QUANTITATIVE STUDIES ON THE SECRETION OF BLOOD GROUP SUBSTANCES IN HUMAN SALIVA

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ROSS W. MILNE

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ABSTRACT

The quantitative hemagglutination method of Dybkjaer (1966) has been adapted to the determination of ABH blood group substances (BGS) in saliva.

Using M/15 phosphate buffered saline (PBS) as diluent, at least 5 dilutions of the saliva sample were made. Each dilution was then preincubated for about one hour with the appropriate antiserum before addition of a 1% red cell suspension of the same ABO group as the saliva. The mixtures were rotated for 20 hours (incubation). The free and agglutinated cells were separated by centrifugation against a 20% dextrose solution at 45 x g for 30 seconds. The upper layers were pipetted off and both free and agglutinated cells were washed with PBS. Drabkin's solution was added to each tube and the O.D. determined at 542 nm. The percent agglutination was calculated from the ratio of O.D. agglutinated to O.D. free plus O.D. agglutinated. The concentration of saliva resulting in 50% agglutination was determined by probit analysis. A parallel inhibition test on a solution of hog gastric mucosal BGS of known concentration was included as reference. Coefficients of variation between 2% and 6% were obtained with replicate assays performed on the same saliva sample.

The final equilibrium of free and agglutinated cells was not influenced by the duration of preincubation of antiserum and saliva (15 minutes to 4 hours). With no preincubation, the percent agglutination initially exceeded the equilibrium value and slowly

fell thereafter towards the equilibrium value. Higher hemagglutination-inhibition occurred when the ionic strength of the assay system was decreased.

Lower blood group activities were found in aliquots of submandibular saliva which had been boiled than in untreated aliquots. However, the boiled aliquots retained their activity well when stored frozen, whereas unboiled aliquots did not.

Ultra-filtration studies of submandibular saliva indicated the presence of low molecular weight substances showing blood group activity but in too low a concentration to affect the assay.

The effect of salivary flow rate and duration of sour lemon drop (SLD) stimulation on the concentration of blood group A substance in the submandibular saliva of four group A secretors was studied. Up to sixfold changes in BGS concentration were observed with prolonged SLD stimulation. No fatigue effect was noted over 15 minutes of stimulation and no marked flow rate effect was seen over the range of flow rates which were tested. Although there was individual variation in the pattern of response to stimulation and also differences when identical experiments were performed on the same subjects on different occasions, there was a maximum of a twofold variation in the concentration of BGS in unstimulated submandibular saliva collected from a given subject on different days.

In two subjects, there was a decrease in the ratio of A activity to H activity in submandibular saliva upon prolonged

stimulation. A third subject showed an increase in the A to H ratio, while the fourth subject showed little change.

The blood group A activities in various salivary secretions of ten group A and two group AB secretors were examined. Very high concentrations of BGS were found in the minor mucous gland secretions and in sublingual saliva. Lower concentrations occurred in whole saliva and submandibular saliva whereas very little A activity was detected in parotid saliva. Using reported values for the proportional contributions of the individual salivary secretions to the whole saliva volume, it can be calculated that the sublingual and minor mucous glands contribute about 70% of the BGS in whole saliva.

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Blood group antigens were first recognized as cell-bound structures at the beginning of the twentieth century. Somewhat later it was observed that the secretions of some individuals could also show blood group activity. Since this finding, many studies have been done on the quantitation, inheritance, structure and synthesis of secreted antigens.

Most studies on quantitation of the salivary blood group substances (BGS) have employed hemagglutination-inhibition as an assay procedure. The agglutination has usually been read visually. Although it is a sensitive qualitative test it is too subjective for an accurate quantitative assay. Therefore one of the objectives of this study was to develop a convenient, sensitive and reproducible quantitative technique for determination of salivary blood group substances.

It is known that many variables affect the composition of saliva. In order to compare results obtained by different workers, or results obtained within the same laboratory, it is necessary to define and control the collection conditions. In previous studies on the blood group activity of saliva, little attempt has been made to standardize the protocol for the saliva collection. If the blood group activity of saliva were found to be sensitive to changes in the saliva collection procedure, it could explain some of the variability which has been observed between individuals and within the same individual when tested on different occasions. With the quantitative hemagglutination-inhibition assay we have attempted to find the effect of some of these variables

upon the concentration of BGS in saliva.

Most quantitative studies on salivary BGS have involved the whole saliva. Based on the results of several of these studies it has been suggested that the amount of BGS secreted into the saliva is under genetic control. However, whole saliva is composed of the secretions from a number of different salivary glands as well as some non-salivary components. It is known that the proportional contributions by the individual secretions to the whole saliva volume show considerable person to person variability and can be influenced by the conditions of the saliva collection. Therefore, if differences existed between the different salivary secretions, any conclusions concerning the genetic control of BGS secretion based only on data from whole saliva would be very questionable. We have undertaken an examination of the individual secretions to determine their respective concentrations of BGS.

CHAPTER II

REVIEW OF THE LITERATURE

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I The ABO System

In 1900, Landsteiner first observed that some human sera would agglutinate the red blood cells of certain other individuals. On the basis of these reactions Landsteiner categorized individuals into one of three groups, I, II and III (now B, A, and O). A fourth group, IV (AB), was discovered in 1902 by Decastello and Sturli. The assignment of individuals into the four groups depended upon whether their red cells possessed either one, or the other, neither or both of two antigens, now designated A and B. The agglutination reactions were explained by a reciprocal relationship between the presence of these antigens on the red cells and the presence of corresponding antibodies in the serum; that is, a person's serum does not normally contain antibodies to those antigens present on his own red cells. This relationship is shown in Table II - 1.

It was soon recognized that the ABO blood groups were inherited (Epstein and Ottenberg, 1908; von Dungern and Hirszfeld, 1910) and the mode of inheritance was suggested by Bernstein (1924). The ABO type is determined by three independent genes, A, B and O, where A and B are dominant and O is recessive. One gene is present on each member of one of the chromosome pairs. When an A gene and a B

Table II - 1

The Relationship between the Presence of Isoantigens and Isoagglutinins in the ABO System

Blood Group	Antigens on Red Blood Cells	Antibodies in the Serum
O	-	anti-A anti-B
A	A	anti-B
B	B	anti-A
AB	AB	none

gene are inherited together, they are co-dominant.

(a) The A Antigen

In 1911 von Dungern and Hirszfeld postulated the existence of two subgroups, now called A_1 and A_2 within blood group A. They found that they could absorb anti-A serum with weakly reactive A cells (A_2) without removing the ability of the serum to agglutinate strongly reactive A cells (A_1). Other authors (Lattes and Cavazutti, 1924; Schiff and Hubener, 1926) claimed that the division into subgroups was unwarranted, as they suggested that there was a continuous progression from weakly to strongly reactive cells. However, Friedenreich and Worsaae (1929) showed conclusively that A_1 and A_2 cells fell into two distinct subgroups and they found no evidence of intermediate forms.

Ever since their discovery, there has been a controversy as to whether the differences between the subgroups are quantitative or qualitative. Lattes and Cavazutti felt that the differences in reactivities were due only to differences in the number of antigenic sites per cell. In contrast to von Dungern and Hirszfeld, they were able to show, by using sufficiently large numbers of A_2 cells, that all the antibodies could be absorbed out, including anti-A_1. Greenbury (1963) also showed that all the anti-A antibodies in the serum of rabbits which had been immunized with A_1 cells could be removed by exhaustive absorption with A_2 cells. They did feel, however, that a qualitative difference existed, which was too subtle for their

system to detect.

Mäkelä et al., in 1969, attempted to explain the lack of agglutination of A_2 cells by anti-A_1, without assuming a qualitative difference between A_1 and A_2 determinants. They suggested that anti-A_1 antibodies were of the multi-valent, IgM type and were of low affinity. For these low affinity antibodies to bind to red cells firmly enough to take part in bridging, the binding would have to involve more than one valence of the antibody molecule per cell. This in turn would require a high density of A receptors on the red cells, which they suggested that A_1 cells possessed. Antibodies of the bivalent, IgG type were of higher affinity and would agglutinate cells of both subgroups. In support of Mäkelä's hypothesis, Greenbury et al. (1963) and Economidou et al. (1967) had previously reported that A_1 cells have 4 times as many accessible A determinants as A_2 cells.

Watkins and Morgan (1956b) suggested that A_1 individuals are more efficient in converting precursor (H) material into the A product. This was consistent with the higher H activity shown by A_2 cells. Bar-Shany et al. (1970), who studied water soluble A_1 and A_2 substances, proposed that steric hinderance between A and H structures accounts for the difference in reactivities.

Dolichos biflorus extract, a plant lectin, preferentially agglutinates A_1 cells (Bird, 1952). However, when this reagent was tested in an Ochterlony system with A substance purified from ovarian cyst fluid of A_1 and A_2 secretors, a reaction of identity

between A_1 and A_2 substances was seen (Bird, 1959). The interpretation of the reaction was that the Dolichos biflorus detected a determinant common to the A substance of both subgroups. The author suggested that the lectin does not detect any qualitative difference between A_1 and A_2 antigens.

Other authors have presented evidence that the A_1 and A_2 cells differ qualitatively. Von Dungern and Hirszfeld (1911) postulated the existence of an A antigen common to both the A_1 cells and the A_2 cells and an A_1 antigen found only on A_1 cells. They further postulated two corresponding agglutinins; α (anti-A), reacting with the A antigen and therefore agglutinating both A_1 and A_2 cells and an α_1 (anti-A_1), which reacts with the A_1 determinant and would therefore agglutinate only A_1 bloods.

Landsteiner and Witt (1926) found that by several absorptions with A_2 cells and elutions they were able to separate anti-A serum into two fractions showing different proportional abilities to agglutinate A_1 and A_2 bloods. These two fractions were suggestive of α and α_1. Landsteiner and Levine (1926) showed that anti-A_1 could be present in the serum of certain A_2 and A_2B individuals. The inability of Friedenreich and Worsaae (1929) to demonstrate forms intermediate in strength between A_1 and A_2 is also indicative of a qualitative difference. Recent chemical and immunochemical studies present more direct evidence of A_1 and A_2 cells being qualitatively different. These will be discussed in detail in later sections. However, at present the true nature of the differences between the two subgroups is still in doubt.

Juel (1959) summarized the current knowledge of A_1 and A_2 when he concluded that A_1 cells will selectively bind α agglutinins before the α_1 fraction and that there was a very limited affinity between α_1 antibodies and A_2 cells.

In 1936 Friedenreich described a third subgroup within A which was designated A_3. This was thought to be controlled by a fifth allele at the ABO locus. In Canada, the phenotype appears with a frequency of about 4.8×10^{-5} (Reed, 1964). The reaction with anti-A serum is characterized by small agglutinates and many free cells. There is controversy as to whether anti-A is bound to the free cells (Reed, 1964; Cohen and Zuelzer, 1965). A_3 appears to be dominant with respect to the O allele but recessive to A_1, A_2 or B (Gammelgaard, 1947, cited in Reed, 1964).

There are other weaker forms of the A antigen which are very rare. Race and Sanger (1968) classified most of the weak variants into two main categories, A_x and A_m. The red cells of A_x individuals gave a weak or negative reaction with anti-A (B serum) but a good reaction with anti-A + B (O serum). The serum of A_x individuals usually contains anti-A_1 but not anti-A and, if the secretor gene is present, H, but not A is found in their saliva. A_m individuals have red cells which give a weak or negative reaction with anti-A or anti- (A + B). Neither anti-A nor anti-A_1 is present in their serum. If the A_m individual is a secretor the saliva shows both A and H activity. Most of these weak forms are inherited as dominant characters.

(b) The B Antigen

Friedenreich and With (1933) demonstrated that the human group B antigen shares specificities with red cells of other species. Besides having a component B_1, unique to human B cells, the antigen contains a component B_2, also found on rabbit cells, and a component, B_3, common to both rabbit and guinea pig red cells. Owen (1954) showed some cross-reaction between opposum red cells and anti-B serum which indicated a possible fourth component, shared by human B cells and opposum red blood cells.

The quantity of literature on varients of the B antigen is much less than that for the A antigen. These weak forms of B may or may not have anti-B in the serum or B substance in secretions. They, like A varients, appear to be inherited as dominant characters.

Salmon et al. (1964) demonstrated three weak type B antigens with the aid of quantitative hemagglutination and thermodynamic techniques. These were named B_{80}, B_{60} and B_0, indicative of the degree of agglutination with respect to normal B (B_{100}) cells. Their results suggested that differences existed in the number of antigenic sites per cell. Enthalpy measurements also indicated a possible qualitative difference between the varients. $A_{1(80)}$, a varient of the A antigen, analogous to B_{80} was demonstrated in the same manner (Salmon et al., 1965).

(c) Blood Group O Cells

The original identification of group O cells was by their lack of agglutination in the presence of anti-A or anti-B serum. In 1927 Schiff demonstrated that certain normal cattle sera, after absorption with human AB cells, would preferentially agglutinate human group O cells. Landsteiner and Witt (1926) had previously described "irregular" agglutinins (α_2) in the sera of some normal individuals which would agglutinate O and A_2 cells. The "anti-O" of Schiff and the α_2 of Landsteiner and Witt were later shown to give identical reactions (Friedenreich and Zacho, 1931). Eisler in 1931 demonstrated that a similar serum could be obtained from goats immunized with Shigella dysenteriae.

The reactions of these sera were not, however, limited to group O cells. Strong reactions were achieved with A_2 cells and weaker reactions with A_1 and B cells. Thomsen (1932) concluded that these anti-O sera were reacting with the product of the O gene and that the reactions with the cells of other groups could be explained by heterozygosity.

Boorman et al. (1948) described a serum which reacted with O, A_2 and A_2B cells but failed to agglutinate A or B cells. This type of serum was not inhibited by secretions of group O secretors (Morgan and Watkins, 1948). On this basis they differentiated sera into two categories; those which are inhibitable by secretions, anti-H, and those which are not, anti-O. They

stated that anti-O serum was reacting with the product of the O gene and that anti-H was reacting with a precursor substance common to all groups. Sanger (1952) showed that individuals possessing anti-O antibodies were Le(a-) whereas those having anti-H antibodies were Le(a+). She suggested that, as the Lewis system was related to the secretor system, it may be the presence or absence of the secretor gene which determines the type of antibody in the serum.

Race and Sanger (1962) stated that several of the sera reported to be anti-O are in fact directed against the I antigen. Gold (1964) showed that some anti-O antibodies required the co-presence of both the O and, either the I or i antigen, thus indicating a structural relationship between the ABO and I blood group systems. This has been confirmed in subsequent immunochemical studies which will be discussed in a later section.

The relationship of anti-H to anti-O, and, likewise, the O and H antigens, is still not clear. Wiener et al. (1966) postulated a structural difference between the H substance found in secretions and that found on the red cells. Ceppellini (1959) accounted for the different reactions of the anti-H and anti-O sera by normal heterogeneity of anti-H antibodies. He also concluded that no serum then available appeared to react specifically with the product of the O gene. This still appears to be true.

(d) The Bombay Phenotype

In 1952 Bhende et al. described three Indian individuals whose cells failed to agglutinate when mixed with anti-A, anti-B or

anti-H serum. Their serum contained anti-A, anti-B and anti-H. They were Le(a+) and their secretions showed no ABH activity. This was designated the Bombay Phenotype.

Levine et al. (1955) described a similar patient whose cells were not agglutinated by anti-H, anti-A or anti-B and who had anti-H in her serum. From a three generation family tree it was evident that she must possess a blood group B allele. The authors postulated that the lack of expression of the B gene was due to a rare suppressor gene, X (now h), which in the homozygous state suppresses both the ABO phenotype and ABH secretion. Lanset et al. (1966) were able to demonstrate the presence of the suppressed antigen on the cells of an individual having the Bombay phenotype by fixation-elution tests. It therefore followed that the antigen was present in some form.

II The Secretor System

In 1924 Schiff demonstrated the presence of BGS in serum by specific precipitation (cited in Race and Sanger, 1968). Moss (1910) had previously described isohemolysin inhibition by serum; however, he postulated that the phenomenon was due to anti-antibodies.

Yamakami (1926) showed a substance to be present in human semen and saliva of A, B and AB individuals which had the ability of inhibiting the agglutination of red cells by isoagglutinins. The specificity of the substance in the individual's secretions was the same as that found on the red cells. Group O saliva was shown to

act similarly in a system of Group O cells and cattle anti-O serum (Schiff and Sasaki, 1932).

Lehrs (1930), who studied human saliva, noted a wide variability between individuals in the quantity of group-specific substances. It was observed in the same year that the saliva and other secretions of some people showed no inhibitory activity. Putkonen, 1930; Schiff and Sasaki, 1932, demonstrated conclusively in a study involving 50 families that the ability to secrete blood group substances was inherited as a Mendelian dominant (Se). Non-secretors were individuals homozygous for the recessive allele (se). Friedenreich and Hartmann (1938) suggested that the water-soluble blood group substances in secretions originate in the glandular cells, rather than being excreted as degradation products of the alcohol-soluble red cell antigen. This work was extended and a dissociation between water-soluble and alcohol-soluble activity was noted (Hartmann, 1941). Whereas water-soluble blood group activity was dependent upon the presence of the Se gene, the alcohol-soluble activity could be demonstrated regardless of secretor status.

III The Lewis System

Ueyama, in 1939, described an antibody (anti-T) in the serum of some normal chickens, which reacted with an antigen (T) found in the saliva of non-secretors only. However, due to the Second World War, as well as to language difficulties, this work was largely overlooked in the West.

In 1946, Mourant demonstrated a "new" antibody in the serum of a Mrs. Lewis which agglutinated about 25% of English bloods. He proposed the name Lewis (now called Le(a)) for the corresponding red cell antigen and showed it to be an inherited character. Grubb (1948) demonstrated that the saliva of most non-secretors could inhibit anti-Le(a) serum. This system is now recognized to be the same as the T-anti-T system of Ueyama.

Andresen (1948) detected an antibody which reacted with an antigen found on the red cells of about 70% of A_2 and O individuals. This antigen, now called Le(b), could not be demonstrated to be co-present with the Le(a) antigen.

In 1948 Grubb made the important observation that individuals possessing the Le(a) antigen on their red cells were also non-secretors of ABH material. Based on this and subsequent observations he proposed, in 1951, a theory for the Lewis system. Grubb envisioned a serological group system of water-soluble mucoids rather than a true blood group system. According to the theory, the presence of the Le(a) antigen is controlled by a pair of alleles; Le, le. The Le(b) antigen was the direct product of the dominant Se gene at the secretor locus. The secretor and Lewis loci were considered to be independent.

Ceppellini and Siniscalco (1955) confirmed the independence of the secretor and Lewis loci. They modified Grubb's theory by proposing that the Le(b) antigen was the product of an interaction of the dominant Se gene and the dominant Le gene (Ceppellini, 1955 and Ceppellini and Siniscalco, 1955). This discrepancy was

apparently due to the less specific anti-Le(b) used by Grubb. The modified theory has now been largely substantiated by biochemical studies.

Sneath and Sneath (1955) transfused cells from an Le(a+b-) donor into an Le(a-b+) recipient. When a post-transfusion sample of blood was separated into donor and recipient cells by differential Rh agglutination and examined for Le antigens, it was found that the donor cells had become Le(a+b+). "In vitro" incubations of cells and plasma gave similar results. The authors suggested that red cells absorb Lewis antigens from the serum. Makela and Makela (1956) demonstrated that the ability to transform Le(b-) into Le(b+) was a property of the plasma rather than the cells. Marcus and Cass (1969) showed that the plasma substance was a glycolipid rather than a glycoprotein such as is found in secretions.

The Lewis antigens are not detectable on the red cells at birth, when tested by agglutination with anti-Lewis serum (Andresen, 1948), although they can be detected in some cord blood by the more sensitive antiglobulin reaction (Cutbrush et al., 1956). The Le(a) antigen becomes apparent on cells during the first post-natal weeks, and during the first months has a frequency of about 90% (Jordel, 1956). The frequency decreases to the adult level of about 22% by about two years. There is a lower frequency of Le(b) antigen in individuals younger than 6 years of age than that in the adult. The frequency of the Le(b) antigen increases concomitant with the decrease in the frequency of Le(a). A transient Le(a+b+)

state is commonly observed in infants. Despite their absence on the red cells, the Lewis antigens are present in saliva at birth.

Andresen and Jordel (1949) described an antibody now known as anti-Le(x) which agglutinated Le(a+b-) and Le(a-b+) cells but failed to agglutinate Le(a-b-) cells. It had the unique property of agglutinating about 90% of cord blood which indicated that anti-Le(x) was not simply a mixture of anti-Le(a) and anti-Le(b). Andresen (1961) proposed a theory for the inheritence of the Lewis genes to include Le(x), but the theory has little current acceptance. Sturgeon and Arcilla (1970) have since modified the theory of Grubb and Ceppellini to account for Le(x). They suggested that the Le gene has two cistrons for which one product is Le(a) and the other Le(x). Under the influence of the secretor gene, the Le(a) substance is converted to Le(b) whereas the Le(x) substance remains unchanged.

