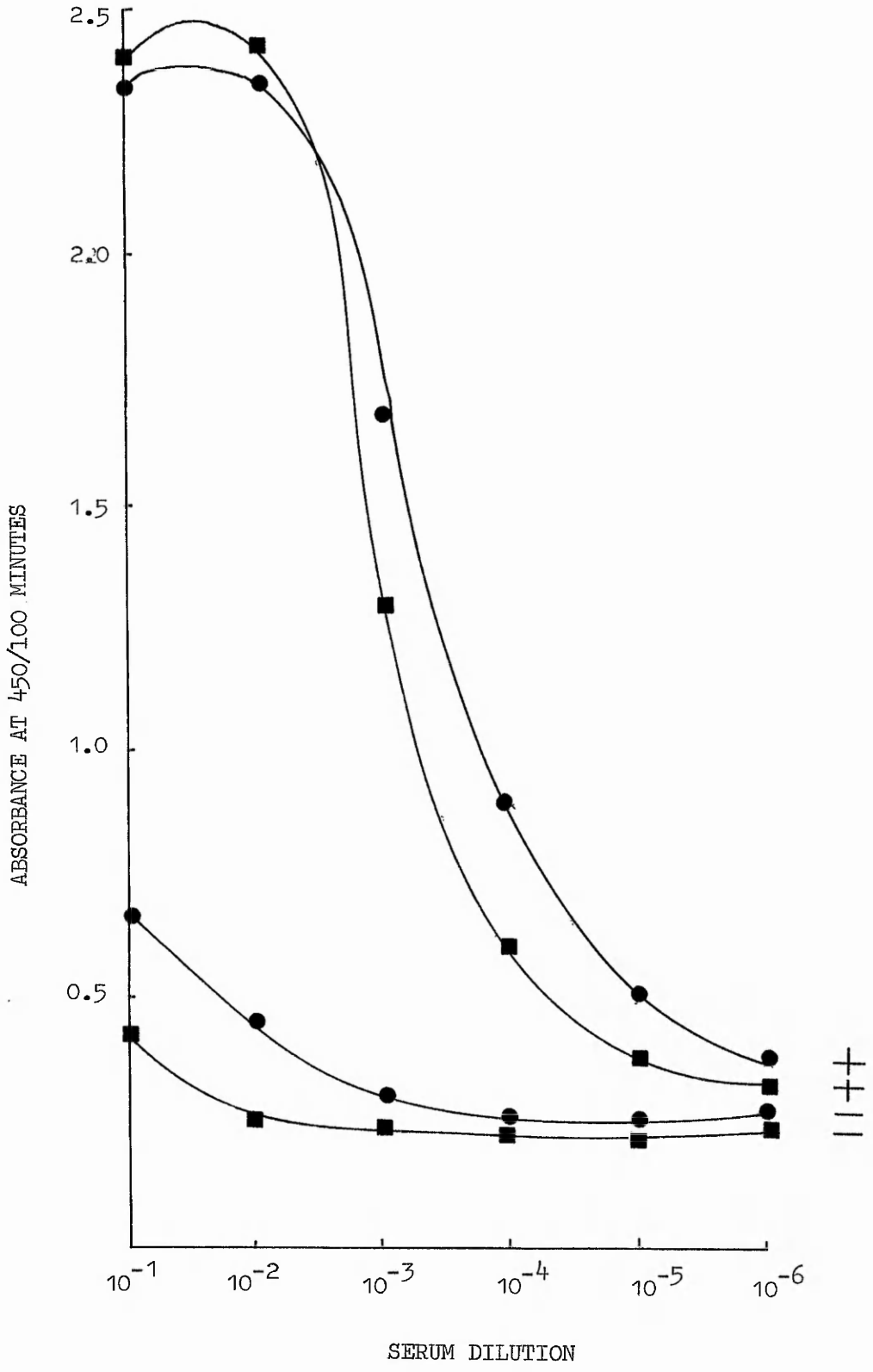
Fig. 38

Typical Titration Curves for positive and negative sera using both Anti IgG and Anti IgM conjugates

● Anti Mouse IgG as conjugate

■ Anti Mouse IgM as conjugate

Fig. 38


2.5
2.0
1.5
1.0
0.5
ABSORBANCE AT 450/100 MINUTES
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
SERUM DILUTION
++
+

S. salivarius. Each serum was assayed unabsorbed, and absorbed with homologous sucrose or glucose-grown cells, to determine the quantity and specificity of antibodies specific for sucrose-grown cells.

Figs (39 to 44) indicate, in detail, the results of assaying sera raised against sucrose and glucose grown cells of S. mutans, S. sanguis and S. salivarius using six different plate-coating antigens. These assays yielded results which were the averages of five separate experiments performed with sera which were pooled from ten mice. The results are summarised in Table (8) which shows the lgG activity as ELISA units, of each serum, and the residual activities after absorption with sucrose and glucose-grown cells are shown in Table (9). Table (8) shows that, for unabsorbed sera raised against sucrose-grown S. mutans, the highest activities were directed against dextran, S. mutans C.A.P., S. sanguis C.A.P. and S. salivarius C.A.P. It was also noted that the activities of sera raised against glucose-grown S. mutans were higher than would be expected for each of the coating antigens.

Unabsorbed sera raised against sucrose-grown cells of S. sanguis showed low activity against C.A.P. from each of the three species, with the highest activity measured against S. salivarius C.A.P. The activities measured for sera raised against glucose-grown S. sanguis were higher for all the coating antigens, than those measured for sera raised against sucrose-grown cells.

Unabsorbed sera raised against sucrose-grown cells of S. salivarius showed the highest activity against levan, and,

of the C.A.P. preparations, against S. salivarius C.A.P. For clarity, the results of the absorbance experiments were transformed first into residual activities (as a percentage of unabsorbed sera values) after absorption with sucrose and glucose-grown cells (Table (9).

These data were then transformed in order to determine the IgG activity of each serum specific for sucrose-grown cells. This was done by calculating the percentage loss of activity caused by sucrose cell and glucose cell absorption (Table 10). The loss of activity caused by glucose-grown cell absorption was then subtracted from that caused by sucrose-grown cell absorption. This revealed the specific IgG activity of sera raised to sucrose and glucose grown cells, against sucrose grown cells i.e. antibody populations directed against determinants present at the surface of sucrose-grown, but not glucose-grown cells (Table 11). It can be seen that sera raised against glucose-grown cells appeared to have some activity specific for sucrose-grown cells. This activity was assumed to be the result of either incomplete absorption of sera by bacterial cells, or by cross-reacting antibody populations, present in both sera, directed against a common antigenic determinant on cells grown on both sugars. In order to compensate for this non-specific activity against sucrose-grown cells in sera raised against glucose-grown cells, it was subtracted from the activity against sucrose-grown cells in sera raised against sucrose-grown cells. This value was then taken to be a measure of specific antibody activity directed against antigens present only at the surface of sucrose-grown cells.

Fig. 39

IgG Assay of Antisera to Sucrose and Glucose-Grown Cells of S. mutans, S. sanguis and S. salivarius Using Sucrose-Grown S. mutans C.A.P. as the Plate-Coating Antigen

- Pre-Immune sera
- Non-Absorbed Post-Immune sera
- Post-Immune sera absorbed with homologous sucrose grown cells.
- Post-Immune sera absorbed with homologous glucose grown cells.

Fig. 39

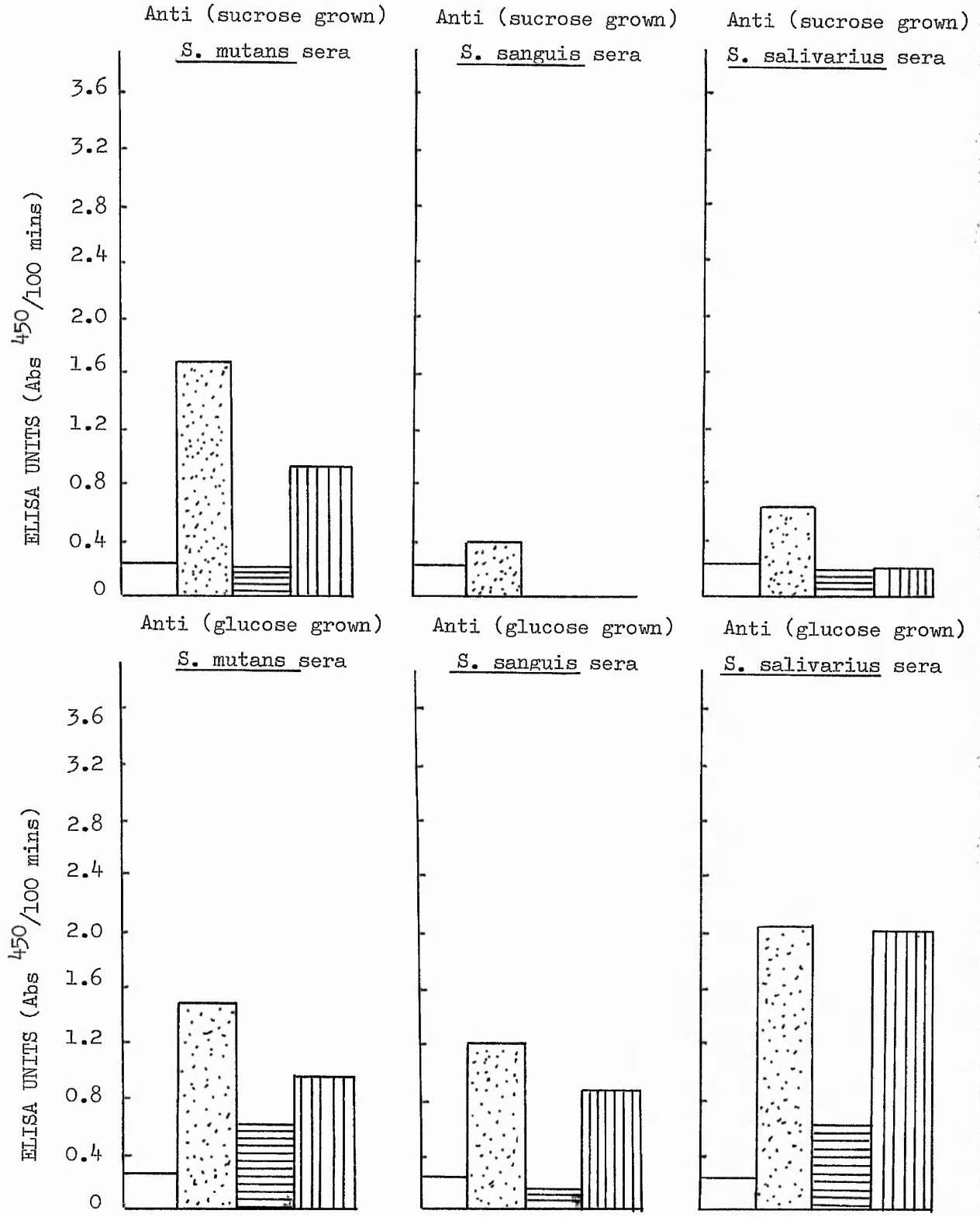

Anti (sucrose grown)
S. mutans sera
Anti (sucrose grown)
S. sanguis sera
Anti (sucrose grown)
S. salivarius sera
Anti (glucose grown)
S. mutans sera
Anti (glucose grown)
S. sanguis sera
Anti (glucose grown)
S. salivarius sera
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0