An antibody, anti-Le(c), has recently been demonstrated which reacts with Le(a-b-) cells of non-secretors (Gunson and Latham, 1972). The corresponding antigen is present in saliva from Le(a-b-) non-secretors and to a lesser extent in the saliva of Le(a+b-) non-secretors and Le(a-b-) secretors.

IV Blood Group I

In 1956 Wiener et al. described a cold agglutinin in the serum of a patient suffering from acquired hemolytic anemia. On testing 22,000 blood samples with this serum, they found only 5 which were non-reactive. They suggested that the antibody was

directed against a very common antigen which they designated I. Bloods showing little of the I antigen were designated i. Titration experiments of group I bloods indicated that the strength of the antigen was normally distributed.

Jenkins et al. (1960) found anti-I in the serum of a healthy group i subject. Marsh (1961) demonstrated the presence of anti-i in the sera of two patients. Using this serum he was able to show that all individuals possess both I and i (reciprocally related) on their red cells. The anti-i was found to react strongly with cord cells. Marsh suggested that the lack of the I antigen in the adult involved a genetically controlled factor essential for the proper development of I. Without this factor, the cells maintain a weak I and a strong i, similar to the foetus.

Dzierzkowa-Borodej et al. (1970) identified a substance in saliva which specifically inhibited the agglutination of I red cells by a cold anti-I autoagglutinin. I substance appeared in the saliva of all individuals independent of age and the Ii status of the red cells. A similar substance was demonstrated in milk but in higher concentration (Marsh et al., 1970). With an Ochterlony system Feizi and Marsh (1970) found a reaction of identity between an OI erythrocyte extract and a milk-extract, which indicated that the two shared at least one common determinant. Marsh et al. (1971) examined a number of different anti-I sera and suggested that the I antigen consisted of two components. One component, which they designated I_{foetal} would be present on all human red cells including adult i cells and cord cells. The second

component $I_{developed}$ would be found on adult I cells and would develop in a gradual, transitional process.

V Origins of Anti-A and Anti-B Isoagglutinins

Two opposing theories have been put forward to explain the presence of anti-A and anti-B isoagglutinins in serum. Isoagglutinins were originally thought to be inherited, natural antibodies whose presence was independent of influences of the environment (Bernstein, 1925; Furuhata, 1927).

Dupont (1934) proposed that these antibodies were produced in response to environmental antigens with determinants which cross-react with blood group antigens. Springer (1956, 1966, 1970) has demonstrated that many bacteria, viruses and higher plants show blood group specificity.

Much support for the acquired origin of anti-A and anti-B has come from the experiments of Springer on germ-free chickens (Springer et al., 1959). By the age of about 20 days, chickens usually have anti-human B antibodies in their serum (Bailey, 1923). Springer et al. (1959) found that chickens raised under germ-free conditions lacked these anti-B antibodies at 45 days after hatching, whereas chickens raised under normal conditions had the usual anti-B titres. Anti-B agglutinins were produced by the germ-free chickens after they were fed group B-active meconium or blood group B-active bacteria (E. coli O_{86}).

VI Lectins

In 1888 Stillmark found that extracts of the castor bean (Ricinus communis) contained hemagglutinins. Landsteiner (1945) found that plant agglutinins from different sources did not always agglutinate the erythrocytes of different species equally. Renkonen (1948) and Boyd and Reguera (1949) demonstrated that some plant agglutinins were specific for some human blood group antigens. For those which showed this specificity, the collective name "lectin" was proposed (Boyd and Shapleigh, 1954).

Renkonen (1949) tested 57 species of plants for their ability to agglutinate human red cells. Amongst those tested he found extracts of Vicia cracca to be specific for group A cells, and extracts of Laburnum alpinum, Cytisus sessilifolius and Lotus tetragonolobus to be specific for the H antigen. Boyd and Reguera, who reported results on saline extracts of 262 varieties of plants, found 25 which showed specificity towards blood groups. Extracts from several varieties of lima beans agglutinated only group A cells.

Bird (1952) found that an A agglutinin of Dolichos biflorus was 500 times more potent as an anti-A_1 reagent than as an anti-A_2 reagent. This reagent is now used commonly as a means to distinguish A_1 from A_2 bloods.

Cazal and Lalaurie (1952) demonstrated anti-H activity in extracts of Ulex europaeus. Boyd and Shapleigh (1954) showed the usefulness of this and other anti-H lectins in the determination of secretor status. Plato and Gershowitz (1961) compared inhibition

titres of anti-H from Ulex europaeus and Cytisus sessilifolius extracts. They found that A and O salivas had higher titres when tested with Ulex extracts than with Cytisus extracts. The opposite was found to be the case for B and AB saliva. Bhatia and Randeria, (1970) who studied the phenomenon of abberant secretion, obtained different results when different anti-H reagents were used. These included human and animal anti-H as well as plant lectins. Differences in the inhibition reactions were also seen between the anti-A lectins Dolichos biflorus and Phaseolus lunatus (Hakim and Bhatia, 1956). Matsubara and Boyd (1963) found that after they covalently coupled p aminophenyl-N-acetyl-D-galactosaminide to an A-specific lima bean lectin (Phaseolus lunatus), the ability to agglutinate group B cells was increased. D-galactose specifically inhibited the agglutination of B cells while not affecting anti-A activity. N-acetyl galactosamine inhibited both A and B agglutination. The authors suggested that the carbohydrate moiety of lectins and possibly antibodies may play a role in their specificity. However, Rigas and Osgoode (1955) showed that removal of the carbohydrate moiety of Phaseolus vulgaris left the haemgglutinating activity intact.

The function of these molecules in the plant is unclear. There is no evidence to indicate that they serve a purpose analogous to animal antibodies. Boyd (1970) has suggested that they may play a role as a carbohydrate catcher in the seed or assist in transportation of carbohydrate to the seed.

VII Blood Group Frequencies

In 1919 Hirszfeld and Hirszfeld found differences in the ABO phenotype frequencies amongst human populations of different races. The large number of studies made subsequent to this finding were tabulated by Mourant in 1958. This discovery and the differential racial distribution of other genetic markers provided an extremely useful tool for anthropologists (see review by Pollitzer, 1970).

Cavalli-Sforza et al. (1968) tabulated gene frequencies for Caucasians to be: A, 0.236; A_2, 0.068; B, 0.066; O, 0.630. All the North American Indians included in the data appeared to possess only the O allele and no Australian Aborigines possessed the A_2 allele. Renwick (1969) reported a frequency of 0.75 for the Le gene and 0.52 for the Se gene amongst Europeans. ABO, Rh foetal-maternal incompatibility, differential sperm counts between ABO groups (Grieve et al., 1967), prezygotic selection in heterozygotes (Matsunaga and Hiraizumi, 1962) and differential susceptibility to disease have been proposed at various times to be factors responsible for the maintenance of the characteristic frequencies.

VIII Linkages and Associations

In 1951 Mohr reported an apparent linkage between the loci controlling the Lewis and Lutheran blood group characters. It was later shown that the actual linkage was between the secretor and

Lutheran genes (Sanger and Race, 1958). The frequency of recombination was found to be about 0.15 (Greenwalt, 1961) and appeared to be higher in females than in males (Cook, 1965). Several other autosomal linkages involving blood groups have since been discovered: ABO and nail-patella syndrome (Renwick and Lawler, 1955); Duffy and a congenital cataract loci (Renwick and Lawler, 1963); ABO and adenylate kinase loci (Rapley et al., 1967).

As well, a number of associations between blood groups and other characters and diseases have been found in man. The best documented and most easily rationalized of these are relationships between hemolytic disease of the new born and incompatibilities of Rh or ABO blood types between mother and foetus.

Another well-accepted association is that involving ABO blood groups, the secretor gene and serum alkaline phosphatase. The electrophoretic patterns of the sera of some individuals show only one zone of alkaline phosphatase activity (Zone A) while others also show a more slowly migrating band (Zone B) (Beckman, 1968). The alkaline phosphatase of zone B appears to originate in the intestine and the faster zone A from the liver and/or bone (Cunningham and Reimer, 1963). Several other bands with alkaline phosphatase activity are observed during late pregnancy (Boyer, 1961). Arfors et al. (1963a) found that the frequency of individuals of blood group A was very low amongst those who showed both zone A and zone B activities. The negative correlation between intestinal alkaline phosphatase and blood group A was confirmed in subsequent

studies (Beckman, 1964; Shreffler, 1965; Bamford et al., 1965). The intestinal alkaline phosphatase appeared to be present more frequently in the sera of individuals of blood group AB than in the sera of those of group A and also more often in the sera of A_2 and A_2B individuals than in A_1 and A_1B individuals, respectively (Beckman, 1968).

Arfors et al. (1963b) found an absence of individuals of the phenotype Le(a+b-) amongst those who possessed both zone A and zone B alkaline phosphatase activities. This was shown to be an association between intestinal alkaline phosphatase activity and the secretor gene (Beckman, 1964). This was confirmed in subsequent studies (Bamford et al., 1965; Shreffler, 1965). No mechanism for the associations has been generally accepted; however, it is unlikely that the ABO or secretor status affects synthesis of intestinal alkaline phosphatase, as no association was observed between blood groups and the alkaline phosphatase of the intestinal mucosa.

Hope and Mayo (1969) found no association between ABO blood groups and the alkaline phosphatase activity in whole saliva. The results which they obtained with whole saliva may have included alkaline phosphatase activity from the bacterial flora. Higher alkaline phosphatase activities were found in whole saliva than in submandibular or parotid salivas (Cornish and Posen, 1968) which may have reflected a bacterial contribution. Bacterial activity would tend to mask any existing relationships.

Hope et al. (1968) found higher concentrations of protein in the whole saliva of secretors than in that of non-secretors. Higher protein concentrations were also observed in the saliva of

A_2 individuals than in the saliva of individuals of other blood groups. Waissbluth and Langman (1971) found no variation in the serum protein concentrations among subjects of different blood groups.

In 1953, Aird et al. found a slight association between blood group A and cancer of the stomach. Following this report, a large number of papers have appeared concerning associations between blood groups and various diseases. This work has recently been reviewed by Vogel (1970). Many of these studies have been criticized by Wiener (1970) for the methods used in the collection and treatment of data. Many studies do, however, indicate that associations exist between various neoplasias and blood group A and between gastro-duodenal ulcers and group O (Vogel, 1970). The mechanism for the associations between non-infectious diseases and blood groups is not yet known.

A rationale for an association between blood groups and some infectious diseases does exist. Springer (1956, 1966, 1970) has shown that bacteria, viruses and higher plants can possess blood group specificities. It would seem reasonable that an individual of a given blood group would be more susceptible to infection by an organism which had the same specificity, as no antibodies to the determinant would be present or elicited (Vogel, 1970). The studies so far have been inconclusive. Different strains of the same organism differ from each other antigenically and therefore, individual strains may not show blood group specificity (Springer, 1966). Wiener (1970) suggested that in many types of infection one must deal with a heterogeneous series of cases and that significant

data on associations would tend to be diluted out with nonsignificant data. Wiener (1970) further suggested that organisms may possess many specificities, among which the blood group specificities may not be the most important.

IX Distribution of Blood Group Substances in the Body

The independence of the alcohol-soluble form of the ABO antigens from the effect of the secretor gene was noted by Hartmann in 1941.

Szulman (1960), who studied the distribution of blood group substances A and B in autopsy and surgical specimens by immunofluorescence, found staining in the endothelia of vessels and in stratified epithelium regardless of secretor status. The staining could be abolished by prior exposure of the tissue to alcohol. The mucous-secreting cells located in the salivary glands, gastro-intestinal tract, the larynx, trachea and bronchus, the gall bladder, the uterine cervix and the pseudomucinous ovarian cysts contained antigen only when the secretor gene was present. The relative staining of the respective salivary glands depended upon the percentage of cells which were mucous secreting. There was no mucous-bound antigen in non-secretor specimens, with the exception of the deeper parts of the gastric foveolae, in deeper parts of crypts of Lieberkühn of bowel mucosa, and in Brunner's gland of the duodenum. Antigen was present in the breast, prostate, and endometrial glands in secretor specimens and in the pancreas and sweat glands of all

specimens. Similar patterns of distribution were found in the three foetuses studied (4 to 7 months gestation). The distribution of H substance paralleled that of the A and B substances in O, A, B and AB tissues (Szulman, 1962).

In secretors, Hartman (1941) found that in the various secretions which he studied, gastric juice had the highest blood group activity and this activity decreased through saliva, bile, semen and urine respectively. The concentration of blood group substance in the various salivary secretions was found to be highest in sublingual saliva, less in submandibular saliva, and extremely low in parotid saliva (Wolf and Taylor, 1964). This appeared to correlate with the relative percentages of mucous-secreting cells in the glands.

X Quantitative Studies of Blood Group Substances in Saliva

It has long been recognized that there is considerable variation amongst secretor individuals in the quantity of ABH substance in the saliva (Lehr 1930; Putkonen, 1930). Changes have also been observed within the same individual when samples are taken at different times (Lehr, 1930; Putkonen, 1930).

Clarke et al. (1960), in a study which involved 151 sib pairs, found a positive correlation between sibs and propositi in the concentration of salivary blood group substances. They suggested that the amount of A, B, or H material secreted by an individual was, in part, inherited and that this control appeared to be polygenic.

Conflicting reports are present in the literature concerning

the concentration of A substance in the salivas of A_1 and A_2 secretors. Wiener and Kosofsky (1942) who used "boosted" human anti-A, and Baer et al. (1961) who used immune chicken anti-A, found no significant difference in the saliva of the two subgroups. However, with normal human anti-A (Gammelgaard, 1942 and Boettcher, 1964) or with extract from the seeds of Dolichos biflorus (Boettcher, 1967a and b), the inhibition titres were significantly higher with A_1 than with A_2 salivas.

H substance is present in the saliva of secretors of all blood groups and its concentration is highest in group O secretors, decreasing through A_2, A_1, B and AB respectively (Plato and Gershowitz, 1961).

McNeil et al. (1957a and b) described secretors of groups A and B who had A and B, but no detectable H in their salivas and other secretors, of blood group A or B, with H, but no salivary A or B. The authors referred to these individuals as abberant secretors and suggested that there was an association between this phenomenon and that of spontaneous abortion. Subsequent studies have indicated that abberant secretors appear to be at the extreme ends of a unimodal distribution of A:H or B:H ratios (Clarke et al., 1960; Bhatia and Randeria, 1970). Bhatia and Randeria (1970) found that identification of abberant secretors depended upon the type of anti-H used. They suggested that these individuals may secrete only a part of the ABH complex and may be missing only that part of the complex which reacts with the antiserum which was being tested. An alternative explanation is that the rates of formation of the

products of the H and ABO loci are independent and that abberant secretors are individuals in whom these rates are greatly different (Boettcher, 1967a; Bhatia and Randeria, 1970).

XI Structural Studies

(a) Water Soluble Blood Group Substances

Values for the molecular weights of blood group substances, which vary from 2×10^5 to 5×10^6, have been reported (Fiori et al., 1971a). The BGS was heterodisperse by both gel filtration (Fiori et al., 1971a) and density gradient centrifugation (Creeth and Denborough, 1970). Fiori et al. (1971a) suggested that the main fraction of salivary BGS has a molecular weight within the range of 2×10^5 to 5×10^6. In their gel filtration studies these authors also detected a second peak which showed activity that eluted in a fraction consistent with a molecular weight of slightly greater than 10,000. It was not seen in the saliva of all individuals and its presence did not correlate with secretor status. Dializable activity was also found in the saliva of some individuals, again independent of secretor status. These were probably oligosaccharides similar to those previously demonstrated in milk (Grollman and Ginsberg, 1967) and urine (Lundblad, 1967).

Blood group substances are composed of about 80% carbohydrate and 20% amino acids (Marcus, 1969). Regardless of specificity, the four sugars D-galactose, L fucose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine are present. Varying

amounts of sialic acid are present, but do not appear to take part in the ABH or Le specificities. The amino acid composition of the peptide backbone has been shown to be independent of the specificity (Pusztai and Morgan, 1963). Serine and threonine constitute about 40% of the total amino acids present.

The first evidence that immunodominant sugars were involved in the specificity of blood group substances came with the finding that agglutination of human group O cells by an eel, anti-H could be inhibited by the simple sugar α-fucopyranoside (Watkins and Morgan, 1952). Agglutinins from other sources, including human anti-H were not inhibited by fucose which indicated that a more complex determinant was involved. Similarly, N-acetyl galactosamine was shown to inhibit the reaction of A cells with the plant agglutinin Vicia cracca (Morgan and Watkins, 1953). The disaccharide Oα-N-acetyl-D-galactosaminoyl-(1→3) galactose, obtained as a hydrolysis product of human A substance was effective in decreasing agglutination by human anti-A (Côté and Morgan, 1956). Kabat and Leskowitz (1955) obtained similar results in a precipitation-inhibition system and further showed that α-D-galactoside decreased the precipitation of B substance by human anti-B. The same sugars were also shown to interfere specifically with the action of enzymes capable of destroying blood group activity. This presumably is an example of product inhibition (Watkins and Morgan, 1955).

The methods of agglutination and precipitation-inhibition were extended by employment of well-defined oligosaccharides. A pentaose which contained the moiety (a) inhibited agglutination by anti-Le(a) serum.

(a) O-α-L-fucosyl
|
(1-4)
Oβ D galactosyl-(1-3)-N-acetyl-D-glucosamine

Maximum inhibition in an Le(b) system was obtained if an oligosaccharide was included having the Le(a) structure with an additional fucose residue joined by an α 1-2 linkage to the galactose (Watkins and Morgan, 1957; Watkins and Morgan, 1962). Horse antiserum directed against pneumonococcus type XIV polysaccharide cross-reacts with human blood group substances. Precipitation in this system was inhibited strongly by the disaccharide O-β-D-Galactosyl-(1-3)N-acetyl glucosamine (Watkins and Morgan, 1956a).

Iseki and Masaki (1953) found that treatment of blood group A substance with a bacterial A destroying enzyme, resulted in an increase in H activity concomitant with the loss of A character. Similar results were observed with B substance (Gibbons and Morgan, 1954). Exposure of ovarian cyst H substance to an H decomposing enzyme resulted in an unmasking of Le(a) activity (Watkins, 1962). The reactivity with anti-pneumonococcal type XIV polysaccharide generally increased in all these enzymatic procedures (Watkins, 1956).

More direct evidence for the structure of blood group substances has been obtained by chemical degradation procedures. Partial acid hydrolysis of blood group B substance yielded two serologically active trisaccharides (b and c) and one active disaccharide (d) (Painter et al., 1962, 1963).

(b) O-α-D-galactosyl-(1-3)-Oβ-D-galactosyl-(1-3)-N acetyl-D-glucosamine

(d) O-α-D-galactosyl-(1-3)-O-β-D galactosyl-(1-4)-N acetyl-D-glucosamine

(d) O-α-D-galactosyl-(1-3) galactose

The corresponding active fragments were found as hydrolysis products of A substance with N-acetyl-D-galactosamine substituted for the galactose at the non-reducing end (Cheese et al., 1961; Côté and Morgan, 1956). Numerous non-active oligosaccharides were identified, many of which were common to both A and B substance. The fragments which contained a β 1-3 linkage between the penultimate galactose and the N-acetyl-D glucosamine were designated as type I chains and those with the β 1-4 linkage as type II chains (Rege et al., 1963). Several fragments which possessed fucose were obtained despite its acid-labile linkage.

Rege et al. (1964a), who used an alkali degradation procedure, obtained an H active trisaccharide (e) from H substance.

(e) O-α-fucose
|
(1-2)
O-β-D-galactosyl-(1-4)-N acetyl-D-glucosamine

Alkali degradation of A and B substances yielded several active oligosaccharides (f and g) (Painter et al., 1965; Lloyd and Kabat, 1967). Fragments of the type I chain were also obtained.

(f) O-α-fucose
|
(1-2)
O-α-(N-acetyl)-D-galactosaminyl-O-β-D-galactosyl-(1-4)-N-acetyl-D-glucosamine

(g) O-α-L fucose

(1-2)

O-α-D galactosyl-(1-3)-O-β-D-galactosyl-(1-4)-N-acetyl-D glucosamine

An Le(a) active oligosaccharide (h) was obtained by alkali degradation of Le(a) substance (Rege et al., 1964b). The corresponding type II structure was inactive.