Fig. 40

IgG Assay of Antisera to Sucrose and Glucose-Grown cells of S. mutans, S. sanguis and S. salivarius, Using sucrose-grown S. sanguis C.A.P. as the Plate Coating Antigen

- ☐ Pre-Immune sera
- ⊠ Non Absorbed Post-Immune sera
- ▤ Post-Immune sera absorbed with homologous sucrose-grown cells.
- ▥ Post-Immune sera absorbed with homologous glucose-grown cells.

Fig. 40

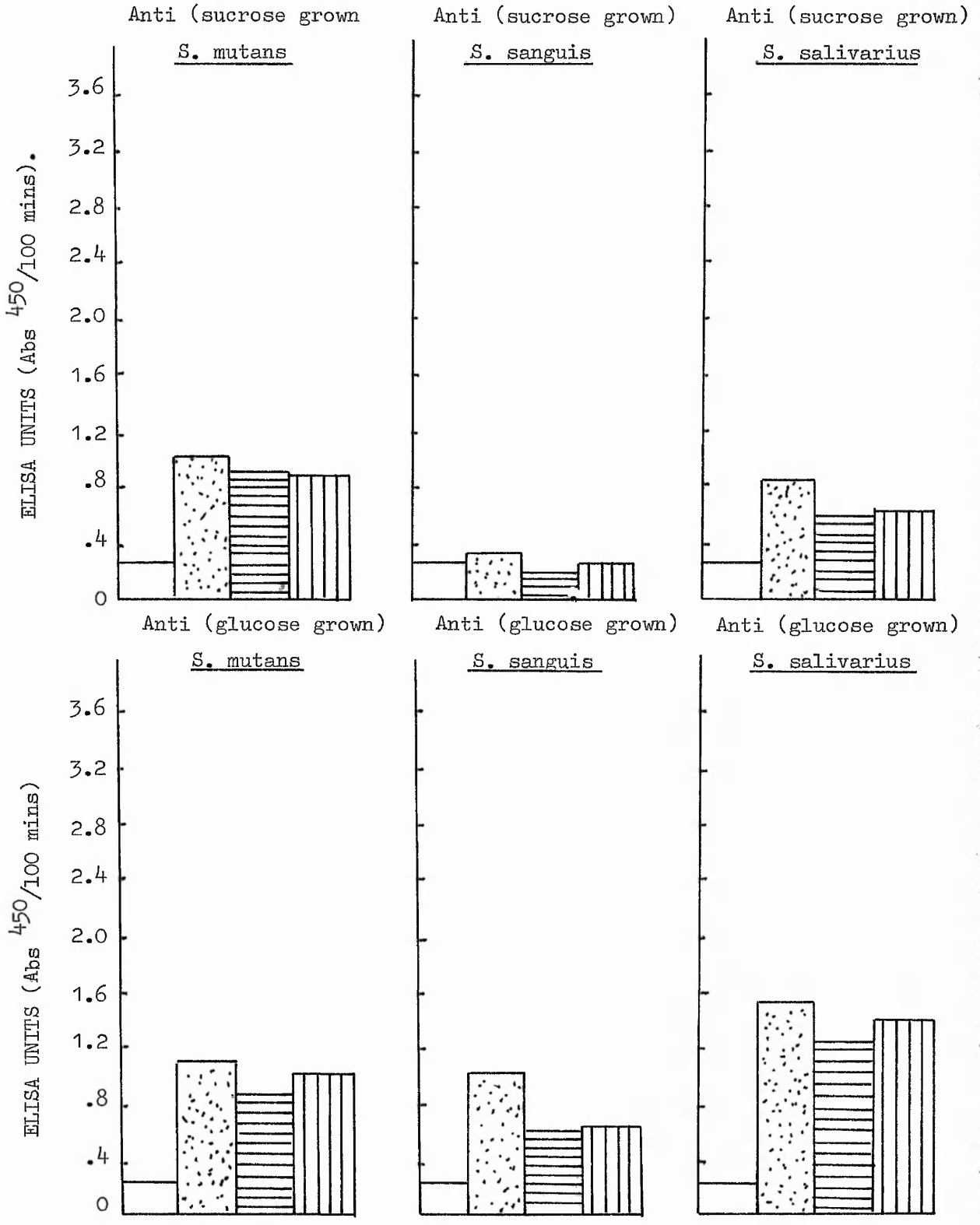

Anti (sucrose grown
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins).
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)

Fig. 41

IgG Assay of Antisera to Sucrose and Glucose Grown cells of S. mutans, S. sanguis and S. salivarius, Using Sucrose-Grown S. salivarius C.A.P. as the Plate Coating Antigen


Pre-Immune sera


Non-Absorbed Post-Immune Sera


Post-Immune sera absorbed with homologous sucrose-grown cells.


Post-Immune sera absorbed with homologous glucose-grown cells.

Fig. 41

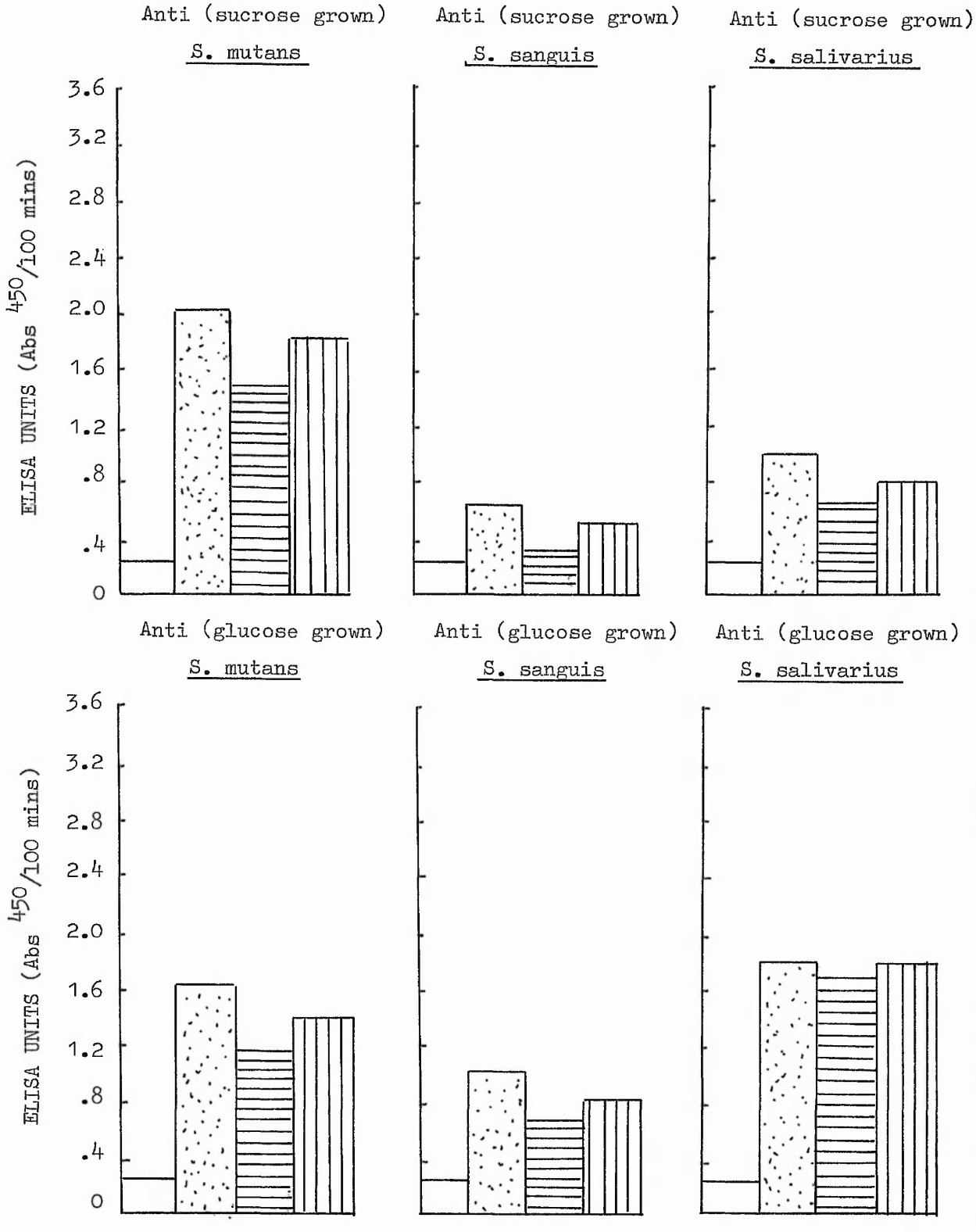

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
0

Fig. 42

IgG Assay of Antisera to Sucrose and Glucose Grown cells of S. mutans, S. sanguis and S. salivarius, Using Dextran as the Plate Coating Antigen