(h) O-α-L fucose

(1-4)

O-β-D galactosyl-(1-3)-N-acetyl-D-glucosamine

From Le(b) substance the active fragment (i) was obtained (Marr et al., 1967). Again the corresponding type II structure was inactive.

(i) O-α-L fucose O-α-L fucosyl

(1-2) (1-4)

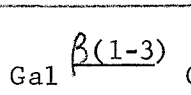
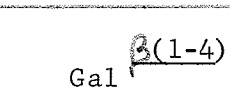
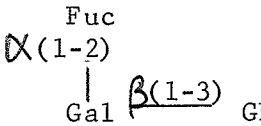
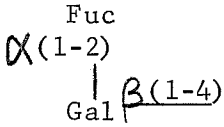
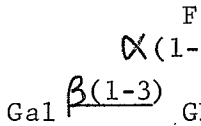
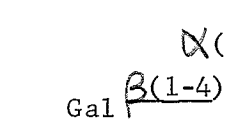
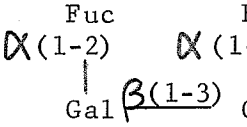
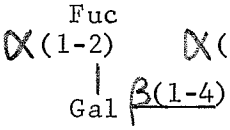
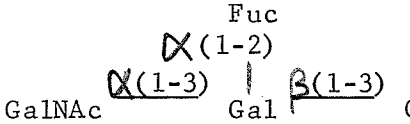
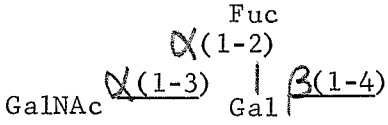
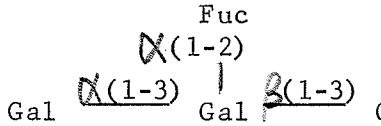
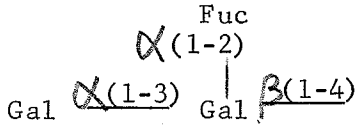
O-β-D galactosyl-(1-3)-N acetyl-D-glucosamine

Table II - 2 summarizes our current concepts of the structures involved in the specificities at the non-reducing ends of the carbohydrate portion of blood group substances.

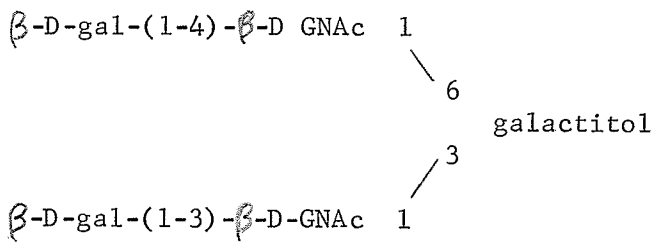
Lloyd and Kabat (1968a and b) isolated a branched oligosaccharide (j). They concluded that the carbohydrate chain had a branched structure, with one branch being a type I chain and the other a type II. Moreno et al. (1971) suggested that the A_2 substance lacked the terminal N-acetyl-D-galactosamine on the type I structure whereas the A_1 substance could have both complete chains.

Table II - 2

The Serologically-Active Non-reducing End Groups
of Certain Blood Group Substances

Type 1 Chain	Specificity	Type 2 Chain	Specificity
	-		Type 14
	H		H
	Le^a		-
	Le^b		-
	A		A
	B		B

(j)



Feizi et al. (1971 a and b), by periodate oxidation, showed that the I specificity is concealed interior to the A, B, H, Le(a) and Le(b). One anti-I serum which was tested was inhibited by the disaccharide moiety β-D-gal-(1 4)-β-D-GNAc-(1-6). However, as other anti-I sera were not inhibited, it would indicate that the I specificity is somewhat heterogeneous.

Information gained from alkaline degradation indicated that the carbohydrate is attached to the protein backbone by a glycosidic linkage between N-acetyl galactosamine and either serine or threonine (Kabat et al., 1965; Donald et al., 1969). Oligosaccharides isolated from this linkage region (Lloyd et al., 1968b; Aston et al., 1968) enabled Lloyd et al. (1968 a and b) to suggest a composite structure of the carbohydrate chain (k).

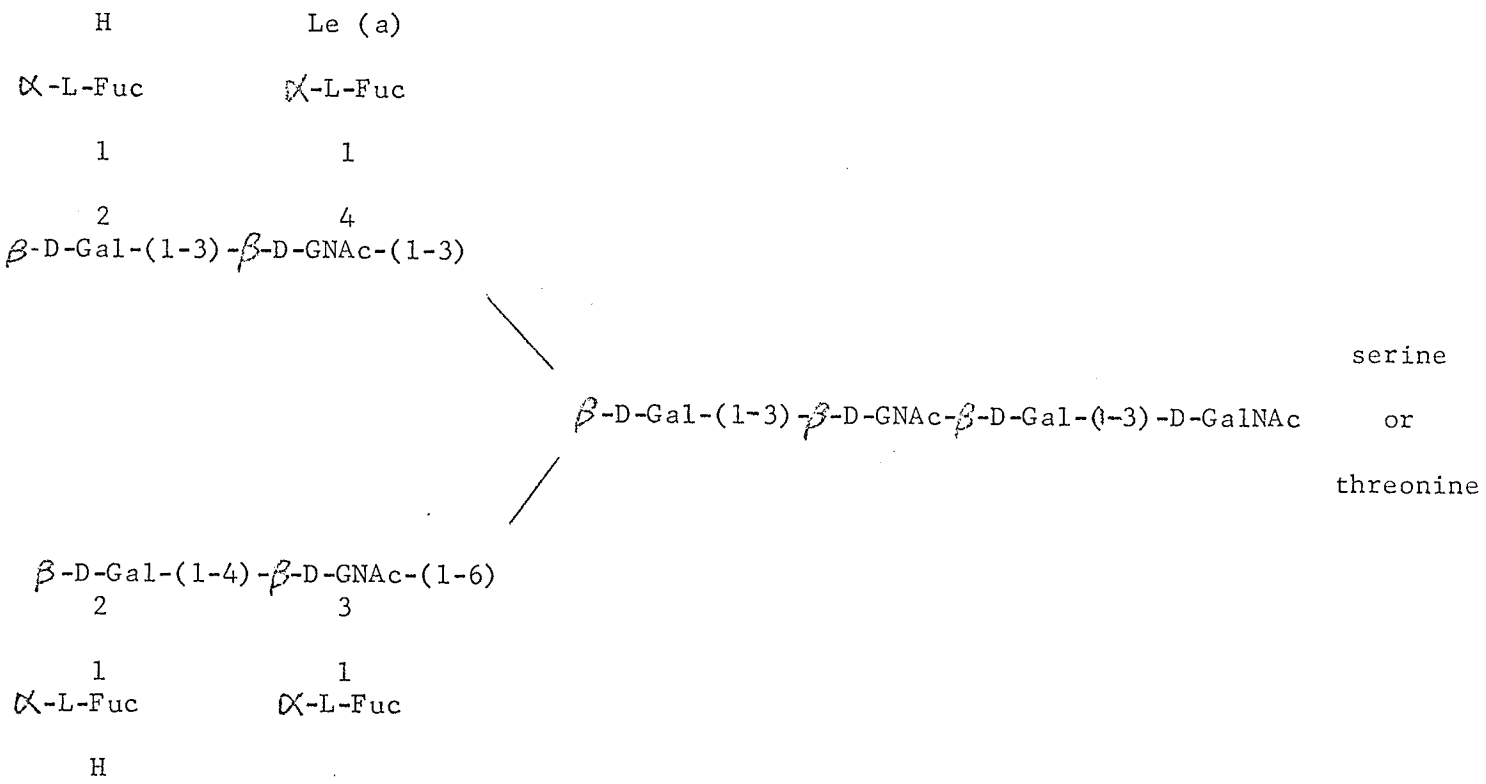
(b) Glycolipid Blood Group Antigens

Blood group active lipids were first isolated from erythrocytes by Masamune who used fractionation with organic solvents (cited in Hakomori, 1970). They were found to be water-soluble glycolipids which contained amino sugar.

Hakomori and Strycharz (1968) separated the glycolipid fraction of A_1 cells into three components which all carried A

(k)

Le (b)


H
Le (a)
α-L-Fuc
α-L-Fuc
1
1
2
4
β-D-Gal-(1-3)-β-D-GNAc-(1-3)
β-D-Gal-(1-3)-β-D-GNAc-β-D-Gal-(1-3)-D-GalNAc
serine
or
threonine
β-D-Gal-(1-4)-β-D-GNAc-(1-6)
2
3
1
1
α-L-Fuc
α-L-Fuc
H

specificity. When A_2 cells were used as the source of glycolipid, only two of the corresponding components could be detected. Two B active glycolipids were isolated from B erythrocytes. A component capable of inhibiting agglutination of O cells by Ulex europaeus was isolated from group O cells. This fraction also showed strong Le(b) activity. The two activities could not be separated.

Hakomori and Jeanloz (1964) isolated a glycolipid which contained fucose, glucosamine, glucose, and galactose (1:1:1:2) from a human adenocarcinoma. It was shown to inhibit Le(a) and H agglutination (Hakomori et al., 1967). Later work indicated that Le(a) and Le(b) activity could be found together in tumor tissue and glandular tissue but not in parenchymatous organs such as kidney and liver (Hakomori and Andrews, 1970). In these latter organs the Lewis specificity is that of the erythrocytes. In tumor tissue no A or B activity was observed.

Hakomori (1970) suggested that a tetrahexosyl ceramide (1) is the common skeleton of all the blood group glycolipids.

(1) gal 1-β-3 GNAc 1-β-3 Gal 1-4 glu-ceramide

This was obtained after Smith degradation of A or B active glycolipids or after weak acid hydrolysis of Le(a) active material. The molar ratios of the sugars in A and B materials indicated that their specificity resides in a terminal N acetyl galactosamine or galactose, respectively. Hakomori (1970) suggested that a fucose residue is probably attached to the penultimate galactose of the A or B structure. Koscielak et al.

(1970), however, found this galactosyl residue to be unsubstituted.

XII Synthesis and Genetics of Blood Group Substances

In 1959, Watkins and Morgan (Watkins, 1959) introduced a theory for the synthesis and genetics of the water-soluble blood group substances. From the known structural, serological and genetical data they concluded that the genes involved in blood group specificity acted through the production of specific glycosyl transferases which added sugars sequentially to the non-reducing ends of carbohydrate chains of a preformed glycoprotein molecule and thereby endowed it with its characteristic serological identity.

A glycoprotein with a chemical composition similar to that of blood group substances has been shown to be present in the secretions of non-secretors who are also Le(a-b-). This molecule cross-reacted with antiserum prepared against pneumococcal type XIV polysaccharide. The role of the Le gene was envisioned as the production of a fucosyl transferase which added an α fucose to this inactive precursor material to give it Le(a) specificity. More recent structural studies have indicated that the addition of the fucose is to the C-4 of the penultimate N-acetyl glucosamine of the type I chain of the precursor molecule (Rege et al., 1964b). The presence of the corresponding fucosyl residue on the type II structure may be a new and presently undefined genetic marker

(Lloyd and Kabat, 1968a).

The H gene was also thought to code for a fucosyl transferase which, in this case, added an α fucose to the C-2 position of the terminal galactose of either a type I or type II chain. The secretor gene appeared to control the expression of the H gene. The dominant Se gene was considered inactive while the homozygous sese prevented the expression of the H gene. The Le(b) specificity was thought to result from an interaction of the Le and H genes. This was a slight modification of the scheme originally put forward by Cepellini in which Le(b) was due to an interaction of the Le and Se genes.

The N-acetyl galactosamine transferase and galactose transferase, considered to be the products of the A and B genes respectively, were thought to require the H structure to serve as acceptor for the addition of the sugar. Thus, the secretor gene would control the presence or absence of A or B activity in secretions by the control of the expression of the H gene.

The demonstration of the appropriate glycosyl transferases in glandular tissue, ovarian cyst epithelium, and milk have supported this hypothesis for the synthesis of blood group substances. The milk of secretor individuals has been shown to contain a soluble fucosyl transferase which can transfer L-fucose from labelled GDP-L fucose to a D galactose residue of milk oligosaccharides. This enzyme can not be demonstrated in the milk of non-secretors (Shen et al., 1968). An N-acetyl galactosaminyl transferase has been

shown to be present in the milk of group A and AB individuals but not in the milk of group B or O individuals. It could transfer N-acetyl galactosamine from UDP-N-acetyl-galactosamine to some milk oligosaccharides (Kobata et al., 1968a). The enzyme was subsequently purified 55 fold and shown to require an acceptor which had a terminal galactosyl residue substituted at the C-2 position with an L fucose (Kobata and Ginsburg, 1970). The specificity of the enzyme and its presence in the milk of A and AB individuals regardless of secretor status supported the hypothesis of Watkins and Morgan that the presence or absence of ABH substances in secretions is controlled at the level of the H specificity. A galactosyl transferase which showed similar properties was demonstrated in the milk of group B and AB secretors (Kobata et al., 1968b). The presence of appropriate fucosyl transferases has been demonstrated in the milk of individuals who have the Le gene whereas they were not detectable in individuals homozygous for the le gene. Similar results were obtained when gastric mucosa, submandibular gland tissue or ovarian cyst epithelium was used as a source of enzymes (Ziderman et al., 1967; Race et al., 1968; Hearn et al., 1968; Race and Watkins, 1970; Hearn et al., 1972). Hearn et al. (1970) demonstrated a particle-bound enzyme in human submandibular glands and stomach mucosal tissue which could transfer N-acetyl-D-galactosamine from UDP-N-acetyl-D-galactosamine to a peptide acceptor isolated from human blood group substance.

It had been assumed that this synthetic scheme would be

the same for the red cell antigens. In support of this, Schenkel-Brunner and Tuppy (1970) found that after group O or B red cells were incubated with an enzyme preparation from the gastric mucosa of a group A individual, the cells were rendered agglutinable by anti-A serum. Similarly, an enzyme preparation from group B gastric mucosa conferred some group B specificity upon group A_1, A_2 and O erythrocytes. In both cases the appropriate nucleotide-sugars were required in the incubation mixture.

However, contrary to the assumption of similarity in the synthesis of the alcohol-soluble and water-soluble forms of the antigen, Koscielak et al. (1970) isolated A-active glycolipids which lacked the fucose residue on the penultimate galactose. This would suggest that the specificity of the enzymes involved in the synthesis of the glycolipid antigens may differ from those which synthesize water-soluble blood group substance.

XIII Quantitative Hemagglutination

The first attempt to estimate the relative concentration of antibody in serum by the technique of serial dilution of the serum was made by Foerster in 1897. This has since become a standard procedure in blood-grouping laboratories. The reciprocal of the highest dilution of the antiserum which gives detectable agglutination is designated the titre of the serum. Agglutination has been read either macroscopically or microscopically and is

therefore a rather subjective judgement. Callender and Race (1946) assigned values between 1 and 10 with respect to the degree of clumping with each of the antiserum dilutions. The sum of the values for the series of dilutions was called the titration score. In some cases it allowed a differentiation amongst sera which had the same titre.

Radioactively-labelled antibodies have been used mainly to estimate the number of antigenic sites present on red cells. Boursnell, Coombe and Rizh (1953) used ^{131}I tagged anti-Rho(D) serum to estimate the number of Rho(D) sites per cell. They found considerable nonspecific binding of labelled serum proteins to the cells. This could be reduced by the inclusion of non-labelled serum proteins in the reaction mixture (Hughes-Jones et al., 1962) or by the use of antibodies purified by absorption and elution (Masouradis, 1959). Hughes-Jones (1962) has used this technique to study the kinetics of antibody-red cell interactions.

The use of microscopic cell counting techniques in the study of hemagglutination originated with Ashby in 1919. The method, as currently used, is based on the standard serial dilution assay. Following incubation of the antiserum dilutions with the cell suspension, the reaction mixture is transferred to a hemocytometer chamber and the number of unagglutinated cells per unit volume is counted. This value is then calculated as a percent of the free cells in an unreacted test cell suspension and the percent agglutination can be computed. In several studies electronic particle counters have been employed (Goodman, 1962;

Gibbs and Becker, 1963; Bowdler and Swisher, 1964; Gibbs et al., 1965; Greendyke and Swisher, 1968).

Separation of free cells from agglutinated cells has been obtained by differential settling rates and by centrifugation (Renton and Hancock, 1964; Dybkjaer, 1966). After separation, the cells were hemolyzed and the quantity of released hemoglobin was determined photometrically (Dybkjaer, 1966). From the respective optical densities found for the free and agglutinated cell fractions, the percent agglutination was calculated. The quantitative hemagglutination studies which use the auto-analyzer involve the similar principle of removing agglutinates by settling and, after hemolysis, passing either the free or agglutinated cells through a colorimeter (Rosenfield et al., 1964; Greenwalt and Steane, 1970).

Filitti-Wurmser and colleagues have used the hemocytometer cell-counting method to study some physico-chemical aspects of hemagglutination. They found that, at a constant temperature, when increasing numbers of red cells were mixed with a fixed quantity of serum, the number of agglutinated cells increased to a maximum and then plateaued. When this reaction was carried out at 4^{o}C. the maximum was called N_4 or if carried out at 37^{o}C. it was called N_{37}. They used this value as a measure of the concentration of agglutinins in the serum.

The maximum number of cells agglutinated at 4^{o}C. (N_4) is greater than that at 37^{o}C. (N_{37}). The displacement of equilibrium by changes in temperature appeared fully reversible (Filitti-Wurmser and Jacquot-Armand, 1947).

Filitti-Wurmser et al. (1954) found that the ratio of N_4:N_{37} for an anti-B serum was characteristic of the genotype of the donor.

A second approach which the authors used was to measure the residual antibody (N'_4) which followed the reaction of a known amount of antiserum (N_4) with a known quantity of red cells (N_t). The difference of N_4-N'_4 was the antibody which was fixed (N_f). As they had shown that the reaction was reversible they were able to use the mass action law. From theoretical calculations it was found that a straight line should be obtained when Nt/N_f was plotted against $1/N'_4$ and that its slope should be proportional to the equilibrium constant of the reaction. When experimental values were used, the slope was shown to be characteristic of the ABO genotype of the anti-B donor. The ratios of slopes found at two different temperatures were substituted into the van t'Hoff equation and the enthalpy calculated.

Salmon and his colleagues have extended this technique to the study of weak forms of ABO antigens and modifications of antigens and isoagglutinins during the course of disease (review by Salmon, 1969).

Wilkie and Becker (1955a and b) have developed an alternative method to quantitate hemagglutination. They found that when the agglutination of constant quantities of red cells by increasing dilution of serum was measured, and the values obtained were plotted in terms of the logarithm of the concentration of the serum, a sigmoidal curve was obtained. Their reference point was the HD_{50}. This was defined as the concentration of serum

required to agglutinate 50% of the cells of a test suspension. When the percent agglutination was converted to probit values and these were plotted against the logarithm of the concentration of antiserum, a straight line was obtained between values of 12% and 97% agglutination. This probit transformation facilitated determination of the HD_{50}.

A shift of the probit line to the left is indicative of an increase in the potency of the antiserum, whereas a shift to the right indicates a decrease in potency. However, there is at present no theoretical explanation for differences in the slopes of probit lines (Salmon, 1969). Ellis et al. (1964) suggested that differences in slope are due to qualitative differences. They found that the reaction of A_2 cells with anti-A serum consistently produced lower slopes in probit plots than when A_1 cells were used. On the basis of similar slopes they assigned the weak A varients, A_4, A_o, and A_m into one group A_x.

The classical procedure for the quantitation of soluble blood group substance has been by hemagglutination-inhibition (Yamakami, 1926). Increasing twofold dilutions of the secretion to be tested are incubated with a constant amount of antiserum. After a set length of time a blood suspension of the appropriate group is added to each of the reaction mixtures. If soluble antigen of the proper specificity is present it will decrease agglutination of the test cell suspension by forming complexes with antibodies in the serum. The titre of the secretion is the highest dilution of the secretion which completely inhibits visual

agglutination.

Gibbs et al. (1961a) employed hemocytometer counts and probit analyses to quantitate more accurately inhibition of agglutination. The reference point which they used was the concentration of the inhibiting substance which resulted in 50% agglutination of the indicator red cells (HID_{50}).

The reactions of human anti-A and hog A substance indicated differences between antibody found in individuals who had had no deliberate antigenic stimulus and in those who had been immunized with blood group substances (Gibbs et al., 1961b). The naturally occurring isoagglutinins had a lower slope for the probit lines, and required larger doses of blood group substance for inhibition. The authors suggested that the decreased slope when non-stimulated antiserum was used was due to the less specific nature of the natural antibodies with respect to hog A substance. Equilibrium studies also showed that the antibody-blood group substance complexes, formed with non-stimulated sera, dissociated more readily than those formed with sera from immunized donors.

These findings were confirmed when pre- and post-immunized sera were studied (Gibbs et al., 1971). The rate and magnitude of these changes could be seen to differ amongst individuals. The authors suggest that this variation may reflect primary and secondary immune responses. However, they admit that the theoretical interpretation for changes in the slope of the inhibition assay curve is uncertain.

Bar-Shaney et al. (1970) used another method to quantitate hemagglutination-inhibition. They absorbed the blood group substance in saliva onto lytron particles. These coated particles were then allowed to react with the antiserum. Following the incubation period, the lytron beads, which now had blood group substance-antibody complexes bound, were centrifuged. The supernatant which contained the residual antibody was used to measure hemagglutination. This modification to the method prevents dissociation of antibody-blood group substance complexes after the cell suspension has been added. The hemagglutination was assayed with an autoanalyzer. In their study they found a semilogarithmic relationship when percent inhibition was plotted as the response to the logarithm of the concentration of the inhibitor saliva.

XIV Saliva

(a) Composition of Saliva

The whole saliva is made up of the secretions from 3 pairs of large glands, the parotid, submandibular and sublingual, as well as those from numerous small mucous glands found on the lingual, buccal and palatal surfaces of the oral mucosa. The individual salivary secretions differ from one another in their composition and relative contribution to the whole saliva. The whole saliva may also contain gingival crevice fluid, leukocytes, bacteria, dental plaque, and food debris.

The principal electrolytes of salivary secretions are sodium, potassium, calcium, chloride, bicarbonate and phosphate. Submandibular and sublingual saliva are similar in their electrolyte compositions but differ considerably from that of parotid saliva. Submandibular and sublingual saliva have higher concentrations of calcium and lower concentrations of potassium than does parotid saliva (Chauncey et al., 1966). The minor mucous glands have virtually no bicarbonate and little phosphate and have chloride as the principal anion (Wood and Dawes, 1968).

The non-dialysable protein concentration is higher in parotid than in submandibular saliva (Daniels and Newbrun, 1966; Newbrun, 1967; Caldwell and Pigman, 1965a). The protein concentration of lip mucous secretion is in the same range as that of parotid saliva (Wood and Dawes, 1968; Mandel, unpublished results). The carbohydrate concentration is higher in parotid saliva than in submandibular saliva (Mandel et al., 1964). However, the ratio of carbohydrate to protein was similar in the two secretions (Newbrun, 1967), or slightly greater in submandibular saliva (Mandel et al., 1964). The concentration of carbohydrate in the secretions of the minor mucous glands appears to be higher than that in the secretions of the major glands (Mandel, unpublished results).

A large number of bands are seen following electrophoresis of saliva (Meyer and Lamberts, 1965; Caldwell and Pigman, 1965b; Mandel, 1966). Many proteins in saliva are identical to serum proteins while others are specific for saliva (Mandel and Ellison,

1961; Stoffer et al., 1962). As well, many of the enzymes observed in whole saliva are of bacterial origin (Ferguson, 1968) while others, such as amylase, are secreted by the salivary glands.

From 4 to 8 amylase isozymes can be identified in parotid saliva (Lamberts et al., 1965; Wolf and Taylor, 1967; Kauffman et al., 1970) and these same isozymes appear to be present in submandibular saliva in lower concentration (Lamberts et al., 1971). No amylase activity can be detected in the minor mucous gland secretions (Dawes and Wood, 1973b). Lysozyme is present in higher concentration in submandibular-sublingual saliva than in parotid saliva (Hoerman et al., 1956). Alkaline phosphatase activity is highest in mixed saliva, with much less activity in parotid saliva. The activity in submandibular saliva is intermediate (Cornish and Posen, 1968). No data is available on either the lysozyme or alkaline phosphatase activities of minor mucous gland secretions. The concentrations of secretory IgA in parotid and submandibular saliva are similar and considerably lower than that in whole saliva (Brandtzaeg et al., 1970). Secretory IgA has also been detected in lip mucous gland secretions (Mandel, unpublished results).

Most important to the present study was the finding of Wolf and Taylor (1964) that the concentration of BGS was highest in sublingual saliva, less in submandibular saliva, and present in only trace quantities in parotid saliva.

(b) Factors which Influence the Composition of Saliva

1 Flow Rate

In 1878, Heidenhain demonstrated that the concentration of inorganic salts in the submandibular saliva of dogs varied in proportion to the rate of secretion (cited in Thaysen et al., 1954). It is now accepted that flow rate also influences the composition of human saliva.

As the flow rate is increased slightly above the unstimulated rate, sodium and bicarbonate concentrations and pH increase in parotid saliva, whereas potassium, calcium, phosphate, chloride, urea and protein concentrations decrease. At higher flow rates, sodium, calcium, chloride, bicarbonate and protein concentrations and pH increase, whereas the phosphate concentration decreases. There is little further change in the potassium concentration (Shannon and Prigmore, 1960; Dawes, 1969). The specific activity of amylase was shown to be higher in stimulated than in unstimulated parotid saliva and independent of flow rate (Dawes, 1972a). The concentration of secretory IgA in parotid saliva decreased with an increase in the flow rate, which was probably due to a non-acinar cell origin (Brandtzaeg, 1971; Mandel and Khurana, 1969). When subjected to gel electrophoresis, several protein bands were seen with unstimulated saliva that could not be seen with stimulated saliva. This was not found with submandibular saliva (Dawes, 1972a).

Caldwell and Pigman (1966) found a relationship between protein concentration and flow rate in submandibular saliva. The

highest concentrations of protein-bound carbohydrate were found in unstimulated submandibular saliva. Low levels of stimulation produced the lowest values of glycoproteins and the concentration increased with increased flow rate. The sialic acid : fucose ratio increased in non-secretor individuals when the flow rate was increased, whereas, the ratio was fairly constant in secretors. This would suggest that the proportions of individual submandibular glycoproteins are flow rate dependent. The ratio of carbohydrate : protein decreased with increased flow rate. Mandel et al. (1965) had previously found a correlation between non-dializable carbohydrate and protein in parotid saliva.

2 Duration of Stimulation

Dawes (1967) showed that with gustatory stimulation to produce a constant parotid flow rate, the protein concentration initially fell below that of unstimulated saliva and then increased upon continued stimulation. Bicarbonate concentrations increased continuously with duration of stimulation and chloride showed a reciprocal decrease (Dawes, 1969). The concentration of parotid sodium showed an initial increase upon stimulation but did not change upon further stimulation. After an initial drop in concentration, potassium and inorganic phosphate were subsequently independent of the duration of the stimulation (Dawes, 1969).

3 Nature of Stimulus

There is evidence that the nature of the stimulus can

influence the composition of salivary secretions aside from their effect upon flow rate. Dawes (1966) found higher values for protein and calcium and lower values for potassium and sodium in pilocarpine-stimulated parotid saliva than in lemon drop-stimulated saliva when collected at the same flow rate. Mandel et al. (1967) found higher activities of amylase and lysozyme and a higher concentration of calcium in pilocarpine-stimulated parotid saliva than in saliva stimulated by 2% citric acid. The protein concentration in submandibular saliva showed no difference with the two forms of stimulation. A 30% sodium chloride solution produced higher protein and protein-bound carbohydrate concentrations than other gustatory stimuli (Caldwell and Pigman, 1966).

4 Time of Day

The time of day influences the flow rate and composition of saliva. Circadian rhythms were found in the flow rate and in the sodium and chloride concentrations of resting whole saliva (Dawes, 1972b). No rhythms were seen in the concentrations of protein, potassium, calcium, phosphate or urea. The protein concentration of stimulated parotid saliva was found to have a rhythm of high amplitude with the acrophase in the afternoon. Stimulated parotid saliva also showed rhythms in sodium, potassium, calcium and chloride concentrations but not in phosphate or urea concentrations.

5 Proportional Contributions to Whole Saliva from Different Glands

As the compositions of the individual secretions are not identical, any factors which alter their proportional contributions to the whole saliva would influence the composition of the whole saliva. Gore (1938) found that parotid flow rate increased relative to "mandibular" saliva (submandibular and sublingual) upon mechanical stimulation (wax). Schneyer and Levin (1955a) found that about 69% of the resting saliva was contributed by the submandibular glands, 26% by the parotid, and about 5% by the sublingual glands. Upon stimulation by weak acetic acid the proportional contribution by parotid saliva increased to 34% (Schneyer and Levin, 1955b). Shannon (1962), who used rubber bands as a salivary stimulus, found that the proportional contribution of the parotid saliva increased from 40.6 to 49.1 when the number of rubber bands was increased from 1 to 4. When 16 mg of pilocarpine hydrochloride was administered, the percentage contribution of parotid saliva rose from 40.7 at the onset of the drug effect to 48.1 one hour later. Dawes and Jenkins (1964) found a higher ratio of parotid : submandibular flow rates with mechanical stimuli than with sapid stimuli. Shannon (1967), who used various acidic candies to stimulate salivation, observed an increase in the mean whole saliva flow rate from 0.22 to 1.86 to 2.78 to 3.00 ml./min. when the various stimuli were used. The percentage contribution from the parotid gland increased from 32.9 to 40.7 to 47.2 to 50.4 respectively. Dawes and Wood (1973a) found that the minor salivary glands contributed 8% of the resting saliva and 7% of sour lemon drop-stimulated whole saliva.

During sleep no parotid flow could be demonstrated (Schneyer et al., 1956). A small amount of submandibular and sublingual flow was noted but the authors suggested that this might be due to stimulation by the collecting device. Thus the proportional contribution to whole saliva from individual glands depends upon total flow rate, the nature of the stimulus and on the time of day.

CHAPTER III

METHODS AND MATERIALS

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I Introduction

A method for quantitative hemagglutination-inhibition was developed as a modification of the quantitative hemagglutination technique of Dybkjaer (1966). A series of experiments were performed in an attempt to elucidate the most suitable conditions for the method and to evaluate its accuracy. The technique was subsequently used to test the effect on the concentration of blood group substances in human submandibular saliva of certain variables which are known to influence the composition of saliva. It was also used to examine the relative contributions of the various salivary glands to the content of blood group substance in the whole saliva

II Reagents

(a) Buffer

All dilutions were performed with an isotonic M/15 phosphate buffer prepared with sodium and potassium phosphate salts and sodium chloride and adjusted to pH 7.3. This will be referred to as phosphate buffered saline (PBS).

(b) Anti-A Serum

The human anti-A sera used in the experiments were kindly donated by the Canadian Red Cross.

The sera were received frozen. They were thawed, divided into 0.25 ml aliquots and refrozen at -60°C. Individual aliquots were removed from the freezer as required.

The thawed serum was then diluted with PBS to the desired concentration. For most inhibition experiments a 1/8 dilution was used. With the saliva of one group AB individual (D.S.) a dilution of 1/16 was employed. Dilutions of up to 1/80 were used when parotid saliva was being treated.

(c) Anti-H Ulex europaeus

Seeds of the plant Ulex europaeus were obtained from Thompson and Morgan (Ipswich) Ltd, Ipswich, Suffolk, England. The extracts were prepared by the method of Boettcher (1967b) with slight modification.

The seeds were ground to a fine powder by a mortar and pestle. 200 ml of cold PBS was added to 25 grm of the ground seeds in a 500 ml Erlenmeyer flask. The suspension was left at 4°C. for 24 hours and mixed by a magnetic stirrer. The mixture was then allowed to settle and the supernatant was poured off and filtered at room temperature by gravity through Whatman number 1 filter paper. The filtrate was temporarily stored at 4°C.

A further 50 ml of PBS was added to the sediment and the

mixture was stirred for another 24 hours at 4°C. The solids were again allowed to settle and the supernatant filtered through Whatman number 1 paper. The two filtrates were pooled and centrifuged in the cold for 30 minutes at 12,840 x g. The supernatant was stored at 4°C. for 1 week and again centrifuged at 12,480 x g for 30 minutes. The supernatant was divided into 0.5 ml aliquots and stored frozen until required.

Individual aliquots were thawed when needed and centrifuged in a clinical centrifuge for several minutes. The supernatants were diluted to 1/2 with PBS and used as anti-H reagent.

(d) Anticoagulant

Blood was stored at 4°C. for no more than three weeks in an equal volume of sterile Alsever's solution. Alsever's solution was prepared according to Collins (1967).

Dextrose	2.05 g
Sodium Chloride	0.42 g
Trisodium Citrate ($2H_2O$)	0.92 g
Citric acid ($.H_2O$)	0.059 g
Distilled H_2O to 100 ml	

The weights of the chemicals were adjusted from those of the reference to account for the degree of hydration of the components used.

The solution was autoclaved for 10 minutes at 121°C. and 19 lb pressure and 5 ml aliquots dispensed aseptically into 25 ml

Erlenmeyer flasks which were then stoppered with sterile serum caps.

(e) Blood Suspension

For all experiments, group A_1 blood and group O blood were obtained from two donors (C.D. and R.M., respectively). Venous blood was drawn from the arm of the donor by means of a 10 ml sterile disposable syringe. Five ml of blood was added to a 25 ml Erlenmeyer flask, which contained 5 ml of sterile Alsever's solution, by passing the needle of the syringe through the serum cap. Five ml of air was withdrawn from the flask to maintain the pressure at atmospheric. This precaution was also taken when blood was removed from the flask.

The hematocrit was determined for the blood-anticoagulant suspension (blood). A portion of the blood was placed in a hematocrit tube and centrifuged for 20 minutes at maximum speed in an International Clinical Centrifuge.

The blood was stored at $4^{o}C$. It was not used during the first four days after it was drawn from the donor since it was found that this period was required for the blood to become stabilized. When needed, a small portion of blood was removed from the flask by passing the needle of a sterile syringe through the serum cap. Measured aliquots were then made by micropipettes. These were washed twice with PBS. A 1% suspension was then prepared with a volume of PBS which depended upon the previously determined hematocrit. The 1% suspension was made up fresh for each experiment.

(f) Hog Gastric Mucosal Blood Group Substance

Blood Group A substance purified from hog gastric mucosa was purchased from the Metrix division of Armour Pharmaceutical Company. The solution was lyophilized to dryness and brought to a constant weight in a vacuum desiccator which contained silica gel. A solution of 10 mg% blood group substance in PBS was prepared. It was divided into 0.5 ml aliquots and stored at -60°C. This was used as the undiluted standard solution in both A and H assays.

(g) 20% Dextrose Solution

A 40% dextrose solution was prepared in deionized water. This was diluted to one-half concentration with PBS. This will be referred to as 20% dextrose. The 20% dextrose was prepared immediately prior to use.

(h) Modified Drabkin's Solution

Modified Drabkin's solution was prepared as described by Dybkjaer (1966).

Potassium cyanide	50 mg
Potassium ferricyanide	250 mg
Sodium bicarbonate	1.0 g
Deionized water to 1 litre	

This was not kept longer than three or four days after it was prepared.

(i) Glassware

The 10 x 75 mm test tubes which were used in the hemagglutination-inhibition assays had been previously soaked in a 1% solution of Siliclad (Clay Adams Division of Becton, Dickinson and Company, Parsippany, New Jersey) according to the instructions which were included with the siliclad solution. This was repeated after every 4 to 5 washings of the test tubes in order to reduce adhesion of erythrocytes to the glass surface.

III Saliva Collection

(a) Submandibular Saliva

Submandibular saliva was collected by a Schneyer-type apparatus (Schneyer, 1955). This is an acrylic appliance, divided into three chambers and has a section of polythene tubing leading out from each chamber to the collecting vessels. It is placed in the floor of the mouth and held lightly in position by the tongue. The central chamber covers the openings of both of the submandibular ducts and the two lateral compartments cover the openings of the sublingual ducts.

In one subject (K.K.) the Schneyer appliance could not be used due to the anterior position of the submandibular ducts. In this one subject the left submandibular duct was cannulated directly with polythene tubing.

If the blood group assays were not performed immediately after collection, the saliva samples were placed in a boiling water bath for ten minutes, the resultant coagulum centrifuged down, and the superantants stored frozen at -60°C. All protein analyses were done on the day of collection.

1 Unstimulated Submandibular Saliva

The subject sat motionless with the collection device in place until sufficient saliva for the assay was obtained.

2 Stimulated Submandibular Saliva Collected at a Constant Flow Rate

A technique similar to that used for parotid saliva by Dawes (1967) was employed to collect submandibular saliva at a constant flow rate. With the cannula in place the subject sucked on a sour lemon drop (SLD) while collecting the saliva into graduated centrifuge tubes. A stop-watch was placed in front of the subject and a mirror positioned to enable him to observe the volume of saliva in the tube. The flow rate was regulated by the intensity of sucking on the SLD. A one minute sample was collected into each centrifuge tube. In most experiments, after a sample of unstimulated submandibular saliva was obtained, saliva was collected for 15 minutes at a constant flow rate.

3 Low Flow Rate - High Flow Rate Collections

The subject maintained a constant flow rate with a low level of stimulation which was provided by a small piece of a

previously fragmented SLD. After a small amount of unstimulated submandibular saliva was obtained, five sequential one minute samples were collected at this low flow rate. At this point a whole SLD was substituted for the fragment and 10 more samples were collected at a higher flow rate.

4 High Flow Rate - Low Flow Rate Collections

After collection of unstimulated saliva the subject collected SLD-stimulated submandibular saliva at the highest flow rate which could be maintained constant. At this point the SLD was removed and 5 more one minute samples were collected.

(b) Whole Saliva

Both unstimulated and wax-stimulated whole saliva samples were centrifuged before blood group assays were attempted (1740 x g for 10 minutes). All collections were made before breakfast.

1 Unstimulated Whole Saliva

The subject sat motionless with his head down and allowed the saliva to flow from the lower lip into a funnel inserted in a 10 ml graduated centrifuge tube in ice water.

2 Stimulated Whole Saliva

The subject chewed on a piece of paraffin wax (melting point 52^{o}C.) and spat the saliva into a test tube.

(c) Lip Mucous Gland Secretions

Lip mucous gland secretions were collected by the method of Dawes and Wood (1973a). The subject exposed the lower labial mucosa by everting the lower lip. The mucosa was dried with a cotton roll and droplets of secretion were allowed to form and were then collected by micropipette. Both unstimulated and SLD-stimulated samples were collected.

(d) Palatal Secretions

The subject was seated in a reclining chair which was adjusted to the fully reclined position. The palatal mucosa was dried with a cottom swab. As the droplets of secretion from the palatal glands formed, they were drawn off with a micropipette. Only the collection of stimulated secretion was undertaken. Stimulation was provided by several drops of 2% citric acid applied to the tongue by a Pasteur pipette prior to collection.

(e) Sublingual Saliva

The parotid and submandibular ducts were blocked off with cotton rolls placed in the buccal sulci and in the floor of the mouth. The subject placed his tongue in the left cheek exposing the openings of sublingual ducts on the right side. This area was dried and isolated with cotton rolls. Secretion was stimulated by application of 2% citric acid to the tongue and

the sublingual saliva was collected by micropipette.

(f) Parotid Saliva

Unstimulated and SLD-stimulated parotid saliva was collected with a Lashley cannula (Shannon and Chauncey, 1967). As the blood group activity of parotid saliva was found to be very low, this study was not pursued beyond preliminary experiments.

IV Protein Analysis

Protein concentration was determined by the method of Lowry et al. (1951) with Hammersten casein as a standard. The optical density was read on a Unicam SP 500 spectrophotometer.

V Ultrafiltration of Saliva

A model 10-PA Ultrafiltration Cell (Amicon Corporation, 12 Hartwell Ave, Lexington, Massachusetts, 02172) was used in ultrafiltration studies of saliva samples. This was carried out at $4^{o}C$. The degree of concentration was estimated from the volume of the filtrate. Two membranes have been used: a UM-10 which retains molecules greater than 10,000 M.W. and a UM-05 which retains molecules greater than 500 M.W.

VI Serological Determinations

(a) Determination of Blood Group

Two small samples of blood were obtained by a "finger prick" and immediately mixed with an approximately equal volume of PBS. To one of the samples a drop of anti-A serum was added and to the other a drop of anti-B and the two samples were examined for visual agglutination.

Group A individuals were considered to be of the subgroups A_1 if their red cells were agglutinated by an anti-A serum which had previously been absorbed with A_2 cells.

(b) Determination of Secretor Status

Only subjects of group A and AB were tested. One hundred µl of the saliva in question, which had been diluted to 1/2 in PBS, was mixed in a 10 x 75 mm test tube with 100 µl of anti-A serum which had been diluted to 1/8. After 15 minutes, 100 µl of a 1% suspension of group A cells was added. The tubes were capped with Parafilm-covered rubber stoppers (size 000), placed on a rotator (Multi-Purpose Rotator, Scientific Industries Inc., Springfield, Massachusetts, 01103) and allowed to rotate at 10 revolutions/minute. Complete inhibition of agglutination after several hours of rotation indicated that the individual was a secretor of A substance.