- Pre-Immune Sera
- Non-Absorbed Post-Immune sera
- Post-Immune sera absorbed with homologous sucrose grown cells.
- Post-Immune sera absorbed with homologous glucose grown cells.

dextran
ex. L. mesenteroides (Sigma)
M.Wt. 60,000 - 90,000

Fig. 42

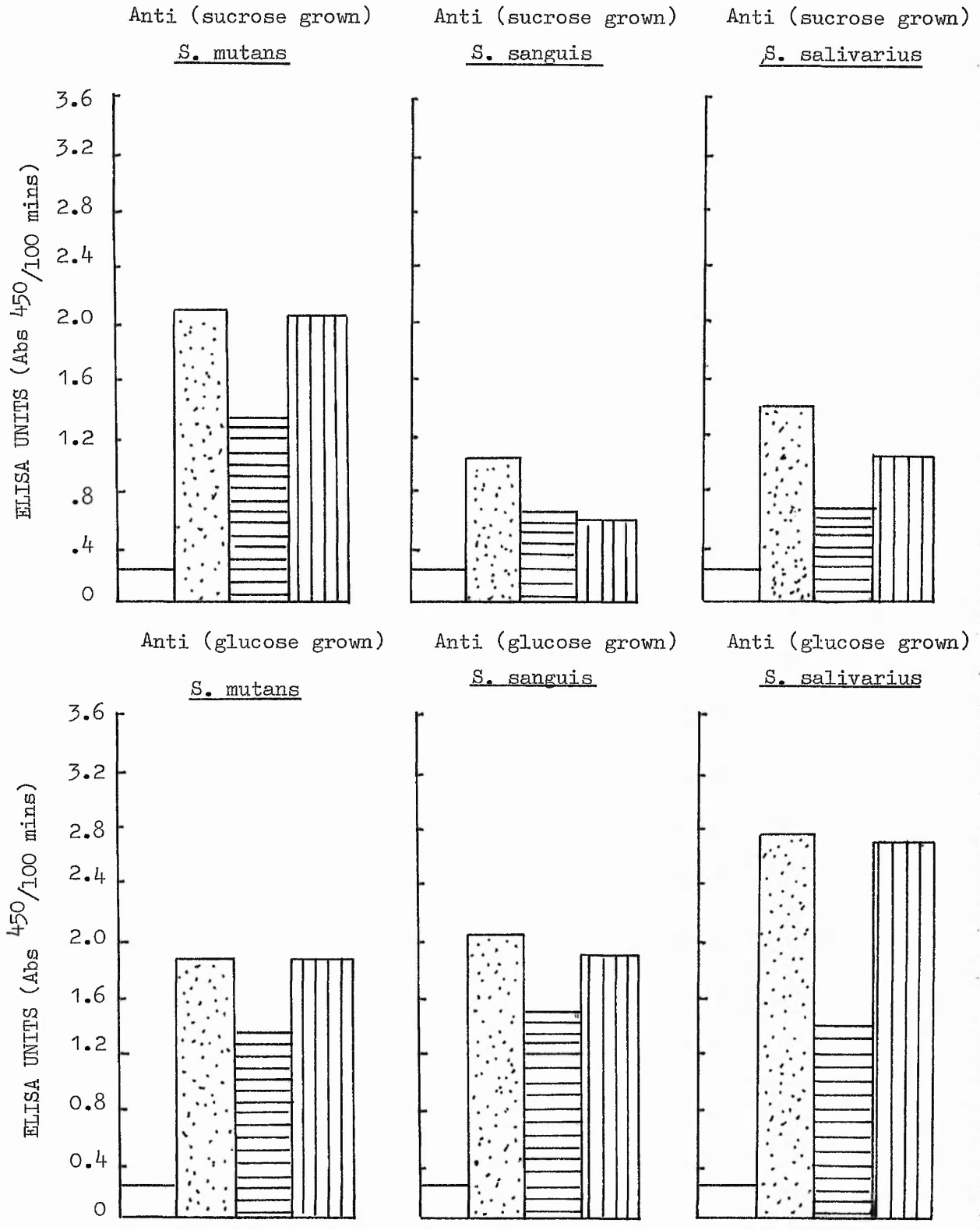

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0

Fig. 43

IgG Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using Glycogen as the Plate-Coating Antigen

- Pre-Immune Sera
- Non-Absorbed Post-Immune sera
- Post-Immune sera absorbed with homologous sucrose grown cells.
- Post-Immune sera absorbed with homologous glucose grown cells.

glycogen

ex. Oyster (Sigma)

Fig. 43

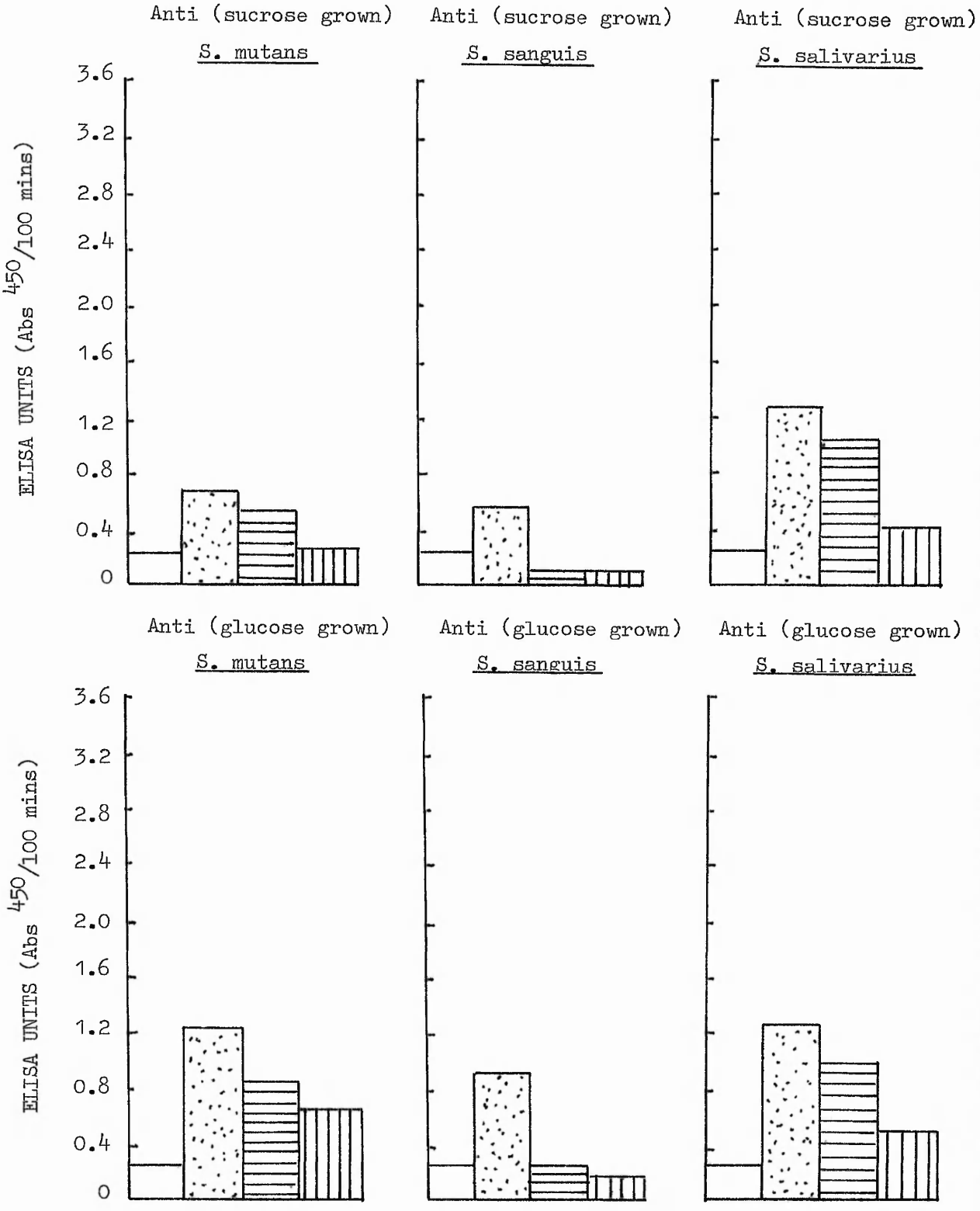

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)

Fig. 44

IgG Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using Levan as the Plate Coating Antigen

- Pre-Immune Sera
- Non-Absorbed Post-Immune sera
- Post-immune sera absorbed with homologous sucrose grown cells.
- Post-Immune sera absorbed with homologous glucose grown cells.

levan

ex. Aerobacter levanicum (Sigma)