(c) Quantitative Hemagglutination-Inhibition Assay

For both the A and H assays, preliminary experiments were performed on subjects who were known to be secretors but whose saliva was being studied quantitatively for the first time. The object of this procedure was to determine suitable dilutions for the quantitative inhibition assay.

One hundred μl serial dilutions of saliva were made in 10 x 75 mm test tubes. 100 μl of diluted anti-A or anti-H were then added and the mixture was allowed to incubate for 1 hour. At the end of this period 100 μl of a 1% suspension of group A or group O cells, respectively, were added. The tubes were stoppered and rotated at 10 revolutions/minute. After several hours, agglutination was examined visually.

For the quantitative assay, dilutions were chosen such that several dilutions were included on either side of the dilution which inhibited 50% of the agglutination.

1 A Assay

Between 4 and 10 suitable dilutions of the saliva sample were prepared with PBS. A 100 μl aliquot of each dilution was transferred to a clean siliclad-coated 10 x 75 mm test tube. 100 μl of diluted anti-A was added to each tube and left for 1 hour. This will be referred to as the preincubation period.

After the preincubation period, 100 μl of a 1% suspension of group A_1 cells were added to each of the tubes. The tubes were

then stoppered and rotated at 10 revolutions/minute for 20 hours (incubation period).

Freshly prepared 20% dextrose was dispensed into clean 10 x 75 mm test tubes in 1.5 ml aliquots. At the end of the incubation period the tubes which contained the reaction mixture were taken from the rotator and 250 µl of the mixture was removed from each and carefully layered on to the 20% dextrose. These tubes were then centrifuged at 45 x g for 30 seconds in an International Clinical Centrifuge with the aid of a rotation counter and a stop-watch (300 revolutions in 30 seconds). The agglutinated cells migrated into the dextrose while the free cells remained in the upper buffer layer.

The upper layers containing free cells were pipetted off and placed in clean 10 x 75 mm tubes. Both free and agglutinated cells were washed with PBS, centrifuged, and the supernatant discarded. One ml of modified Drabkin's solution was added to the packed cells. After at least 10 minutes the optical densities (O.D.) were read on a Unicam SP 500 at 542 nm. The percentage agglutination for each dilution was calculated from the optical densities.

$$\% \text{ agglutination} = \frac{\text{O.D. agglutinated cells} \times 100\%}{\text{O.D. free cells} + \text{O.D. agglutinated cells}}$$

2 H Assay

A similar procedure was followed for the H assay. Ulex

europaeus extract was used as a source of anti-H and a 1% suspension of group O cells were used as the indicator red cell suspension. Volumes of 200 µl of the reactants were employed which made a final reaction mixture of 600 µl. This minimized adhesion of red cells to the sides of the test tube. An incubation period of 6 hours was found to be sufficient for equilibrium to occur.

3 Standard

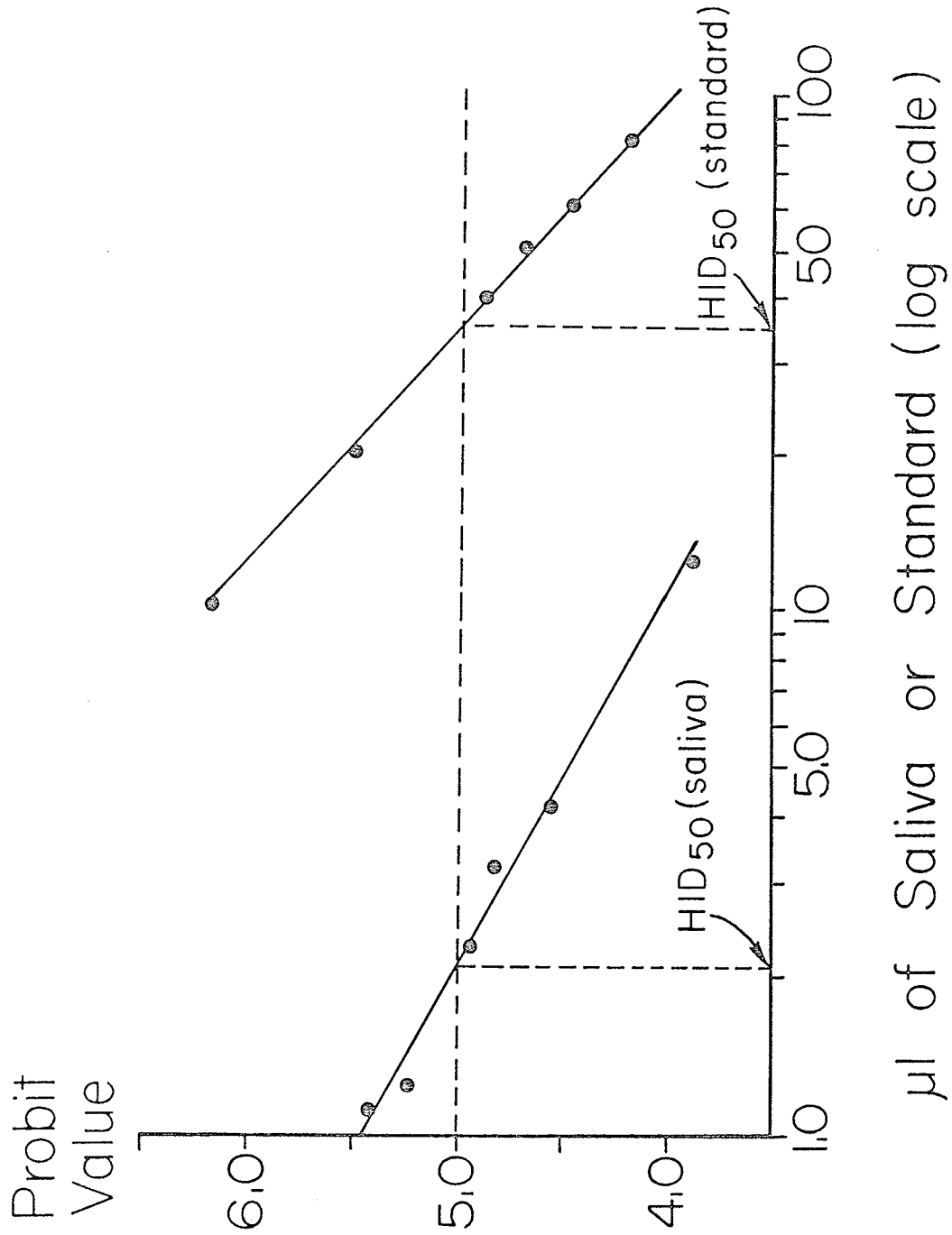
With each A or H experiment a parallel assay was performed on the hog gastric mucosal blood group substance. Seven dilutions of the standard solution were made and analyzed in the same manner as the saliva samples.

VII Probit Analysis

The data obtained by the quantitative hemagglutination-inhibition technique were subjected to probit analysis (Wilkie and Becker, 1955a). The percentage values for the agglutination found with dilutions of saliva or standard were transformed to probit values with the aid of a table of probits (Documenta Geigy, Scientific Tables 6th ed., 1962). The probit values for a series of dilutions were then plotted against the volume of undiluted saliva or undiluted standard solution (µl) which was used in the reaction mixture (Figure 3 - 1).

Linear regression and correlation analyses were done on an Olivetti-Underwood Programma 101. All correlation coefficients were between -0.96 and -1.00.

Figure 3 - 1. Sample inhibition assay curves of hog group A substance and human submandibular saliva from a group A secretor.


Probit Value
6.0
5.0
4.0
1.0
5.0
10
50
100
HID50 (saliva)
HID50 (standard)
µl of Saliva or Standard (log scale)

From the regression data, values for the slope and the y intercept of the probit line were obtained. These were then substituted into the straight line equation and the volume of undiluted saliva or standard which resulted in a probit value of 5.00 was calculated. This is the volume of the undiluted saliva or standard which inhibited 50% of the hemagglutination: that is, the HID_{50}.

$$HID_{50} = \text{antilog} \quad \frac{5.00 - \text{y intercept}}{\text{slope of probit line}}$$

The HID_{50} found for the standard was converted from units of volume (μl) to units of weight (μg) by multiplication of the HID_{50} volume by the concentration of the undiluted standard solution (0.1μg/μl). The concentration of blood group substance in the saliva (μg/μl) was then computed by division of the HID_{50} weight of the standard (μg) by the HID_{50} volume of the saliva (μl). The units of μg/μl were then converted to units of mg% by multiplication by a factor of 10^2.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

I Quantitative Hemagglutination-Inhibition

(a) Preincubation

An equilibrium experiment was performed to examine the reversibility of the hemagglutination-inhibition reaction and to determine if a preincubation of saliva or standard with the antiserum was required prior to the addition of the test cell suspension. Four combinations of reactants were used in the preincubation mixture. After one hour the third reactant was added (Table IV - 1).

The addition of the third reactant was considered to be t_o of the incubation and at intervals thereafter, samples were removed and the percent agglutination was determined.

The results when hog gastric mucosal standard was used as a source of blood group substance (BGS) are shown in Figure 4 - 1. Similar results were obtained with submandibular saliva from secretors of blood group substances.

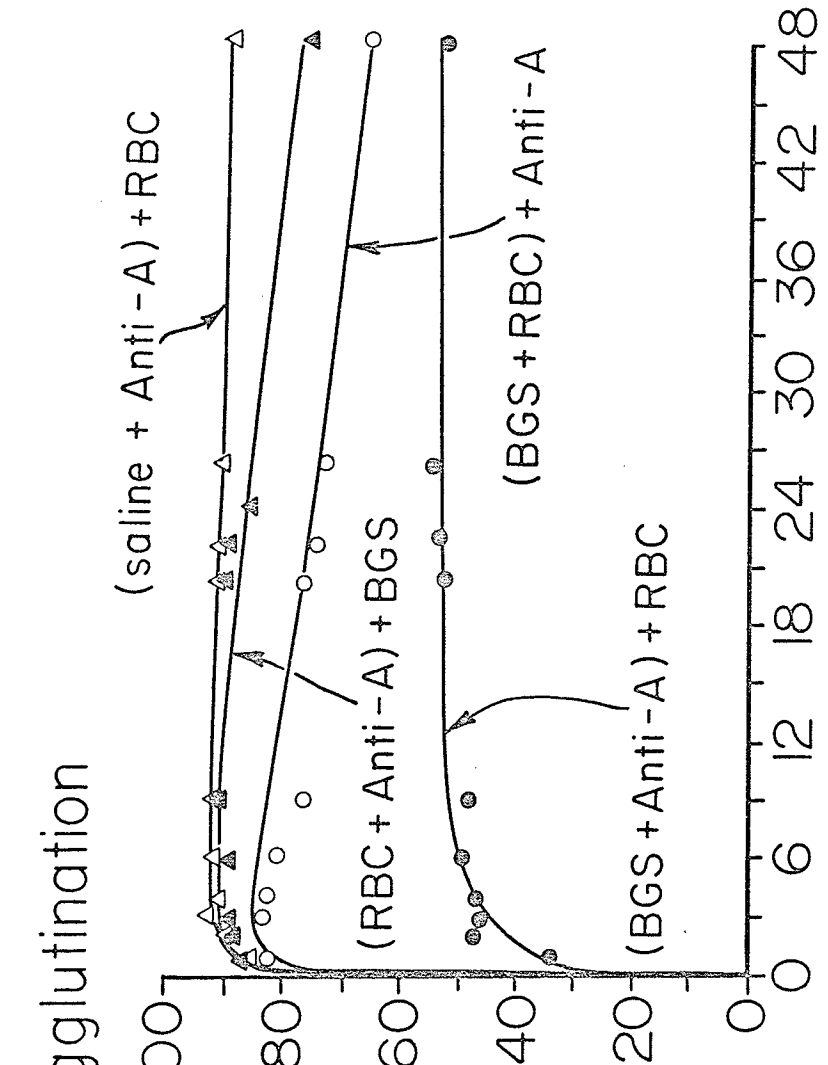
Gibbs et al. (1961a), who had performed a similar experiment, found that when anti-A was added to a mixture of hog group A substance and group A cells, the agglutination increased to, but did not surpass that level obtained when the BGS and anti-A were preincubated before the addition of the test cell suspension. In our system, the agglutination initially rose

Table IV - 1

The Design of an Experiment to Test the Reversibility of the Hemagglutination-Inhibition Reaction and the Requirement for a Preincubation of Antiserum and BGS

Preincubation Mixture	Third Reactant added at t_o
BGS + Anti-A	1% test cell suspension (RBC)
BGS + RBC	Anti-A
RBC + Anti-A	BGS
PBS + Anti-A	RBC

Figure 4 - 1. Equilibrium-time study of a system containing hog group A substance, A_1 red blood cells and human anti-A serum.


% Agglutination
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Hours of Incubation
(saline + Anti-A) + RBC
(RBC + Anti-A) + BGS
(BGS + RBC) + Anti-A
(BGS + Anti-A) + RBC

beyond this equilibrium level and then began to decrease towards it. It would appear therefore, that red cell anti-A complexes formed initially. This was followed by their gradual partial break-up and the formation of BGS-anti-A complexes. With time, the three reactants would come into equilibrium, presumably at the same level as that obtained with preincubation. In a subsequent experiment (not shown), when the duration of the incubation was extended to 96 hours, the agglutination continued to approach, but failed to reach the equilibrium level. However, after about 48 hours, hemolysis became increasingly evident in the reaction tubes, a factor which would influence the equilibrium.

Gibbs et al. (1961b) compared anti-A sera from donors who had no history of BGS stimulation with sera from donors who had been artificially stimulated with hog A substance. They found that with stimulated sera, anti-A-hog A substance complexes and anti-A group A red cell complexes, appeared to dissociate less readily than those formed with unstimulated sera. They concluded that, antibodies in the stimulated sera had a higher avidity than those in the unstimulated sera; that is, antibody with a lower rate of dissociation and a higher reaction velocity. The sera used in our experiments were from group B donors who had been immunized with hog A substance prior to collection and preparation of the sera. According to our equilibrium data the anti-A appeared to be extremely avid. Strangely enough, the avidity appeared greater with respect to the red cells than to hog A substance, the homologous antigen.

An equilibrium experiment was then undertaken to test whether the duration of preincubation of BGS and anti-A had any effect on the final level of agglutination which was reached in the system. After preincubation of BGS and anti-A for periods of 0.25, 1, 2 and 4 hours, a one percent test cell suspension was added. The results are shown in Figure 4 - 2.

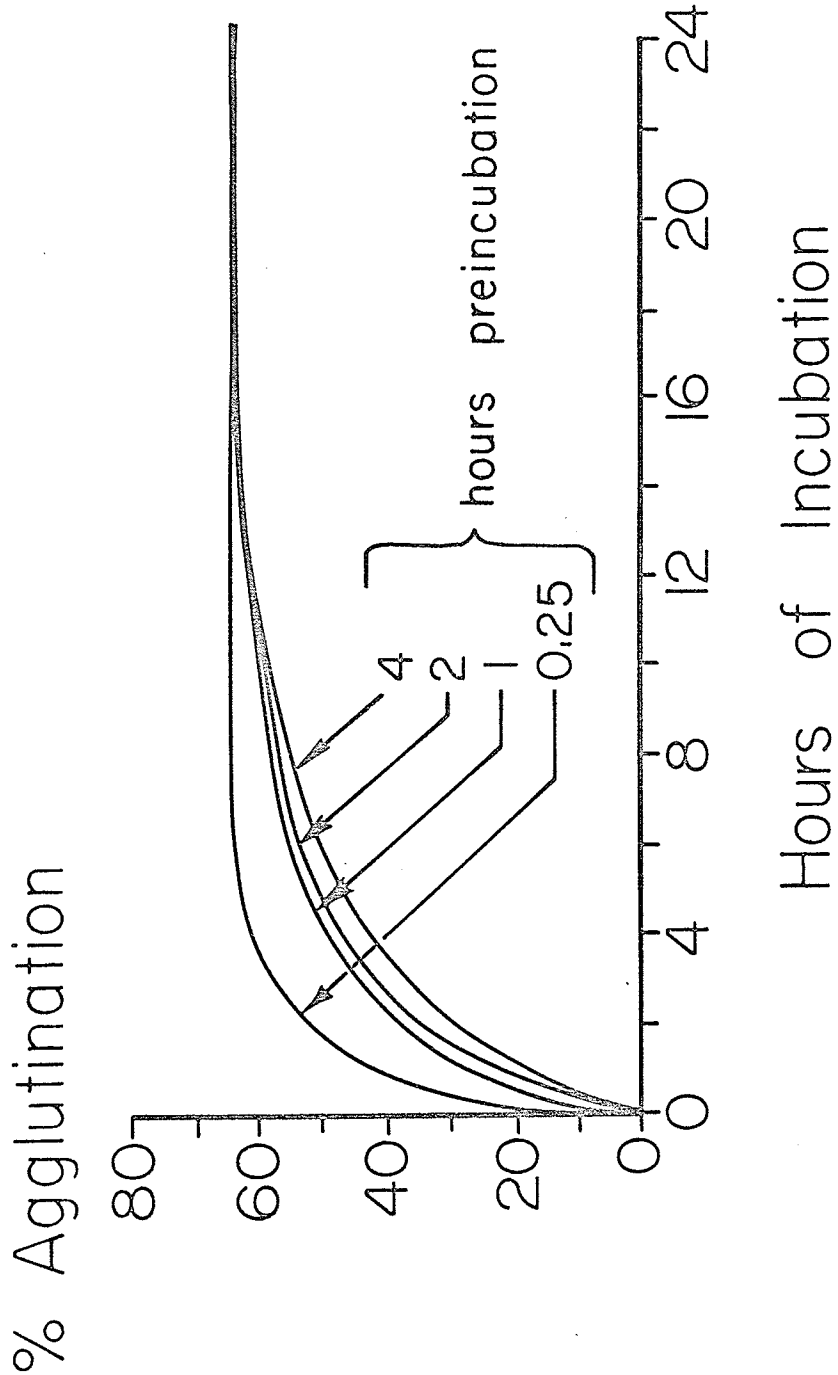
Variation in the duration of preincubation between 0.25 and 4 hours did not influence the final degree of agglutination observed at equilibrium, but only the time required to reach this level. The shorter the preincubation period, the shorter was the time taken to reach the final equilibrium. For all assays, we thus chose a preincubation period of one hour for reasons of technical convenience.

(b) Effect of Boiling and Freezing Saliva Samples

Many workers have recommended boiling saliva samples and then freezing them if assays can not be performed immediately after the saliva is collected. The boiling procedure would presumably destroy both endogenous and bacterial enzymic activity which could alter the specificity of the salivary blood group substances.

Schiff, in 1931, described the spontaneous destruction of blood group antigens in saliva (cited in Hartmann, 1941). Hartmann (1941) demonstrated enzymic activity in human submandibular gland tissue which resulted in the destruction of salivary BGS. He also found that saliva kept at 37°C. usually lost all of its blood

Figure 4 - 2. Equilibrium-time study of a system containing hog group A substance, A_1 red blood cells and human anti-A serum when the duration of preincubation of hog group A substance and anti-A serum was varied between 0.25 and 4 hours.


% Agglutination
80
60
40
20
0
0
4
8
12
16
20
24
Hours of Incubation
hours preincubation
4
2
1
0.25

group activity within 24 hours. However, if the saliva was boiled, it could retain its original activity for over a month. The addition of an anti-bacterial agent also retarded the loss of BGS. Hartmann concluded that most of the spontaneous loss of salivary BGS was due to bacteria; but he did not exclude the possibility of a contribution by endogenous salivary enzymes. Menguy et al. (1970) observed α-L-fucosidase and β-N-acetyl-D-glucosaminidase activities in sterile parotid and submandibular-sublingual salivas. Other glycosidase activities, which included β-D-galactosidase and neuraminidase, were found only in whole saliva and were therefore probably of bacterial origin.

It would appear from these reports that inactivation of these enzymes by boiling the saliva prior to the assay would be desirable. We have therefore tested the effects of both boiling and freezing on the blood group activity of saliva samples.

Submandibular saliva was collected from subjects H.C. (experiment H.C.1) and B.Y.O., vortexed and divided into two aliquots. The first of these aliquots was again divided in two: one being assayed immediately for blood group activity (submandibular saliva) and the other frozen at -60^{o}C. overnight and thawed, centrifuged and assayed the next day (frozen). The second aliquot was placed in a boiling water bath for ten minutes and then centrifuged at 1740 x g for ten minutes. The supernatant was removed, vortexed and divided in two: one portion being assayed immediately (boiled) and the other being frozen at -60^{o}C. and assayed the next day (boiled and frozen). The determinations were done in triplicate and standard was included with each day's

assay.

The effect of boiling was retested later on another sample of H.C. saliva (H.C.2) and on the saliva of another subject, R.K. Determinations were again in triplicate. Results of both experiments are shown in Table IV - 2.