Fig. 44

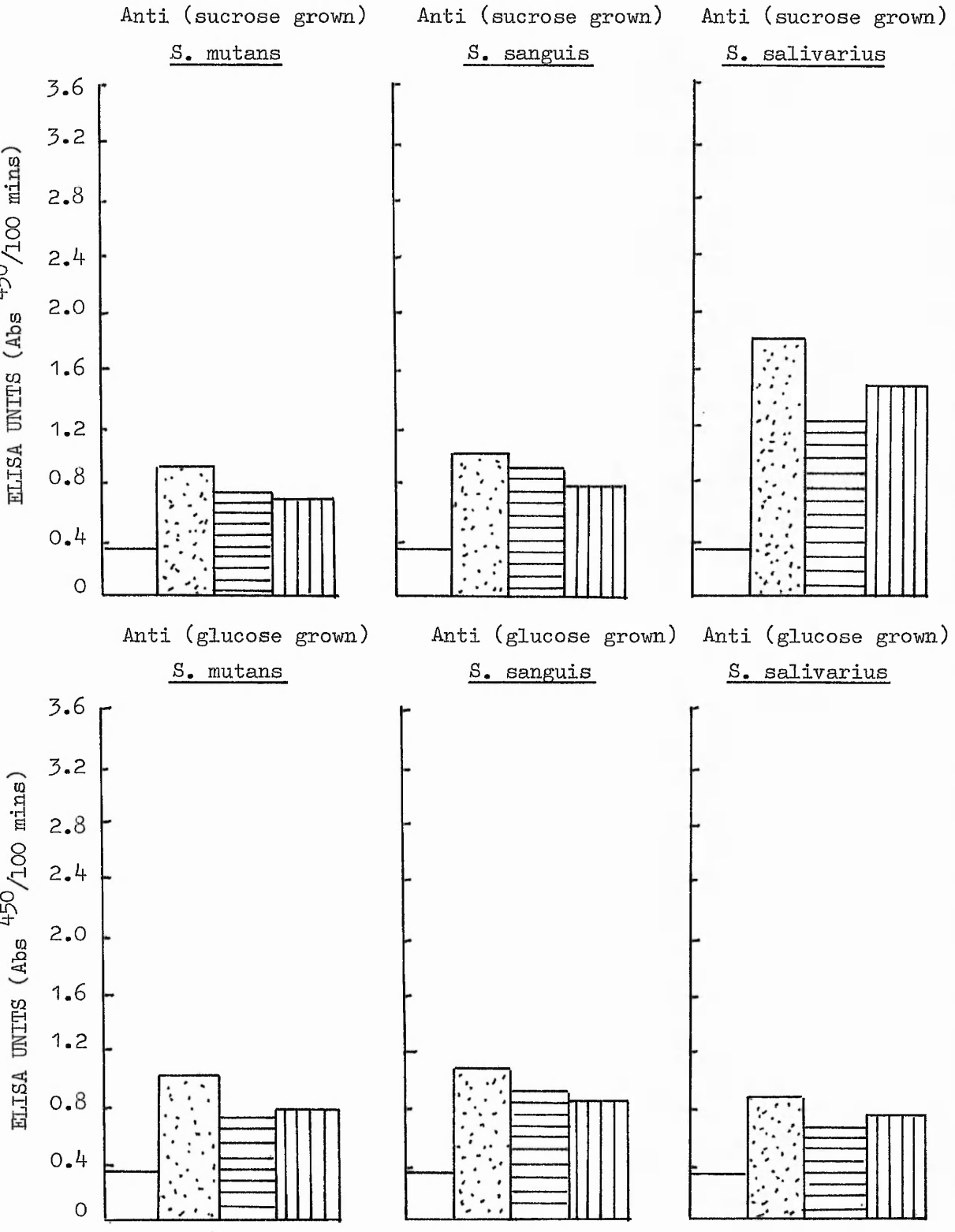

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 mins)

Table 8

IgG Activity of Antisera to Sucrose and Glucose-Grown Cells of S. mutans, S. sanguis and S. salivarius, against Six Coating Antigens. (Abs. $^{450}/_{100\ min}$).

Table 8

Coating Ag.	Absorption	Antisera to S. mutans grown on		S. sanguis grown on		S.salivarius grown on	
		sucr.	gluc.	sucr.	gluc.	sucr.	gluc.
Dextran	None	2.284	1.934	1.070	2.260	1.360	2.78
	suc. cells	1.370	1.272	0.710	1.496	0.764	1.378
	gluc. cells	2.09	2.012	0.648	1.926	1.068	2.738
Glycogen	None	0.730	1.210	0.574	0.870	1.250	1.260
	suc. cells	0.540	0.822	0.118	0.254	1.080	0.942
	gluc. cells	0.268	0.718	0.114	0.180	0.424	0.462
Levan	None	0.850	1.004	1.014	1.094	1.734	0.854
	suc. cells	0.770	0.778	0.930	0.882	1.238	0.692
	gluc. cells	0.750	0.800	0.796	0.852	1.474	0.780
Mutans CAP	None	1.650	1.520	0.400	1.214	0.670	2.250
	suc. cells	0.264	0.572	0	0.120	0.200	0.600
	gluc. cells	0.924	1.018	0	0.880	0.196	2.036
Sanguis CAP	None	1.084	1.174	0.274	1.064	0.814	1.594
	suc. cells	0.918	0.880	0.152	0.692	0.610	1.244
	gluc. cells	0.904	1.056	0.196	0.800	0.652	1.398
Saliv. CAP	None	2.014	1.724	0.680	1.004	0.924	1.800
	suc. cells	1.490	1.120	0.380	0.732	0.676	1.620
	gluc. cells	1.772	1.412	0.462	0.832	0.794	1.800

Table 9

Summary of percentage Residual IgG Activities of Post Immune Sera After Absorption with Homologous Sucrose or Glucose Grown Cells

Table 9

Coating Ag.	Absorption	Anti Serum to:- S. mutans grown on;-		S. sanguis grown on:-		S. salivarius grown on:-	
		sucr.	gluc.	sucr.	gluc.	sucr.	gluc.
Dextran	None	100	100	100	100	100	100
	Suc. cells	59.9	66.0	66.0	66.2	56.2	49.6
	Gluc. cells	91.5	100	60.0	85.2	78.5	98.5
Glycogen	None	100	100	100	100	100	100
	Suc. cells	74.0	67.9	20.5	29.2	86.4	74.8
	Gluc. cells	36.7	59.3	19.8	20.7	33.9	36.6
Levan	None	100	100	100	100	100	100
	Suc. cells	90.6	77.5	91.7	80.6	71.4	81.0
	Gluc. cells	88.2	79.7	78.5	77.9	95.0	91.3
Mutans CAP	None	100	100	100	100	100	100
	Suc. cells	16.0	38.0	0	9.9	29.8	26.6
	Gluc. cells	56.0	67.0	0	72.5	29.2	98.0
Sanguis CAP	None	100	100	100	100	100	100
	Suc. cells	84.7	74.9	55.0	65.0	74.9	78.0
	Gluc. cells	83.4	89.9	71.5	75.2	80.1	87.7
Salivarius CAP	None	100	100	100	100	100	100
	Suc. cells	73.9	64.9	55.9	72.9	73.2	90.0
	Gluc.cells	87.9	81.9	67.9	82.8	85.9	100

Table 10

Loss of IgG Activity (as % Absorbance) on Absorption with Sucrose or Glucose-Grown Cells.

Table 10

Coating Ag.	Absorbing Cells Grown on	Anti Serum to S. mutans grown on sucr.	S. mutans grown on gluc.	S. sanguis grown on sucr.	S. sanguis grown on gluc.	S. salivarius grown on sucr.	S. salivarius grown on gluc.
Dextran	Sucrose	40.1	34.0	34.0	33.8	43.8	50.4
	Glucose	8.5	0	39.4	14.8	21.5	1.5
Glycogen	Sucrose	26.0	32.1	89.5	70.8	13.6	25.2
	Glucose	63.0	40.7	80.2	79.3	66.1	63.4
Levan	Sucrose	9.4	22.5	8.3	19.4	28.6	19.0
	Glucose	11.8	20.3	21.5	22.1	15.0	8.7
Mutans CAP	Sucrose	84.0	62.0	100	90.1	70.2	73.4
	Glucose	44.0	33.0	100	27.5	70.8	2.0
Sanguis CAP	Sucrose	15.3	25.1	44.5	35.0	25.1	22.0
	Glucose	16.6	10.1	28.5	24.8	19.9	12.3
Salivarius CAP	Sucrose	26.1	35.1	44.1	27.1	26.8	10.0
	Glucose	12.1	18.1	32.1	17.2	14.1	0

Table 11

Sucrose-Grown Cell Specific IgG activities as a percentage of Post-Immune Serum Activity

Table 11

Coating Ag.	Anti Serum to:- S. mutans grown on		S. sanguis grown on		S. salivarius grown on	
	sucrose	glucose	sucrose	glucose	sucrose	glucose
Dextran	31.6	34.0	0	19.0	22.3	48.9
Glycogen	0	0	0	0	0	0
Levan	0	2.2	0	0	13.6	0
mutans C.A.P.	40.0	29.0	0	62.6	0	71.4
sanguis C.A.P.	0	15.0	16.0	10.2	5.2	9.7
salivarius C.A.P.	14.0	17.0	12.0	9.9	12.7	10.0

Table 12

Assay Anti IgG Activities Specific for Sucrose-Grown cells and for Anti Sucrose-Grown Cell Serum

Table 12

Coating Ag.	Antisera to:- S. mutans	S . sanguis	S. salivarius
Dextran	0	0	0
Glycogen	0	0	0
Levan	0	0	13.6
mutans C.A.P.	11.0	0	0
sanguis C.A.P.	0	5.8	0
salivarius C.A.P.	0	2.1	2.7

Since antigen preparations used to coat assay plates were de-proteinised, and contained no detectable protein or sugar components other than glucose and rhamnose, this activity was assumed to be due to the presence of anti-glucan antibodies. Table (12) shows these values, obtained for each of the three antisera, when assayed against the six plate-coating antigens tested.

Sera raised against sucrose-grown S. mutans had 11% of their activity directed against sucrose-grown cell specific components of the C.A.P. from S. mutans. This activity was assumed to be mediated by anti-glucan antibodies. This serum contained no anti-dextran activity, however, nor did it show any specific activity to the other coating antigens tested.

Sera raised against sucrose-grown S. sanguis showed specific activity only against S. sanguis C.A.P., and S. salivarius C.A.P., indicating some immunological similarity between these antigen preparations. Similarly, sera raised against sucrose-grown cells of S. salivarius showed specificity only for levan and for its homologous C.A.P. preparation, indicating a degree of cross-reaction between these two antigens.

A more detailed discussion of the results obtained in this section will be undertaken after the presentation of data obtained for the IgM response of mice to these streptococcal glucans.

3.3.4. Investigation of the IgM Response of Mice to Streptococcal C.A.P.

To quantify and determine the specificity of the IgM antibody response of mice to challenge with sucrose and glucose-grown

cells of S. mutans, S. sanguis and S. salivarius, sera were assayed for activity against dextran, glycogen, levan and C.A.P. from sucrose-grown cells of S. mutans, S. sanguis and S. salivarius. Each serum was assayed unabsorbed, and absorbed with homologous sucrose and glucose-grown cells to determine the quantity and specificity of IgM antibodies specific for sucrose-grown cells.

Figs (45 to 50) indicate, in detail, the results of assaying sera raised against sucrose and glucose grown cells of S. mutans, S. sanguis and S. salivarius using six plate-coating antigens. Table (13) summarises the results of these assays. The activity of sera raised against sucrose grown cells of S. mutans was greatest against its homologous antigen preparation, and those of sera raised against sucrose-grown S. sanguis and S. salivarius were highest against levan. For each serum, it was noted, that the activity of sera raised against glucose-grown cells was higher for each antigen than was obtained for sera raised against sucrose-grown cells. The results obtained for absorption experiments were transformed in the same way as those obtained in Section 3.3.3. for IgG activities.

Table (14) shows the residual IgM activity of sera absorbed with either sucrose or glucose grown cells and Table (15) gives the percentage loss of activity produced by these treatments. Table (16) shows the specific antisucrose grown cell activity of each serum against each antigen. These figures were obtained by subtracting the percentage loss of activity caused by sucrose-grown cell absorption from that caused by sucrose-grown cell absorption.

Finally, Table (17) indicates the values for IgM activities

Fig. 45

IgM Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using Dextran as the Plate Coating Antigen

□ Pre Immune Sera

▣ Non Absorbed Post Immune Sera

▤ Post Immune Sera absorbed with homologous sucrose grown cells

▥ Post Immune Sera absorbed with homologous glucose grown cells

Fig. 45

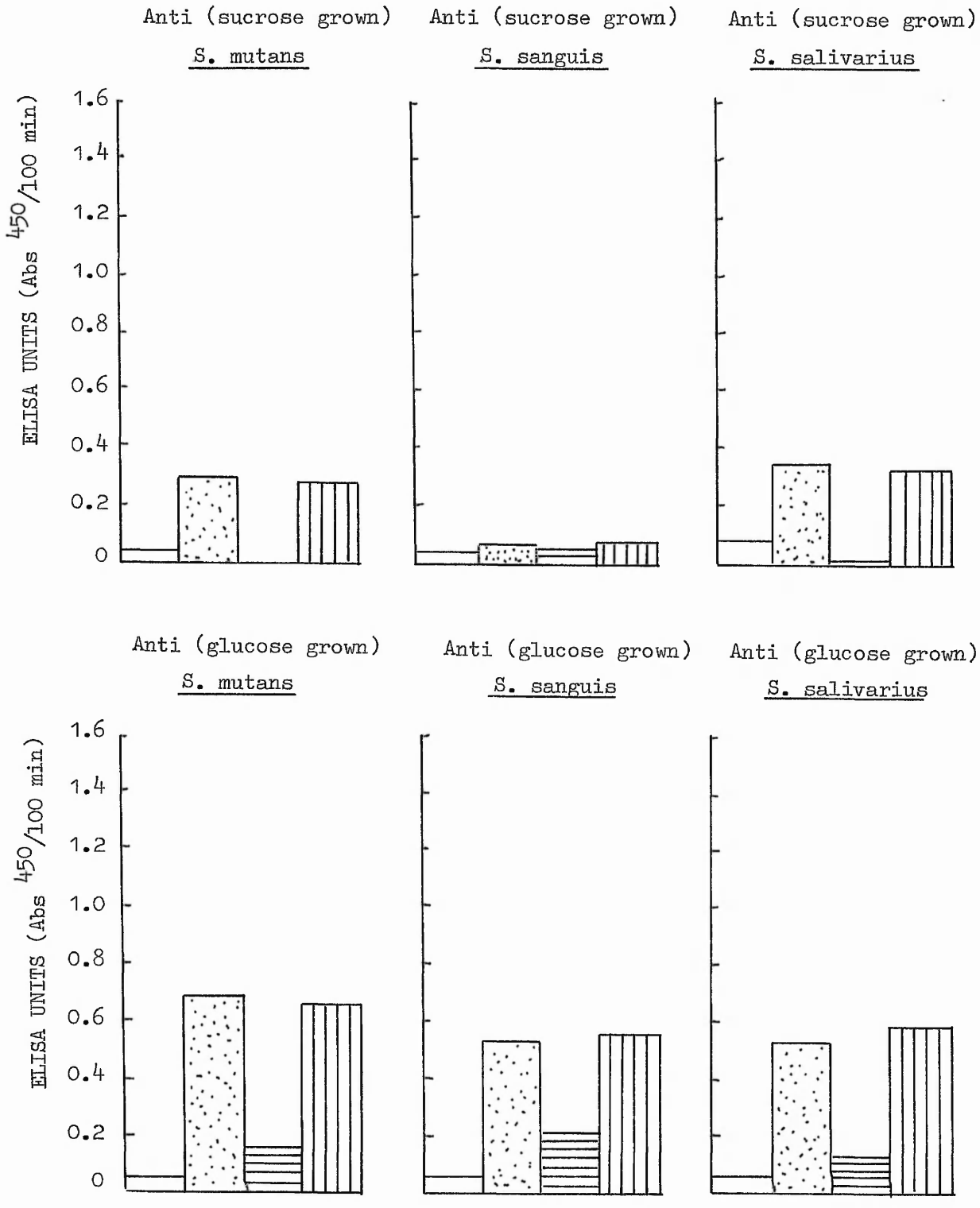

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

Fig. 46

IgM Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using Glycogen as the Plate Coating Antigen

 Pre Immune Sera

 Non Absorbed Post Immune Sera

 Post Immune Sera absorbed with homologous sucrose grown cells

 Post Immune Sera absorbed with homologous glucose grown cells

Fig. 46

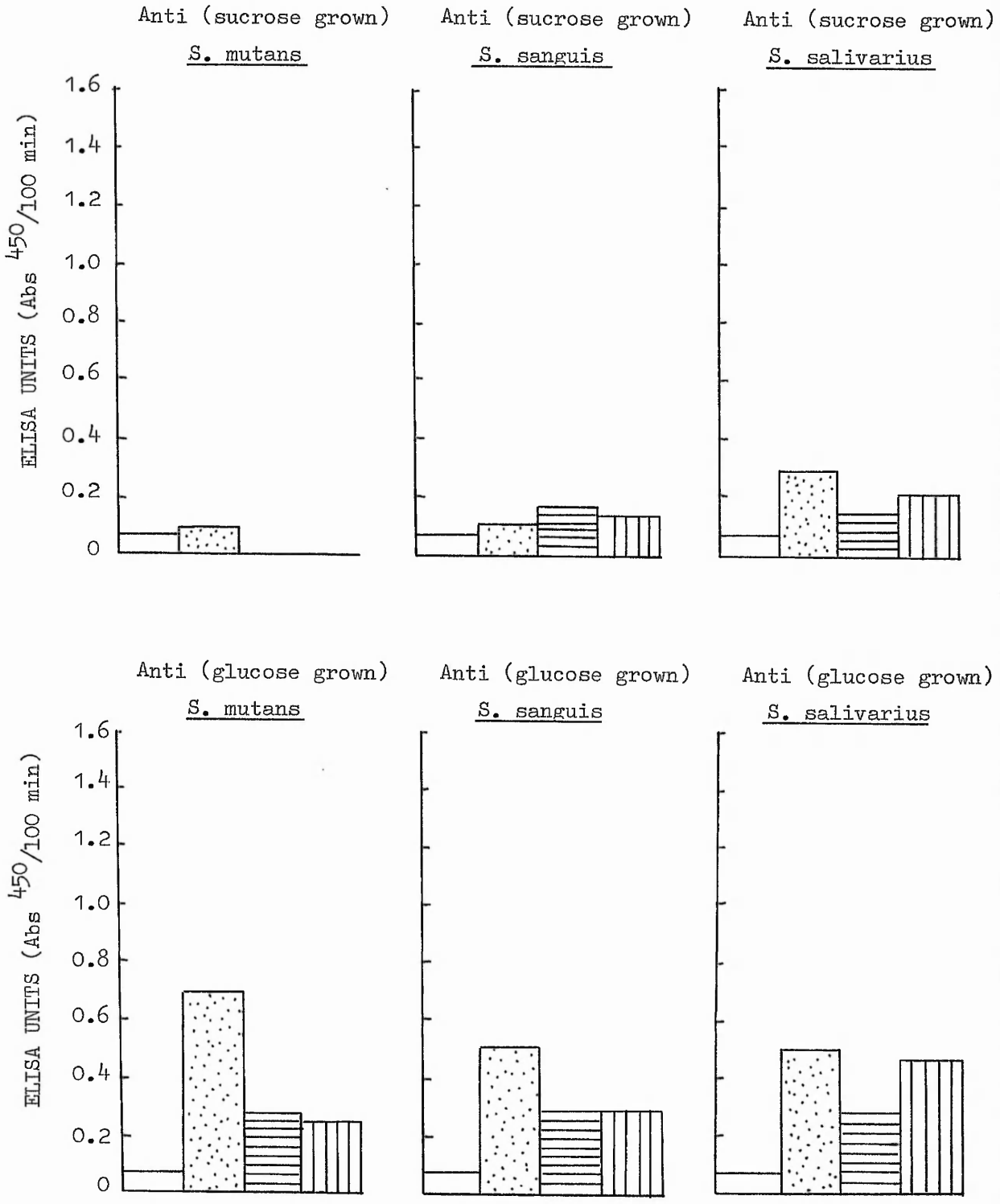

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

Fig. 47

IgG Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using Levan as the Plate Coating Antigen

- Pre-Immune Sera
- Non-Absorbed Post-Immune Sera
- Post-Immune Sera absorbed with homologous sucrose grown cells.
- Post-Immune Sera absorbed with homologous glucose grown cells