We have found that when submandibular saliva was placed in a boiling water bath for ten minutes, it resulted in a lower blood group activity than when the saliva was assayed immediately (Table IV - 2). Kin (1939) reported that salivary BGS was stable at 100°C. for 20 minutes. However, we observed that during the boiling procedure an opaque precipitate appeared which formed a pellet upon centrifugation. The clear supernatant was used for BGS determinations and it may have been that a certain amount of BGS had co-precipitated with this material and was lost. Rex-Kiss (1942) found that chemical removal of mucin from saliva resulted in a loss of blood group activity.

Whole saliva was routinely centrifuged prior to the BGS determination to remove bacteria and cell debris. In one experiment, wax-stimulated whole saliva was collected, vortexed and divided into two aliquots. One aliquot was placed in a boiling water bath for 10 minutes and then centrifuged while the other aliquot was only centrifuged. The boiled-centrifuged sample showed considerably more activity than did the centrifuged sample. This is not surprising since many bacterial enzymes remain in the supernatant after centrifugation of whole saliva (Ferguson, 1968). Although

Table IV - 2

The Effect of Boiling and Freezing on the Blood Group A Activity of Submandibular Saliva

Subject and Experiment	Procedure applied to Submand. Saliva	mg% BGS
H.C.1	untreated	129
"	boiled	104
"	frozen	64.7
"	boiled and frozen	110
B.Y.O.	untreated	148
"	boiled	79.0
"	frozen	93.4
"	boiled and frozen	105
H.C.2	untreated	182
"	boiled	164
R.K.	untreated	43
"	boiled	37.7

no attempt was made to collect submandibular saliva aseptically, it is unlikely that bacteria would greatly influence the results, as duct saliva would lack an indigenous bacterial population.

Those submandibular saliva samples which were frozen immediately after collection contained a precipitate when they were thawed. The samples were centrifuged and the assays of the supernatants indicated a large loss in blood group activity compared with samples assayed immediately (Table IV - 2). Samples which were boiled and centrifuged prior to freezing did not contain a precipitate and the BGS determinations indicated concentrations comparable with those samples which were boiled and centrifuged but not frozen. In order to control to some extent the inevitable loss of BGS with saliva storage, it became routine practice to boil, centrifuge and freeze all submandibular samples which could not be assayed immediately.

(c) The Effect of Ionic Strength and the Concentration of Non-Blood Group Active Salivary Components on the Hemagglutination-Inhibition Reaction

It has been shown that the composition of saliva depends upon variables such as flow rate and duration of stimulation. Amongst those components which can show a change are the concentration of salivary proteins and the ionic strength. As one of the objects of the study was to observe the effect of some of these same variables upon the concentration of blood group substances, it was desirable to see whether changes in the concentration of

salivary components lacking blood group specificity, and changes in ionic strength, affected the hemagglutination-inhibition assay, and whether high protein concentrations could produce non-specific inhibition.

Submandibular saliva was collected at a low flow rate and then at a high flow rate from a group A non-secretor (C.D.). The respective protein concentrations were 135 mg% and 360 mg%. A quantitative hemagglutination-inhibition assay was performed on each of the samples using saliva dilutions between neat and 1/4 and anti-A diluted to 1/125 (experiment A). Even with such highly diluted antiserum there was little or no inhibition when compared with the PBS control. It would therefore appear that salivary proteins which lack blood group specificity show little or no ability to inhibit agglutination.

A second approach was then taken. Submandibular saliva was collected from a group A secretor individual (H.C.) and from a group A non-secretor (C.D.). The H.C. saliva was vortexed and divided into two aliquots. The first was assayed for blood group activity in the normal manner. For the second aliquot, all the saliva dilutions were made up with non-secretor (C.D.) saliva serving as diluent. The anti-A and test cell suspension were prepared with PBS. The assay was performed in duplicate (experiment B).

The experiment was repeated with different samples of H.C. and C.D. saliva. In this case (experiment C) the C.D. saliva was dialyzed overnight against PBS before it was used as diluent. The

assay was done in triplicate.

To test further the effect of ionic strength and the concentration of salivary components other than BGS, an experiment involving 4 different diluents was performed (experiment D). Submandibular saliva was collected from a blood group A non-secretor (C.D.). The potassium and sodium concentrations were determined to allow calculation of the approximate ionic strength and then the saliva was boiled and centrifuged. The supernatant was divided into two aliquots; one was frozen immediately (C.D. saliva) and the other was dialyzed overnight against PBS before being frozen (dialyzed C.D. saliva). The submandibular saliva of a group A secretor (B.Y.O.) was then assayed for blood group A activity using the two samples of the non-secretor saliva as diluent. As well, quantitative hemagglutination-inhibition tests were performed on the same sample of B.Y.O. saliva using as diluents PBS (PBS) and PBS which had been diluted to the same ionic strength as the C.D. saliva (1/4 PBS). The determinations were done in triplicate.

An experiment was also carried out to test the effect of ionic strength alone on the reaction (experiment E). Submandibular saliva was collected from the subject R.K., PBS, diluted with deionized water to strengths of 1/4, 1/2, and 3/4 of the original PBS, as well as undiluted PBS and deionized water were used to dilute the R.K. saliva in hemagglutination-inhibition tests. The assays were performed in duplicate.

The results for experiments B, C, D and E are given in

Table IV - 3.

Even at high flow rates submandibular saliva is hypotonic with respect to serum. Thus, in experiment B, one sees a combined effect on the assay of both a decreased ionic strength and the constituents of the non-secretor saliva. The value for the BGS determination with C.D. saliva as diluent was over twice that with PBS as diluent. When the non-secretor saliva was dialyzed against PBS prior to being used as diluent (experiment C) the values obtained were again higher than the PBS control, but the difference was less than that seen in experiment B.

The actual values from experiments B and C can not strictly be compared as different samples of both H.C. and C.D. saliva were used and the experiments were performed on different dates. However, experiment D did tend to confirm the relationship. In experiment D, when dialyzed non-secretor saliva was used as diluent, a value for the BGS of the secretor saliva was obtained which was intermediate between those values obtained with PBS and undialyzed C.D. saliva used as diluent. An intermediate value was also obtained when saliva dilutions were prepared with 1/4 PBS. Experiment E also demonstrated increased hemagglutination-inhibition when the ionic strength was lowered.

It would appear therefore that decreased ionic strength and the presence of relatively large volumes of non-secretor saliva increased the apparent blood group activity of the secretor saliva. Furthermore, the two effects appeared cumulative.

Table IV - 3

The Effect of Ionic Strength and Non-secretor Saliva on the Estimation of Blood Group A Activity of Submandibular Saliva

Experiment	Diluent	mg% BGS
B	PBS	84.4
	C.D. saliva	172
C	PBS	77.9
	dialysed C.D. saliva	102
D	PBS	52.9
	dialysed C.D. saliva	63.9
	C.D. saliva	73.0
	1/4 PBS	62.1
E	PBS	61.7
	3/4 PBS	65.9
	1/2 PBS	84.4
	1/4 PBS	90.3
	deionized water	142

Agglutination and precipitation are two-stage reactions. The first is the formation of antibody-antigen complexes, followed by the second stage which is the linking together of these complexes to form a lattice. The combination step occurs rapidly whereas lattice formation requires a considerable length of time before an equilibrium is reached.

Jerne and Skousted (1953) found an increased rate of bacteriophage-neutralization by specific antibody when low salt concentrations were employed. Similar results were observed in a luciferase, anti-luciferase system (Tsuji et al., 1962). Barnes (1966) showed increased specific and non-specific binding of anti-D γ-globulin to red cell stroma when the ionic strength was decreased below physiological levels. At a constant ionic strength the species of ions present also influenced the first stage agglutination in an Rh-anti-Rh system in a manner which appeared to be a function of the hydrational properties of the ions (Good and Wood, 1971a and b).

The second stage of agglutination has been shown to be inversely related to the zeta potential of the red cells (Pollack et al., 1965; Pollack and Reckel, 1970). Treatment of red cells with proteolytic enzymes, or an increased ionic strength or dielectric constant of the medium decreased the zeta potential of Rh(+) cells and increased agglutination by specific antibody (purified anti-Rh_o (D) immunoglobulin). The authors suggested that a decreased zeta potential allowed the cells to approach each other more readily so that the distances between the cells were small

enough to be spanned by an antibody molecule.

The hemagglutination-inhibition system is more complex than those which are described above, as three reactants are involved. The preincubation included soluble antigen and antiserum. The ionic strength of the preincubation mixture was lower than that of the final reaction mixture, especially in those assays in which the secretor saliva was diluted with 1/4 PBS or C.D. saliva. However, as the anti-A-BGS reaction is reversible, a new equilibrium would occur at the higher ionic strength of the final incubation mixture. If, in our system, a decreased salt concentration resulted in an increased combination of antibody with antigen, there would tend to be more anti-A neutralized during preincubation by secretor saliva diluted with 1/4 PBS or C.D. saliva than when diluted with PBS. By the same reasoning, however, this effect would tend to be offset by an increased uptake of anti-A by the red cells.

A more likely explanation for the higher inhibition titres in the presence of low ion concentrations would involve the second stage in agglutination. The lower ionic strengths would increase repulsion between the group A cells and consequently result in lower levels of agglutination. This would result in an apparently higher inhibition titre.

The higher inhibition achieved with secretor saliva which had been diluted with dialyzed C.D. saliva, relative to that which had been diluted with PBS can not be explained by an ionic strength effect. It would appear that some factor in saliva which itself has no blood group activity may augment the hemagglutination-inhibition

by BGS. Pollack and Reckel (1970) observed no effect of the viscosity of the medium on the amount of hemagglutination. They do not, however, give any data on the effect of viscosity on the rate of the reaction. When saliva is used as diluent, the increase in viscosity in the system is considerable. The high viscosity may have decreased the rate of reaction such that equilibrium was not achieved in the 20 hours of incubation. This would result in less hemagglutination and an apparently higher blood group activity of the secretor saliva.

In actual hemagglutination-inhibition assays of saliva samples, all dilutions were performed with PBS. In most cases the saliva dilutions were quite high. Therefore, although fairly large differences in ionic strength can occur between saliva samples, the ionic strength in the assay system would be close to that of PBS. Differences in the concentration of non-blood group active salivary constituents between samples would also not be as great as between samples diluted with non-secretor saliva and those diluted with PBS. In any case the changes in the concentration of BGS under varying conditions of stimulation as shown in the data presented in subsequent sections, are too great to be explained by either of these effects.

(d) The Effect of Low Molecular Weight, Blood Group Specific Substances on the Quantitative Hemagglutination-Inhibition Assays

There have been reports that oligosaccharides which show blood group specificity are present in saliva and other body fluids.

We have attempted to find the contribution of these low molecular weight substances to the total blood group activity of submandibular saliva.

Fifteen ml of submandibular saliva was collected from a group A secretor (H.C.). The saliva was concentrated by ultrafiltration with a UM-10 membrane (membrane retains molecules above 10,000 M.W.). The filtrate was again subjected to ultrafiltration with a UM-05 membrane (membrane retains molecules above 500 M.W.). Protein concentrations and blood group A activities were determined at each stage. The results are shown in Table IV - 4.

About 25% of the total blood group activity initially subjected to the filtration procedures was not recovered. The fluid retained by the UM-10 membrane was extremely viscous and some loss of material was inevitable. As well, some BGS may have been denatured by the concentration procedures or may have remained absorbed to the membrane. A 14% loss of protein also occurred during the first filtration (UM-10) and there was a further loss of 12% of protein from the UM-10 filtrate when subjected to the second filtration (UM-05).

A small amount of blood group activity was present in the UM-10 filtrate. This was probably due to low molecular weight oligosaccharides which possessed blood group specificity. However, the contribution to the total blood group activity of the submandibular saliva made by these low molecular weight molecules would be negligible.

Table IV - 4

Fractionation of the Blood Group A Activity of Submandibular Saliva by Ultra-filtration

Sample	Volume	Wt. BGS	Wt. Protein
Unfiltered saliva	15 ml	31.5 mg	25.2 mg
Retained by UM-10	3 ml	23.6 mg	16.4 mg
Passing UM-10	12 ml	not tested	5.2 mg
Retained by UM-05	3 ml	1.3×10^{-3} mg	1.8 mg
Passing UM-05	8 ml	1.2×10^{-2} mg	2.8 mg

(e) Evaluation of the Method

During development of the technique we experimented with different centrifugal forces and times in the centrifugation step. Both were found to be critical to obtain good separation of free from agglutinated cells. Microscopic examination of the two layers indicated that several combinations of time and centrifugal force gave good separation. A 30 second spin at 45 x g was amongst those giving optimal separation. This was the time and force used in the quantitative hemagglutination technique of Dybkjaer (1966), upon which we based our quantitative hemagglutination-inhibition method. We thus used this combination in all experiments.

When the technique was initially developed, multiple assays of the same sample of group A secretor saliva resulted in coefficients of variation of between 9 and 12%. With practice, this was reduced to between 2 to 6%. All precision experiments for H substance had coefficients of variation within this latter range.

A possible source of variation is that due to inaccurate pipeting. Twelve, 100μl aliquots from a single 1% red blood cell suspension were dispensed into tubes, washed once with PBS and hemolyzed with 1 ml of Drabkin's solution. When the optical densities were determined at 542 nm a coefficient of variation of 0.7% was found.

Another source of error may occur in the separation of free cells from agglutinated cells. Aliquots of 250μl were taken from a single equilibrium mixture of BGS standard, anti-A, and indicator red cells. The percentage agglutination was determined

by the usual procedure. When all the tubes were centrifuged together at 45 x g for 30 seconds, a coefficient of variation of about 1% was obtained. The sources of error would include those which occurred in the separation of the layers which contained free cells from those which contained agglutinated cells and in the addition of Drabkin's solution. If the tubes were divided into two groups which were then centrifuged separately, the coefficient of variation increased to 2%. The decrease in precision was assumed to be due to small inconsistencies in centrifugation.

Kabat et al. (1947) were able to obtain between 17 and 127 mg of purified BGS per litre of mixed saliva. Our results with quantitative hemagglutination-inhibition are expressed in terms of mg% of the hog gastric mucosal standard. Our values appear to overestimate the concentration of salivary BGS and must therefore only be considered as relative values.

The activities of hog A substances are known to approximate those of human A substances (Gibbs et al., 1961a). However, the purified hog BGS used in our experiments may have a decreased potency as a result of the purification process. Gibbs et al. (1961a) found that human ovarian cyst BGS, purified by phenol extraction, had much less activity than did an equal weight of desiccated original cyst fluid. If our standard had a lower activity per unit weight of BGS than did saliva, an overestimate of salivary BGS would result. The presence of contaminants in the standard would also contribute to an overestimate.

Gibbs et al. (1961a) compared BGS from a number of sources. The slopes of the assay curves (probit values against concentration of inhibiting substance) varied from day to day but were always similar to that of the hog gastric mucosal standard for that day. They tested two partially purified salivary BGS preparations but they did not present any data for untreated saliva. In our study, we found the slope of the assay curve for submandibular saliva to be consistently lower than that for the BGS standard. The assay curves for different subjects were also not all parallel. For this reason we have, in most cases, refrained from comparing actual BGS concentrations in samples collected on different days or from different subjects; that is, we have studied relative changes and relative differences. An exception to this was the experiments to observe the effect of freezing the saliva sample on the BGS concentration. The results from these experiments suggest that this restriction placed on the technique is justified, as the apparent concentration of BGS in samples which were boiled and frozen was somewhat higher than those which were only boiled.

II Effect of Several Variables on the Concentration of Blood Group A Substance in Submandibular Saliva

(a) Duration of Stimulation

Unstimulated submandibular saliva and one minute samples of stimulated submandibular saliva collected at a constant flow rate over

periods of 12 to 15 minutes were obtained from 4 group A secretors. The BGS and protein assays were performed on the samples. The results from three subjects are shown in Figures 4 - 3, 4 - 4, 4 - 5 and 4 - 6.

In order to show protein concentration and BGS concentration on the same scale, the mean concentration of protein or BGS for all the one minute samples and the unstimulated sample was calculated. The concentrations of protein or BGS for each of the samples was expressed as a percentage of this mean.

Figure 4 - 3 shows the results obtained with the subject H.C. when the saliva was collected at a flow rate of 1.7 $\pm$ 0.04 ml/min. The BGS showed little change in concentration upon continued stimulation. The protein concentration, after an initial drop, rose to a maximum of 2.5 times the unstimulated level during the third minute. Upon continued stimulation it showed little further change.

In a later experiment on this subject (Figure 4 - 4), when the saliva was collected at an average flow rate of 1.9 $\pm$ 0.05 ml/min., the BGS reached a maximum level during the fifteenth minute; a concentration, which was about 2.5 times that found in the unstimulated sample. The protein concentration rose to a maximum value during the ninth minute, approximately 2.5 times the unstimulated level, and then slowly fell over the next six minutes. A third experiment on this subject (not shown) gave results similar to those shown in Figure 4 - 4.

Figure 4 - 5 shows the results for the saliva of the subject R.K. (average flow rate 3.4 $\pm$ 0.1 ml/min.). The concentration

Figure 4 - 3. The effect of duration of stimulation on the blood group A activity and the protein concentration in the submandibular saliva of the subject H.C. A sample of unstimulated submandibular saliva was collected, followed by a 12 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 1.7 ml/min.

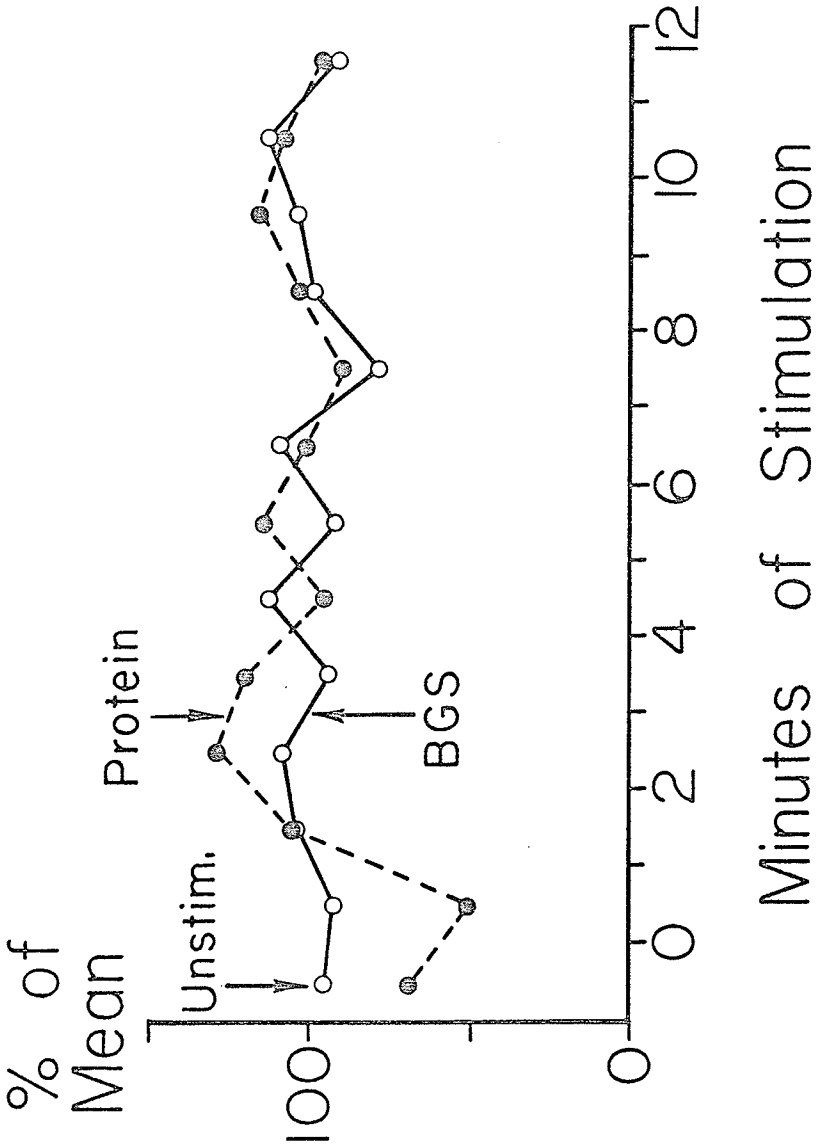

% of Mean
100
0
Unstim.
Protein
BGS
0
2
4
6
8
10
12
Minutes of Stimulation

Figure 4 - 4. The effect of duration of stimulation on the blood group A activity and the protein concentration in the submandibular saliva of the subject H.C. A sample of unstimulated submandibular saliva was collected, followed by a 15 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 1.7 ml/min.