Fig. 47

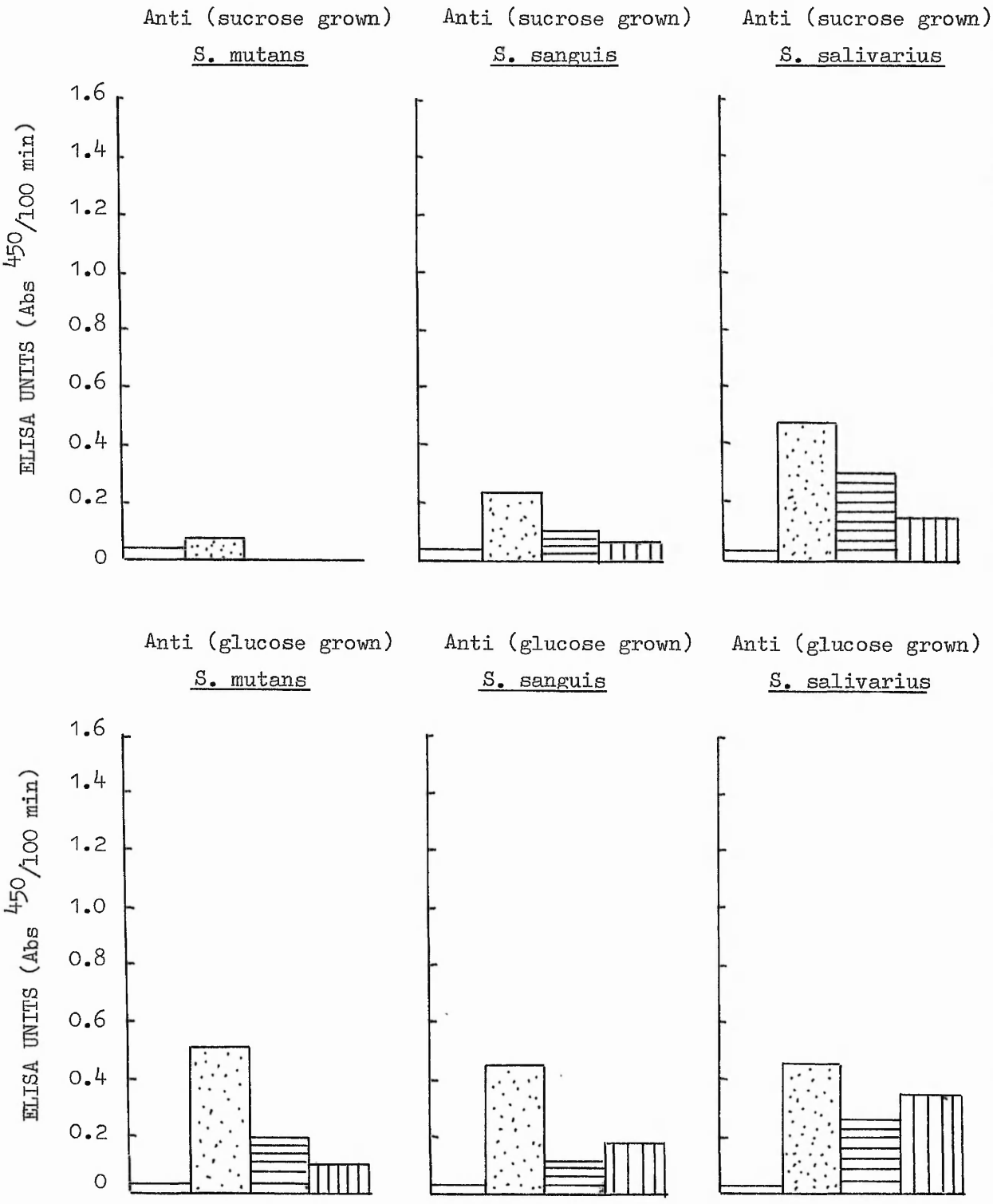

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

Fig. 48

IgG Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using C.A.P. from Sucrose Grown S. mutans as the Plate Coating Antigen

- Pre-Immune Sera
- Non-Absorbed Post-Immune Sera
- Post-Immune Sera absorbed with homologous sucruse grown cells.
- Post-Immune Sera absorbed with homologous glucose grown cells.

Fig 48

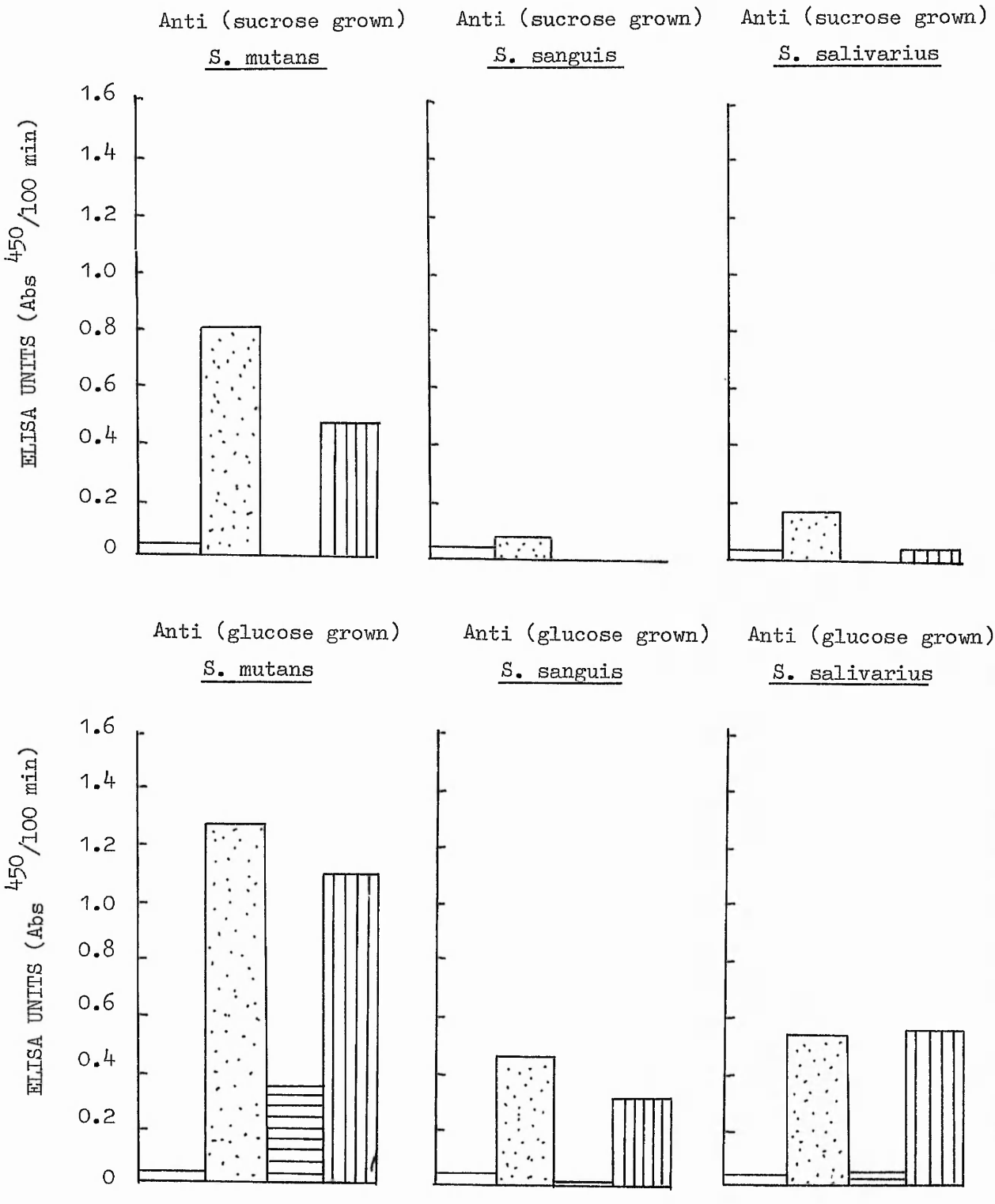

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

Fig. 49

IgM Assay of Antisera to Sucrose and Glucose Grown Cells of S. mutans, S. sanguis and S. salivarius, Using C.A.P. from Sucrose S. sanguis as the Plate Coating Antigen

- Pre-Immune Sera
- Non-Absorbed Post-Immune Sera
- Post-Immune sera absorbed with homologous sucrose grown cells.
- Post-Immune Sera absorbed with homologous glucose grown cells.

Fig. 49

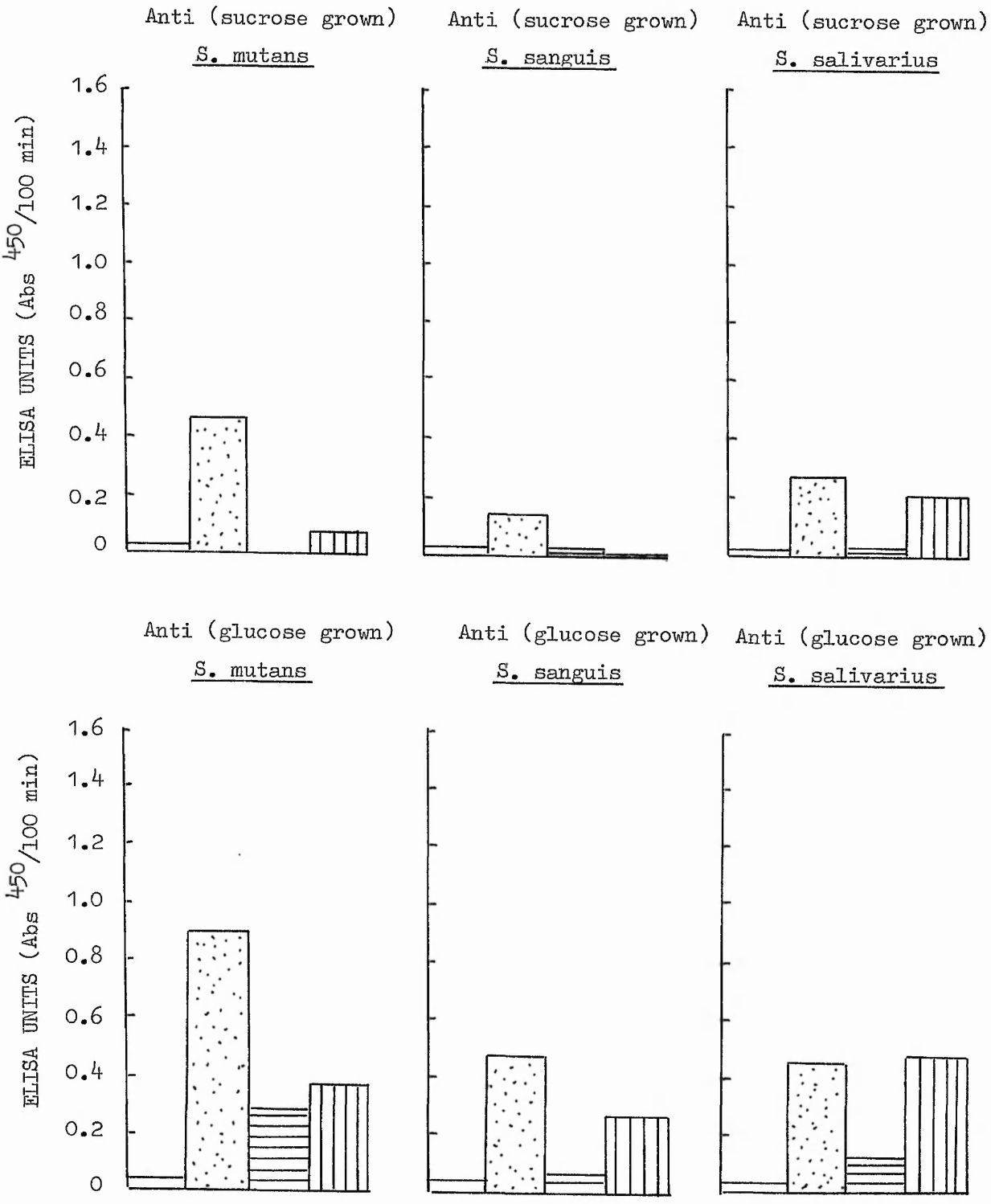

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Anti (glucose grown)
S. mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

Fig. 50

IgM Assay of Antisera to Sucrose and Glucose Grown cells of S. mutans, S. sanguis and S. salivarius, Using C.A.P. from Sucrose grown S. salivarius as the plate coating antigen.

- Pre-Immune Sera
- Non-Absorbed Post Immune Sera
- Post-Immune Sera absorbed with homologous sucrose grown cells.
- Post-Immune sera absorbed with homologous glucose grown cells.

Fig. 50

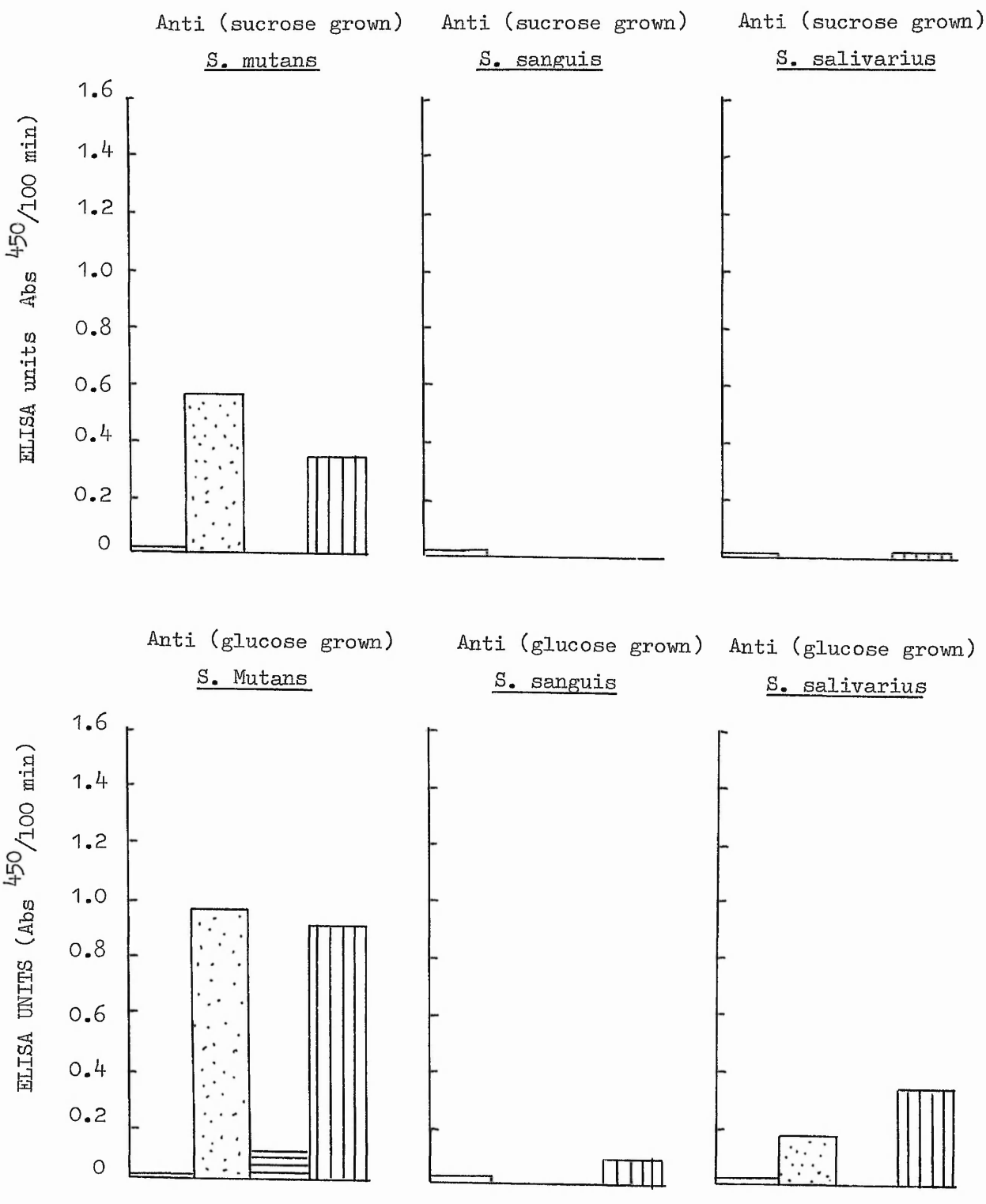

Anti (sucrose grown)
S. mutans
Anti (sucrose grown)
S. sanguis
Anti (sucrose grown)
S. salivarius
ELISA units Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Anti (glucose grown)
S. Mutans
Anti (glucose grown)
S. sanguis
Anti (glucose grown)
S. salivarius
ELISA UNITS (Abs 450/100 min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

TABLE 13

IgM Activity of Antisera to Sucrose and Glucose-Grown Cells of S. mutans, S. sanguis and S. salivarius Against Six Coating Antigens (Abs ^{450}/100 min).

Table 13

Coating Ag.	Absorbing cells grown on	Antisera to:- S. mutans grown on sucr.	S. mutans grown on gluc.	S. sanguis grown on sucr.	S. sanguis grown on gluc.	S. salivarius grown on sucr.	S. salivarius grown on gluc.
Dextran		0.315	0.690	0.075	0.550	0.350	0.570
	Sucrose	0	0.180	0.070	0.210	0.005	0.155
	Glucose	0.305	0.635	0.078	0.580	0.315	0.600
Glycogen		0.105	0.690	0.100	0.495	0.280	0.500
	Sucrose	0	0.325	0.185	0.290	0.165	0.275
	Glucose	0	0.295	0.160	0.290	0.205	0.460
Levan		0.090	0.500	0.230	0.450	0.460	0.435
	Sucrose	0	0.200	0.115	0.120	0.305	0.260
	Glucose	0	0.100	0.080	0.190	0.160	0.350
Mutans CAP		0.800	1.255	0.070	0.440	0.180	0.540
	Sucrose	0	0.365	0	0.015	0	0.050
	Glucose	0.450	1.105	0	0.340	0.050	0.555
Sanguis CAP		0.455	0.900	0.145	0.435	0.280	0.455
	Sucrose	0	0.260	0.045	0.075	0.030	0.130
	Glucose	0.070	0.390	0.015	0.275	0.215	0.480
Salivarius CAP		0.570	0.970	0	0	0	0.180
	Sucrose	0	0.120	0	0	0	0
	Glucose	0.345	0.910	0	0.140	0.025	0.360

Table 14.

Summary of Percentage Residual IgM Activities of Post Immune Sera after Absorption with homologous sucrose or Glucose grown cells.

Table 14

Coating Ag.	Absorption	Antisera to:- S. mutans grown on sucr.	S. mutans grown on gluc.	S. sanguis grown on sucr.	S. sanguis grown on gluc.	S. salivarius grown on sucr.	S. salivarius grown on gluc.
Dextran	None	100	100	100	100	100	100
	Suc. cells	0	26.1	93.3	38.2	1.4	27.2
	Gluc. cells	96.8	92.0	100+	100+	90.0	100+
Glycogen	None	100	100	100	100	100	100
	Suc. cells	0	47.1	100+	58.6	58.9	55.0
	Gluc. cells	0	42.7	100+	58.6	73.2	92.0
Levan	None	100	100	100	100	100	100
	Suc. cells	0	40.0	50.0	26.6	66.3	59.7
	Gluc. cells	0	20.0	34.8	42.2	34.8	80.4
mutans CAP	None	100	100	100	100	100	100
	Suc. cells	0	29.1	0	3.4	0	9.2
	Gluc. cells	56.2	88.0	0	77.3	27.7	100+
sanguis CAP	None	100	100	100	100	100	100
	Suc. cells	0	28.9	31.0	17.2	10.7	28.6
	Gluc. cells	15.4	43.3	10.3	63.2	76.8	100+
salivarius CAP	None	100	100	100	100	100	100
	Suc. cells	0	12.4	0	0	0	0
	Gluc. cells	60.5	93.8	0	100+	100+	100+

Table 15

Loss of IgM Activity (as % Absorbance) on Absorption with Sucrose and Glucose-Grown Cells

Table 15

Coating Ag.	Absorbing Cells Grown On	Antisera to:- S. mutans grown on sucr.	S. mutans grown on gluc.	S. sanguis grown on sucr.	S. sanguis grown on gluc.	S. salivarius grown on sucr.	S. salivarius grown on gluc.
Dextran	Sucrose	100	73.9	6.7	61.8	98.6	72.8
	Glucose	3.2	8.0	0	0	10.0	0
Glycogen	Sucrose	100	52.9	0	41.4	41.1	45.0
	Glucose	100	57.3	0	41.4	26.8	8.0
Levan	Sucrose	100	60.0	50.0	73.4	33.7	40.3
	Glucose	100	80.0	65.2	57.8	65.2	19.6
mutans CAP	Sucrose	100	70.9	100	96.4	100	90.8
	Glucose	43.8	12.0	100	22.7	72.3	0
sanguis CAP	Sucrose	100	71.1	69.0	82.8	89.3	71.4
	Glucose	84.6	56.7	89.7	36.8	13.2	0
saliv. CAP	Sucrose	100	87.6	100	100	100	100
	Glucose	39.5	6.2	100	0	0	0