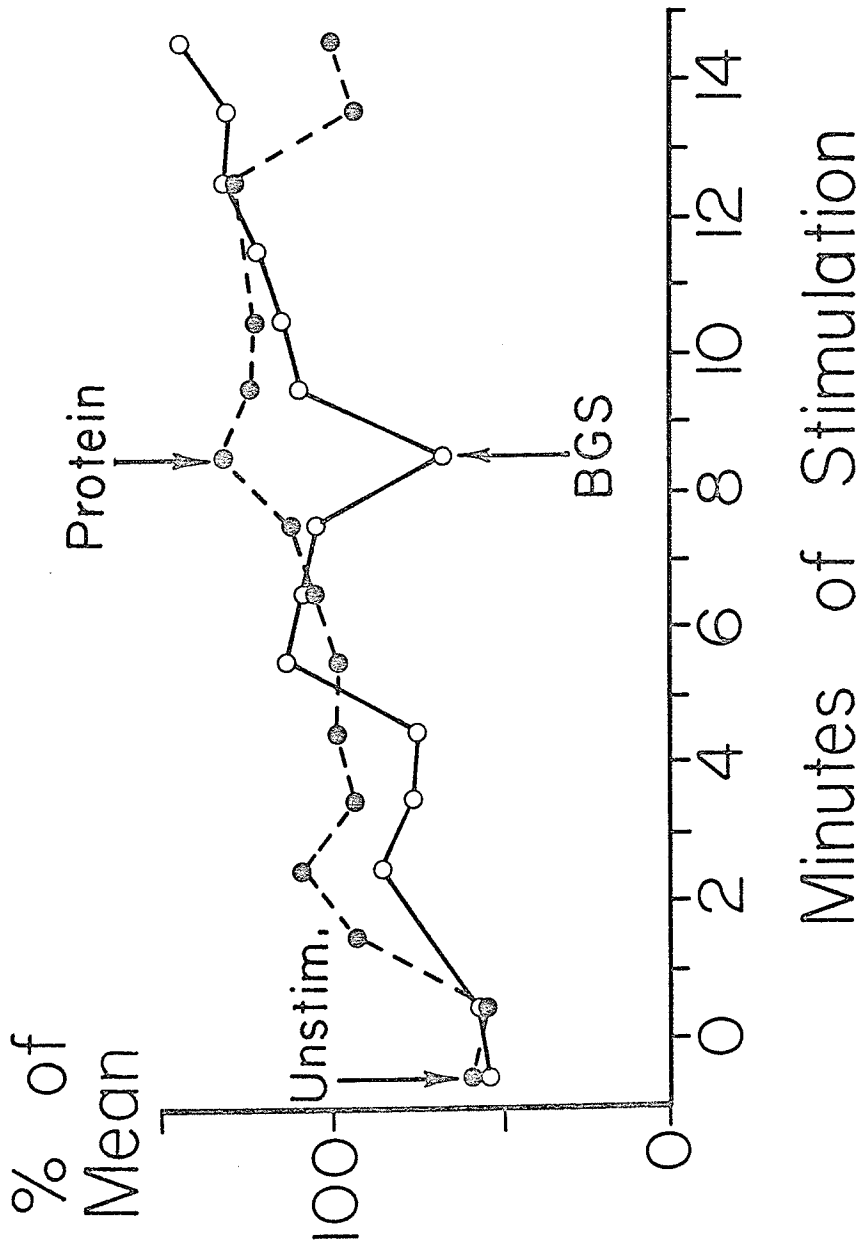

% of Mean
100
Unstim.
Protein
BGS
0
0
2
4
6
8
10
12
14
Minutes of Stimulation

Figure 4 - 5. The effect of duration of stimulation on the blood group A activity and the protein concentration in the submandibular saliva of the subject R.K. A sample of unstimulated submandibular saliva was collected, followed by a 12 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 3.4 ml/min.

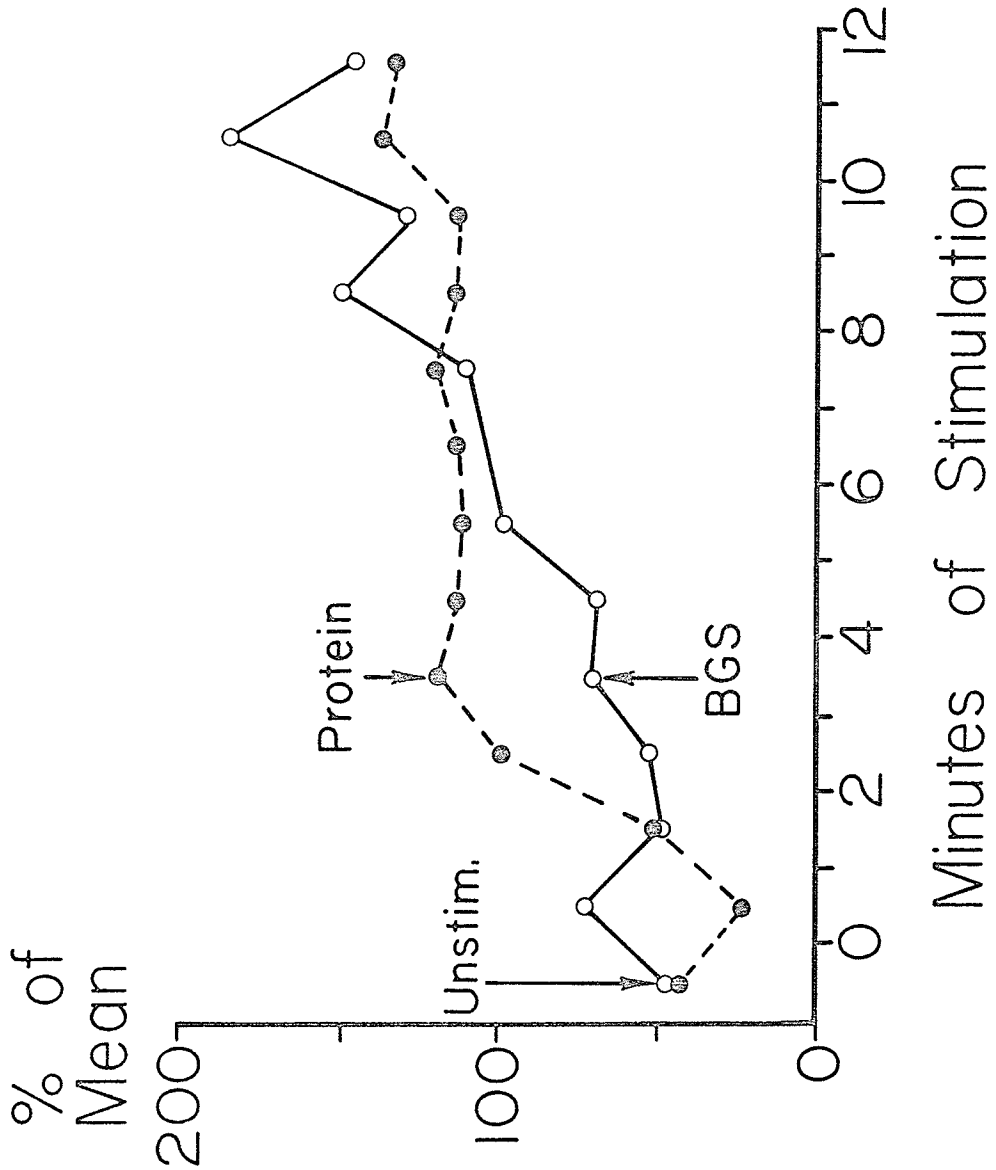
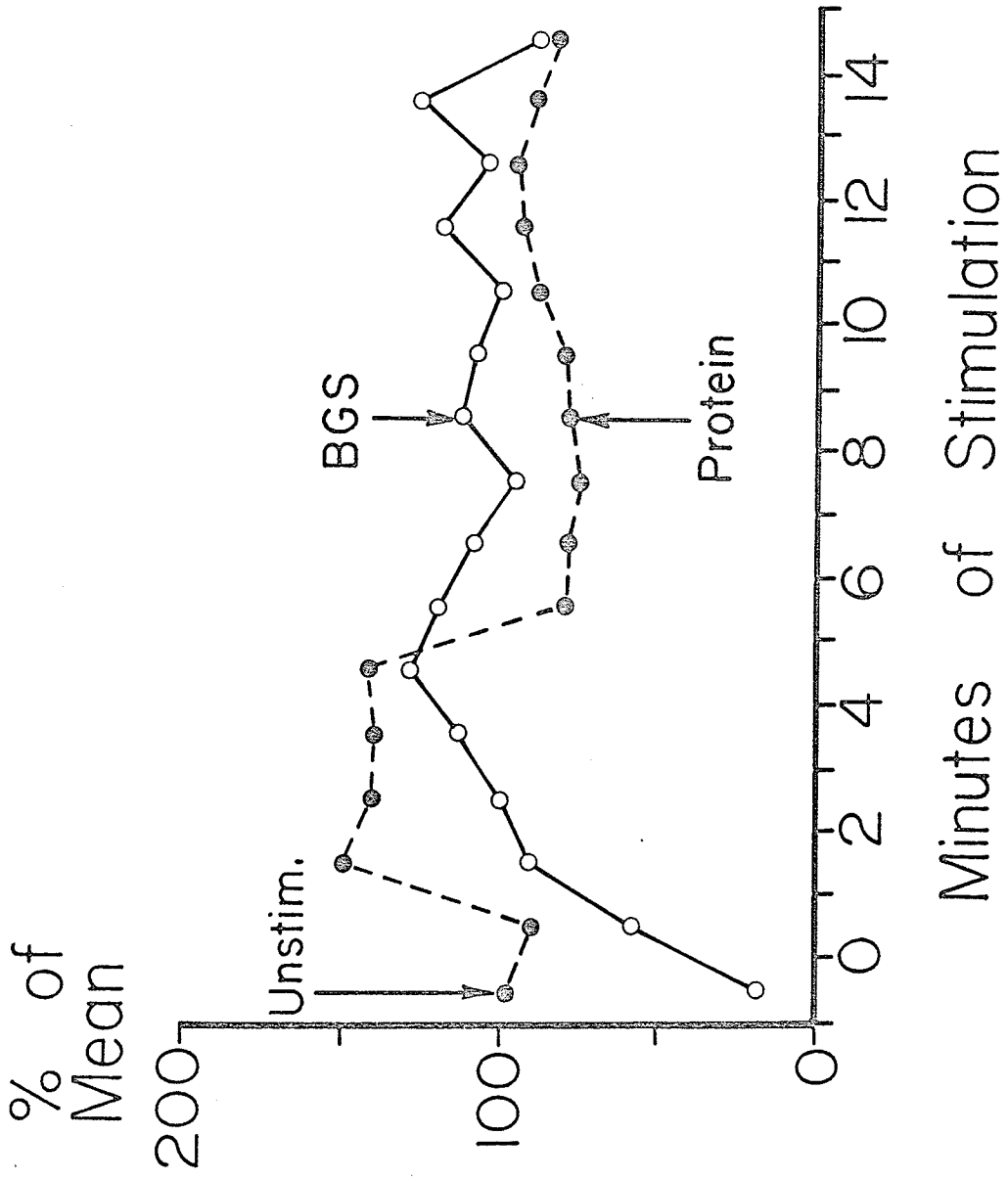

% of Mean
200
100
0
Protein
BGS
Unstim.
0
2
4
6
8
10
12
Minutes of Stimulation

Figure 4 - 6. The effect of duration of stimulation on the blood group A activity and the protein concentration in the submandibular saliva of the subject B.Y.O. A sample of unstimulated submandibular saliva was collected, followed by a 15 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 1.2 ml/min.


% of Mean
200
100
0
Unstim.
BGS
Protein
0
2
4
6
8
10
12
14
Minutes of Stimulation

of BGS increased continuously over the period of stimulation. The maximum concentration (4 times the unstimulated level) was obtained during the eleventh minute of stimulation. The protein concentration increased to about 2.5 times the unstimulated value during the first 4 minutes of stimulation and then showed little subsequent change. A second experiment on this subject showed similar results over 10 minutes of stimulation.

The results for the subject K.K. (Figure 4 - 13) also showed a continuous increase in blood group activity over the period of stimulation to 3 times the unstimulated level.

Submandibular saliva collected from B.Y.O. (Figure 4 - 6) showed a sixfold increase in the concentration of BGS during the first five minutes of stimulation and then the concentration decreased slowly over the next 10 minutes.

Therefore, in most experiments the concentration of BGS in submandibular saliva showed considerable change with the duration of the sour lemon drop stimulation. However, the pattern of the response appeared to be different amongst the different subjects tested. When the experiment was repeated on the same subject at different times, different responses were also seen (e.g. Figures 4 - 3 and 4 - 4). At the flow rates tested, none of the subjects showed a fatigue effect in the concentration of BGS within the period of the collection; in fact, in most cases the concentration of BGS after prolonged stimulation was considerably higher than the concentration in the unstimulated sample. Caldwell and Pigman (1966) found no fatigue effect

with prolonged stimulation at various flow rates in the concentrations of carbohydrate, non-dialyzable nitrogen or total nitrogen. We found that the protein concentration increased with duration of stimulation in most cases. However, a close correlation between BGS and protein was not seen.

(b) Effect of Flow Rate on the Concentration of Blood Group Substances in Submandibular Saliva

1 Collection of Saliva at a Low Flow Rate followed by a Collection at a High Flow Rate

Submandibular saliva was collected for 5 minutes at a low flow rate and for a subsequent 10 minutes at a high flow rate from 3 group A secretors. In the case of a fourth subject, B.Y.O., saliva was collected at a low flow rate for 3 minutes and at a high flow rate for 12 minutes. An unstimulated sample was obtained prior to stimulation for all subjects.

Figure 4 - 7 shows the results obtained for the subject H.U. The average flow rate for the first 5 minutes of stimulation was 1.2 ± 0.05 ml/min. and 1.6 ± 0.06 ml/min. for the next 10 minutes. The BGS concentration rose continuously at the low flow rate. When the flow rate was increased, the level fell and then increased to oscillate about a plateau. The maximum concentration occurred during the twelfth minute of stimulation and was almost four times the unstimulated concentration. The protein concentration

Figure 4 - 7. The effect of flow rate and duration of stimulation on the blood group A activity and the protein concentration in the submandibular saliva of the subject H.U. A sample of unstimulated submandibular saliva was obtained. A 5 minute collection of submandibular saliva was then made at a low flow rate (1.2 ml/min.) followed by a 10 minute collection at a higher flow rate (1.6 ml/min.).

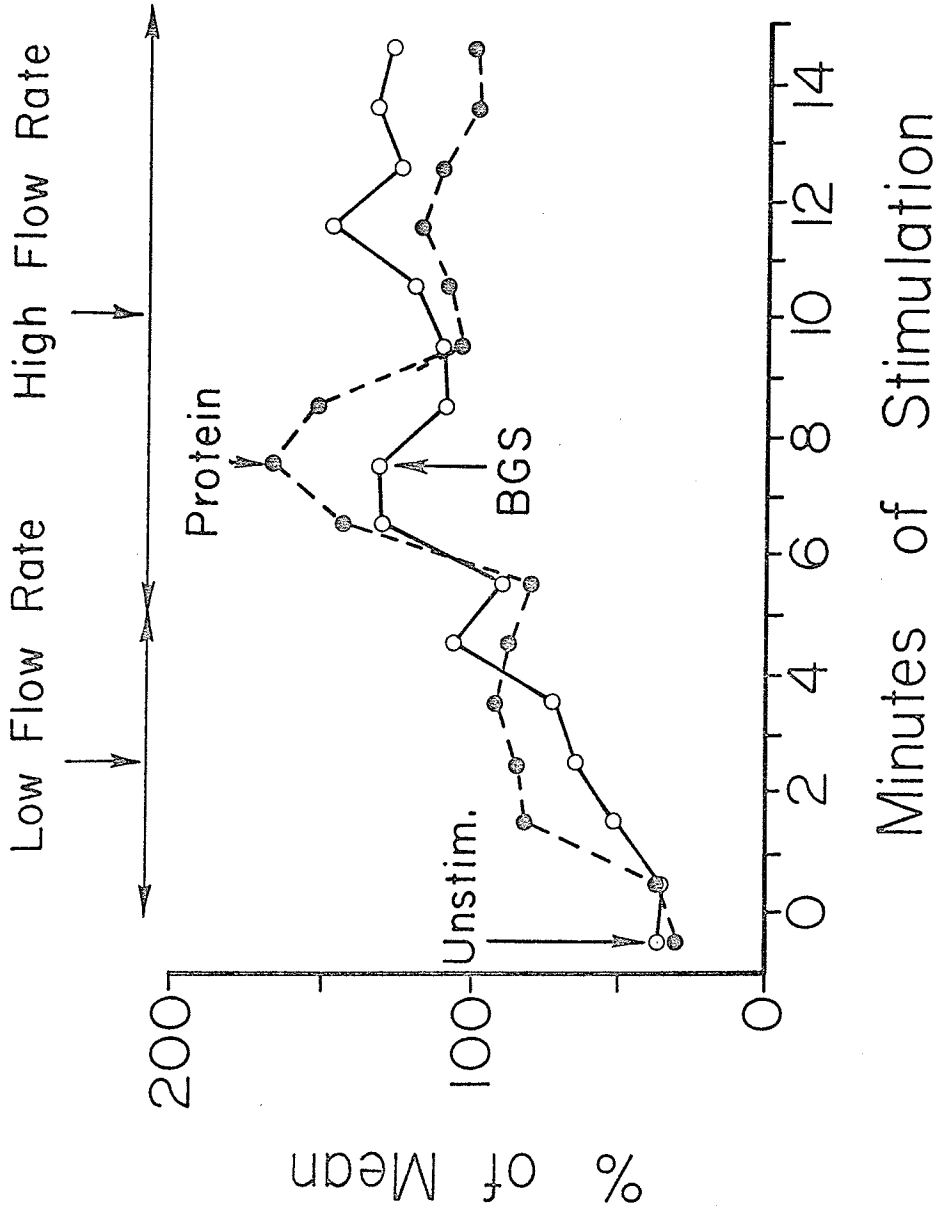
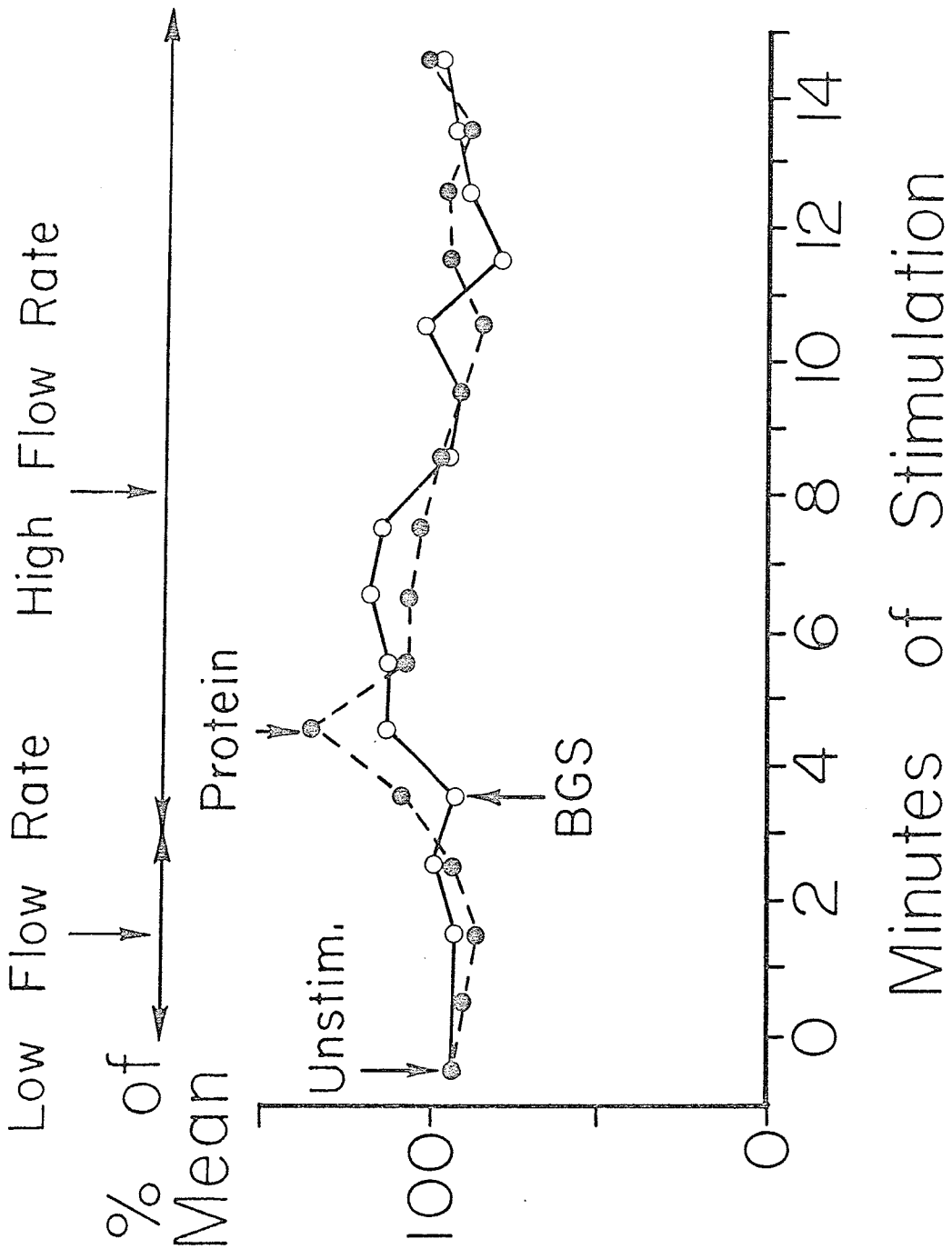

Low Flow Rate
High Flow Rate
Protein
BGS
Unstim.
200
100
0
% of Mean
0
2
4
6
8
10
12
14
Minutes of Stimulation

Figure 4 - 8. The effect of flow rate and duration of stimulation on the blood group A activity and the protein concentration in the submandibular saliva of B.Y.O. A sample of unstimulated submandibular saliva was obtained. A 5 minute collection of submandibular saliva was then made at a low flow rate (1.0 ml/min.) followed by a 10 minute collection at a higher flow rate (2.0 ml/min.).