Table 16

Sucrose Grown Cell Specific IgM Activities as a Percentage of Post-Immune Serum Activity

Table 16

Coating Ag.	Antisera to:- S. mutans grown on		S. sanguis grown on		S. salivarius grown on	
	sucrose	glucose	sucrose	glucose	sucrose	glucose
Dextran	96.8	65.9	6.7	61.8	88.6	72.8
Glycogen	0	0	0	0	14.3	37.0
Levan	0	0	0	0	0	20.7
mutans CAP	56.2	58.1	0	73.9	27.7	90.8
sanguis CAP	15.4	14.4	0	46.0	66.1	71.4
salivarius CAP	60.5	81.4	0	100	100	100

Table 17

Assay IgM Activities specific for Sucrose-Grown Cells and for Anti Sucrose Grown Cell Serum

Table 17

Coating Ag.	S. mutans	S. sanguis	S. salivarius
Dextran	30.9	0	15.8
Glycogen	0	0	0
Levan	0	0	0
mutans CAP	0	0	0
sanguis CAP	1.0	0	0
salivarius CAP	0	0	0

specific for sucrose-grown cells after correction for incomplete absorption, or cross-reactivity.

These values indicate that sera raised against sucrose-grown cells of S. mutans and S. salivarius contain IgM antibodies specific for dextran. S. mutans antisera also showed a small degree of cross-reaction with S. sanguis C.A.P.

It was noted, in many of the assays, using various plate-coating polysaccharide antigens, that the activities of sera raised against glucose-grown streptococci were relatively high, and in many cases exceeded those of sera raised against sucrose-grown cells. This would appear to contradict the proposal that the antibody activities measured were those of anti-glucans. It was considered that there may have been two possible explanations for this effect. Firstly, it was known that the extracted polysaccharides obtained from the three species of streptococci grown on sucrose, contained small but detectable quantities of rhamnose (Section 3.2.3), and it was proposed that this rhamnose originated from contamination of the extracted glucan with serotype specific cell wall carbohydrate antigens. It is likely, therefore, that the glucan samples used as plate-coating antigens contained traces of other cell-wall constituents. Sera raised against whole glucose or sucrose-grown cells would contain antibody populations specific for cell-surface antigenic determinants, and these would bind to contaminating cell-wall constituents in the plate-coating antigens during assay. It would be expected, if this were the

case, that sera raised against sucrose-grown cells would have lower anti-cell wall component specificity than sera raised against glucose-grown cells, because of the "masking" of some cell surface determinants by glucans at the surface of sucrose-grown cells. Where sera raised against glucose-grown cells had higher activities in the assays than sera raised against sucrose-grown cells, this was assumed to be the case. If these cell wall determinants were more potent immunogens than extracellular glucan, the response to these glucans may have been obscured to some extent during our assays. In all the assays performed it was noted that sera raised against glucose-grown cells had higher anti-glycogen activity than did sera raised against sucrose-grown cells. This finding can be interpreted in two ways. It may either be that glucose grown cells contain higher levels of glycogen-like material than do sucrose-grown cells, or that in the blood stream, fewer sucrose-grown cells are lysed to release their intracellular polysaccharides than glucose-grown cells.

Since it is known that species of oral streptococci synthesise similar amounts of intracellular iodophilic polysaccharides when grown on glucose and sucrose (DiPersio 1974; 1978), it may be that sucrose-grown cells are protected from in vivo lysis to some extent by their cell-associated glucans.

A second explanation for the unexpectedly high anti polysaccharide activity of sera raised against glucose-grown cells may be that such cells possess cell-surface components with some degree of immunochemical identity with the plate-coating antigens used during assay. The presence of dextran-

like components at the surface of glucose-grown cells would support this explanation.

It has been reported that glucose-grown cells of S. mutans synthesise cell surface glucan. These glucans were reported to act as receptors for glucosyltransferases which, in the presence of sucrose synthesise adhesive glucans (Hamada and Slade 1980b). It has also been reported that sera raised against glucose-grown cells of S. mutans possess low levels of anti dextran activity (Hamada et al 1983; Czerkinsky 1983). These studies utilised ELISA and RIA techniques respectively to determine the antibody response of mice and rabbits to glucose-grown S. mutans. They detected low levels of activity in these sera apparently directed against dextran, indicating the presence of dextran-like components at the surface of these organisms.

In the present study, it was noted that sera raised against glucose-grown cells of S. mutans, S. sanguis and S. salivarius all had relatively high activities against dextran. It was observed that this activity was significantly reduced by pre-absorption of sera with homologous sucrose-grown cells, but much less so by glucose-grown cells. This would appear to indicate two things, firstly that there is immunochemical similarity between the "dextran" present at the surface of glucose-grown cells and the glucan surrounding the absorbing sucrose-grown cells. Secondly, it would appear that the dextran present at the surface of glucose grown cells is a less efficient absorbent for its homologous antibodies than is the glucan synthesised by sucrose-grown cells.

Since the aim of the present study was an immunological investigation of the cell-associated glucans produced by the oral streptococci from sucrose, it was necessary to transform the data obtained to compensate for the sucrose-independent factors previously discussed. The stages in these transformations and the reasons for them were discussed in Section 3.3.3.

Table (18) represents a summary of these transformed data and illustrates the class and specificity of the antibody response of mice to the sucrose-derived cell-associated glucans of S. mutans, S. sanguis and S. salivarius.

The results obtained showed a clear difference in the class of antibody response between dextran-like determinants and those associated with extracted streptococcal glucans. It was apparent that sera raised against sucrose-grown cells of S. mutans and S. salivarius contained antibody populations of the IgM class with specificity for dextran-like determinants. Sera raised against sucrose grown cells of S. salivarius contained a population of IgG antibodies with activity specific for levan. As would have been expected there was no sucrose-specific antibody response directed towards glycogen, and this also reflects a lack of cross-reaction between dextran, levan and glycogen, indicating immunological non-identity. Interestingly, the sucrose-specific antibody responses of sera raised against all three species, showing specificity for extracted glucan preparations appeared to be IgG mediated. It was thought unlikely that the differences observed in antibody class between the responses to dextran and extracted glucans were due to seroconversion since all immunisations and all

Table 18

Summary of Specific Anti Sucrose Grown Cell Activities of Three Antisera, and Class of Antibody Response (as % Activity of unabsorbed Sera)

Table 18

		Antisera to Sucrose Grown:-		
		S. mutans	S. sanguis	S. salivarius
Dextran	IgG	0	0	0
	IgM	30.9	0	15.8
Glycogen	IgG	0	0	0
	IgM	0	0	0
Levan	IgG	0	0	13.6
	IgM	0	0	0
mutans CAP	IgG	11.0	0	0
	IgM	0	0	0
sanguis CAP	IgG	0	5.8	0
	IgM	1.0	0	0
salivarius CAP	IgG	0	2.1	2.7
	IgM	0	0	0

serum collections were made at the same times in all experiments. If all the activities measured were indeed glucan specific, then the differences in antibody class of response may have been a reflection of different antigenic determinants. If it is assumed that the dextran-like determinants were α1-6 glucosidic link specific, then the non-dextran like determinants eliciting a predominantly IgG response may correspond to other glucosidic linkage-types e.g. the α1-3 linked residues detected by methylation analysis of the streptococcal glucans. Unfortunately, sufficiently pure α1-3 linked polysaccharides were unavailable for this study, since their use as plate-coating antigens would have elucidated the nature of non-α1-6 glucosidic determinants present in the cell-associated polysaccharides of these organisms. Whatever the nature of these determinants it was shown that sera raised against sucrose-grown cells reacted specifically with their homologous antigen preparations, although there appeared to be some immunochemical similarity between the glucan samples derived from S. sanguis and S. salivarius.

In summary, several points can be made about the data obtained in this Section. Inoculation of mice with whole sucrose-grown cells of S. mutans and S. salivarius lead to the production of a population of antibodies, of the IgM class, with specificity for dextran-like determinants. Sera raised against sucrose-grown cells of S. salivarius produced, in addition to these antidextrans, a population of IgG antibodies with specificity for levan-like determinants. Sera raised against the three species of sucrose-grown streptococci reacted specifically via

an IgG mediated response to their homologous antigen preparations. This activity may have been directed against non dextran-like determinants on the glucan samples.

4, Further Work

The aims of the present work were threefold:- to quantify accurately the cell-associated polysaccharides produced from various sugars by the oral streptococci, to investigate these substances biochemically, and to characterise them immunologically. It was felt that such investigations would yield information on the immunochemistry of these clinically important organisms which could be of use in any immunological approach to the elucidation of the structure of their polysaccharides. Data obtained in the present work indicated the difficulties involved in the methylation analysis of streptococcal polysaccharides derived from whole bacterial cells, and emphasised the desirability of a more specific approach.

Immunological investigations of the polysaccharides derived from sucrose-grown cells of S. mutans, S. sanguis and S. salivarius indicated that antibody populations were raised in mice to specific glucosidic linkage determinants.

Before such antibody populations could be used effectively in the elucidation of polysaccharide structure, the problems associated with their polyvalence would have to be overcome. An obvious extension of the present work would therefore be the production of monoclonal antibodies with specificity for the different glucosidic linkage determinants involved in these polysaccharides. This would eliminate the problems encountered in the present project with the production, on immunisation with whole bacterial cells of large numbers of antibody populations with undefined specificity.

Further immunochemical studies could also elucidate the phenomenon of immunological "masking" of cell-wall determinants by cell-associated polysaccharides noted during agglutination studies. This reduction in

immunogenicity observed with sucrose-grown organisms may be relevant in the aetiology of streptococcal endocarditis.

5.

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