Low Flow Rate
High Flow Rate
% of Mean
100
Unstim.
Protein
BGS
0
0 2 4 6 8 10 12 14
Minutes of Stimulation

appeared to follow that of the BGS. More or less similar results were found with the saliva of R.K. (not shown).

The results found with the saliva of B.Y.O. are shown in Figure 4 - 8. The low flow rate was maintained at 1.0 $\pm$ 0.1 ml/min. and the high flow rate at 2.0 $\pm$ 0.05 ml/min. Neither the BGS nor the protein concentration changed markedly over the period of stimulation. Similar results to those in Figure 3 - 8 were obtained with the subject H.C. In both cases the protein concentration correlated well with the BGS concentration.

Caldwell and Pigman (1966) found that when the concentration of protein-bound hexose in submandibular saliva was plotted as a function of flow rate, a continuous curve resulted. Maximum values were found in unstimulated saliva and minimum values were found in saliva collected at low flow rates. As the flow rate was increased, the concentration of BGS also increased. Their analyses were done on pooled 15 ml samples of saliva collected at a constant flow rate. Different flow rates were obtained with the aid of different stimuli.

As the concentration of total glycoprotein in submandibular saliva increased when the flow rate was increased, it might be expected that the concentration of BGS, a glycoprotein, would also increase. In some subjects there did appear to be an increase in the concentration of BGS with the higher flow rate, whereas in others there was little change. It is, therefore, difficult to make a general statement about the effect of flow rate on the concentration of BGS in submandibular saliva. It is

also difficult to dissociate the effects of flow rate from those due to the duration of stimulation. The mean BGS concentration for the 4 subjects in the saliva collected at a high flow rate (191 $\pm$ 8 mg%) was greater than that in the saliva collected at the low flow rate (68 $\pm$ 12 mg%). In the series of experiments to examine the effects due to duration of stimulation, the mean BGS concentration in the saliva collected during the first 5 minutes of stimulation (91 $\pm$ 15 mg%) was similar to that in the saliva which was collected over the remainder of the experiment (109 $\pm$ 10 mg%). However, the two sets of experiments can not validly be compared as the subjects were not the same in the two experiments.

Even if a rise in the concentration of BGS did occur, concomitant with total glycoprotein when flow rate was increased, the large drop in total glycoprotein concentration upon stimulation (Caldwell and Pigman, 1966) did not occur with BGS. One must suppose that on stimulation, there is a differential change in the rate of secretion of different species of glycoproteins. There are precedents for the supposition that under different conditions of stimulation, different relative proportions of glycoproteins can exist.

Dische et al. (1962, 1969) observed an increase in the ratio of fucose to sialic acid in dog submandibular saliva, when the dose of pilocarpine was increased. With increasing stimulation, the electrophoretic and ultracentrifugation patterns indicated

a shift in the proportions of two glycoproteins which had different molecular weights and electrophoretic mobilities. A change was observed in the relative proportions of 2 glycoproteins in cat submandibular saliva when secreted in response to electrical stimulation of either sympathetic or parasympathetic nerves (Dische et al., 1970). The saliva produced after sympathetic stimulation had a consistently higher sialic acid to fucose ratio.

More relevant to this study was the report that with an increased flow rate, the ratio of sialic acid to fucose increased in the submandibular saliva of human non-secretors of BGS (Caldwell and Pigman, 1966). The ratio of sialic acid to fucose in secretors remained constant. The authors concluded that, at least in non-secretors, the relative proportions of the individual glycoproteins can undergo change. In this study we must also assume that this can occur in order to correlate our findings on the concentration of BGS with those of Caldwell and Pigman on total glycoprotein in human submandibular saliva.

2 Collection of Saliva at a High Flow Rate followed by a Collection at a Low Flow Rate

Saliva was collected at a high flow rate for 10 minutes, at which time the sour lemon drop was removed and 5 more 1 minute samples were collected. When the stimulus was removed, the flow rate fell; however, during the 5 minutes it never decreased to the unstimulated flow rate.

Figure 4 - 9 gives the results for the subject R.K. The

average flow rates were 3.2 $\pm$ 0.1 ml/min. for the first 10 minutes and 0.7 $\pm$ 0.04 ml/min. for the last 5 minutes. By the fifth minute of stimulation the concentration of the BGS had increased to a level of about twice that of the unstimulated saliva. When the sour lemon drop was removed the concentration of the BGS fell over the 5 minutes to a level only slightly greater than that of the unstimulated sample. The protein concentration followed a similar pattern.

The concentration of BGS in the saliva of the subject H.U. (not shown) increased over the 10 minute high flow rate collection reaching a maximum of about 7 times the unstimulated level in the tenth minute. When the stimulus was removed, the concentration fell to about 3 times the unstimulated level.

The results for H.C. are given in Figure 4 - 10. The BGS increased over the period of stimulation and continued to rise after the sour lemon drop was removed. The maximum was reached in the twelfth minute; a value of over 4 times that found in the unstimulated saliva. The concentration then decreased only slightly. The protein pattern was similar to that of the BGS.

The subject B.Y.O. (not shown) showed little change in the BGS. The protein concentration increased to reach a level 4 times that of the unstimulated saliva during the sixth minute and then showed a fairly uniform decrease to about twice the unstimulated level in the fifteenth minute.

In 2 of the subjects (H.U. and R.K.) the concentration of BGS fell considerably when stimulation was terminated. The

Figure 4 - 9. The effect of flow rate and duration of stimulation on the blood group A activity and protein concentration in the submandibular saliva of R.K. A sample of unstimulated submandibular saliva was collected, followed by a 10 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 3.2 ml/min. The SLD was then removed and a further five, one minute samples were obtained (average flow rate 0.7 ml/min.).

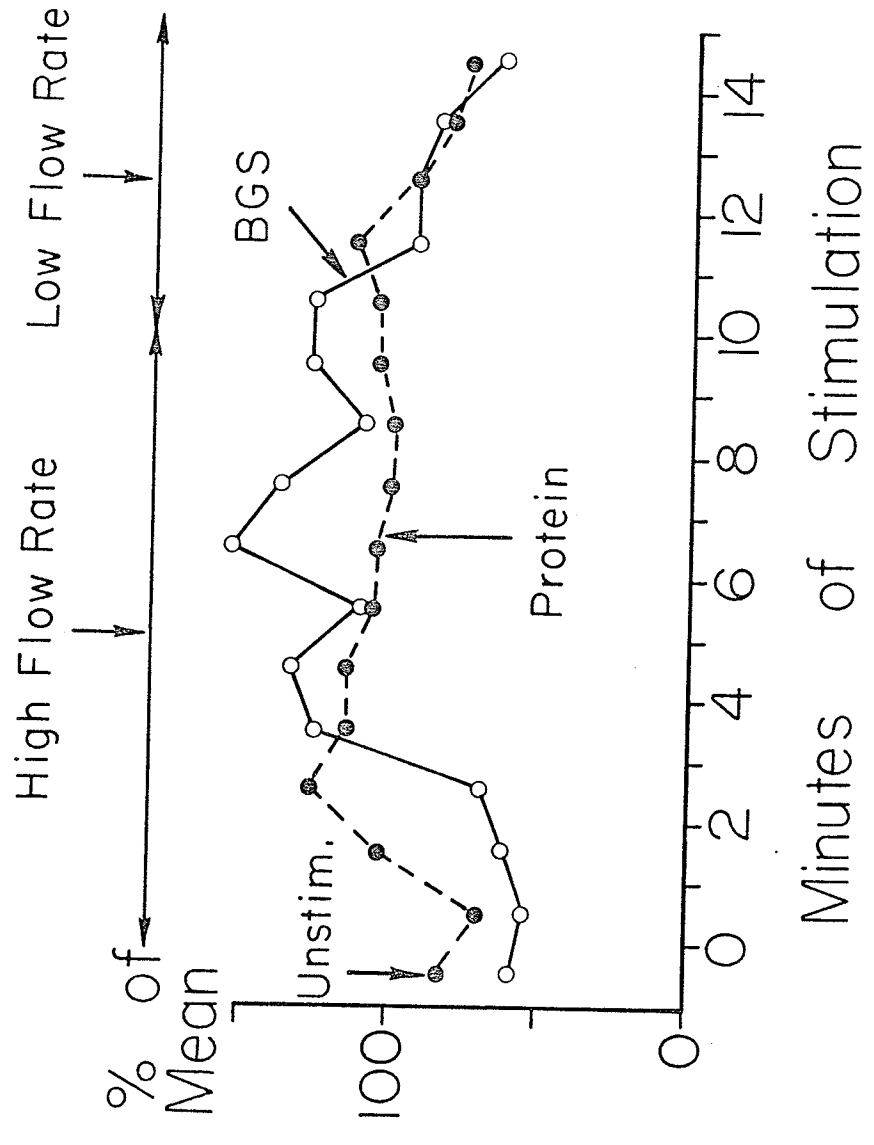
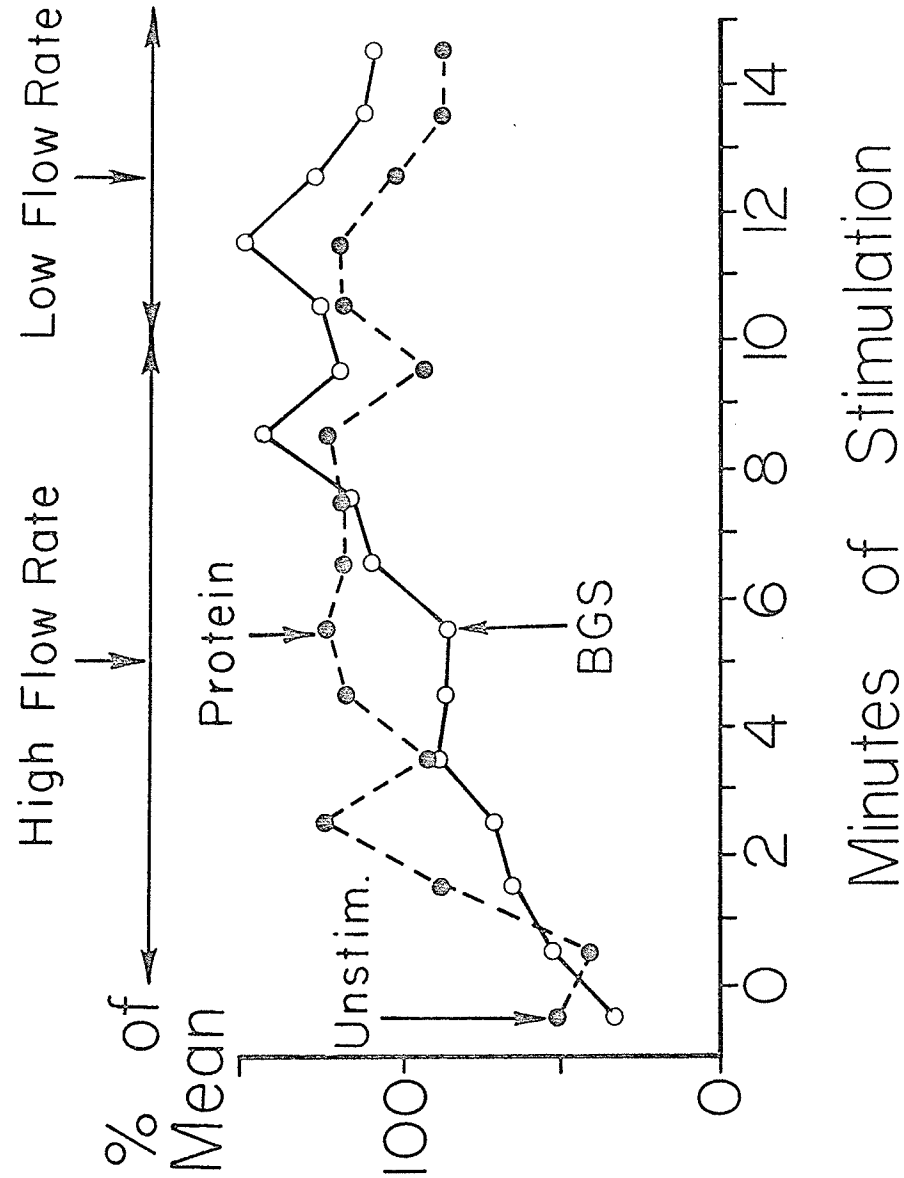

High Flow Rate
Low Flow Rate
% of Mean
100
0
Unstim.
BGS
Protein
0
2
4
6
8
10
12
14
Minutes of Stimulation

Figure 4 - 10. The effect of flow rate and duration of stimulation on the blood group A activity and protein concentration in the submandibular saliva of H.C. A sample of unstimulated submandibular saliva was collected, followed by a 10 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 2.5 ml/min. The SLD was then removed and a further five, one minute samples were obtained (average flow rate 0.8 ml/min.).


High Flow Rate
Low Flow Rate
% of Mean
100
Protein
BGS
Unstim.
0
0
2
4
6
8
10
12
14
Minutes of Stimulation

delay which occurred before the decrease in the BGS concentration in the saliva of the subject H.C. may have been due to residual dissolved sour lemon drop which continued to stimulate secretion. However, the decrease in the flow rate was only slightly more gradual in this subject than in R.K. and H.U.

(c) Effect of the Duration of Stimulation on the Ratio of A to H Activity in Submandibular Saliva

Unstimulated submandibular saliva was obtained from 3 group A secretors followed by 10, one minute stimulated samples which were collected at a constant flow rate. In the fourth subject (K.K.) it was necessary to cannulate the submandibular duct directly with a length of polythene tubing. With the latter procedure reduced quantities of saliva were obtained. Therefore, each sample of stimulated saliva contained the secretion collected over 4 minutes. For this subject the total period of the collection was 12 minutes.

Figure 4 - 11 gives results for the subject B.Y.O. Upon stimulation both A and H activity fell. Both activities increased slightly during the second and third minutes and then remained quite constant for the duration of the collection. The A/H ratio followed a similar pattern.

Continued stimulation for 10 minutes resulted in little change in the A or H activity in the submandibular saliva of the subject H.C. (not shown).

The A activity in the submandibular saliva of the subject R.K. (Figure 4 - 12) did not change markedly during the period of stimulation. The H activity, however, increased during the first 7 minutes to reach a maximum of about 3 times the unstimulated concentration during the seventh minute. The activity decreased slightly during the last 3 minutes. The A/H ratio of the sample collected in the first minute of stimulation was over 4 times the A/H ratio of the sample collected in the seventh minute.

The results for the subject K.K. are given in the Figure 4 - 13. The A activity increased over the period of stimulation to reach a maximum in the sample collected through the eighth to twelfth minutes which was about 3 times the A activity of the unstimulated sample. The H activity increased parallel to the A activity over the first 8 minutes. The H activity in the sample collected over the last 4 minutes, however, was lower than that collected during the fifth to the eighth minutes of stimulation. The A/H ratio of this last sample was over twice that of the unstimulated saliva.

There are several possible explanations for a shift in the A/H ratio with duration of stimulation. One possibility is that there may be a change in the proportional rates of secretion of two or more distinct species of blood group specific molecules which have different ratios of A/H specificities. This would be analogous to the shift in the proportional concentrations of the two glycoprotein fractions in dog submandibular saliva when the animal is given increasing

Figure 4 - 11. The effect of duration of stimulation on the blood group A and H activities in the submandibular saliva of the subject B.Y.O. A sample of unstimulated submandibular saliva was collected, followed by a 10 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 1.2 ml/min. The ratio of A activity to H activity is also shown.

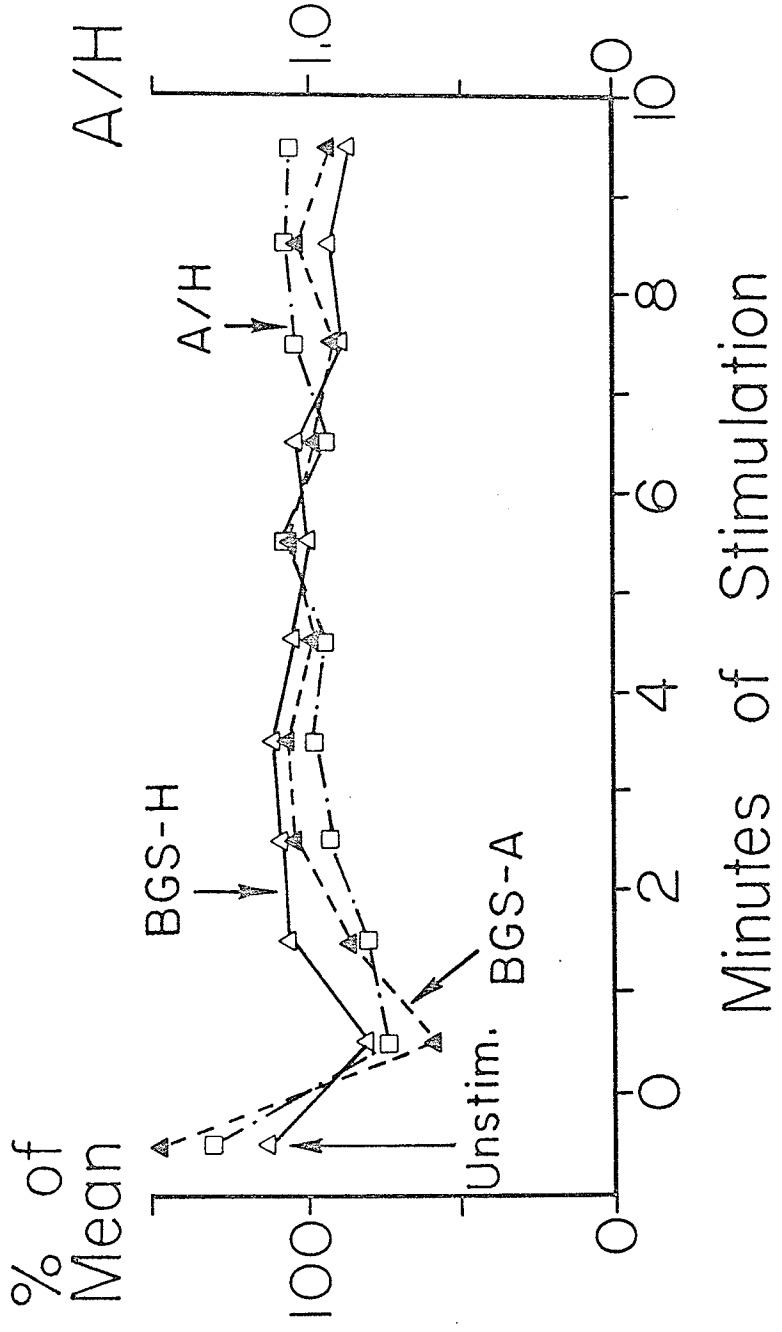

A/H
1.0
0
10
A/H
8
6
4
2
0
Minutes of Stimulation
% of Mean
100
0
BGS-H
Unstim.
BGS-A

Figure 4 - 12. The effect of duration of stimulation on the blood group A and H activities in the submandibular saliva of the subject R.K. A sample of unstimulated submandibular saliva was collected followed by a 10 minute collection of SLD-stimulated submandibular saliva maintained at a constant flow rate of 1.8 ml/min. The ratio of A activity to H activity is also shown.

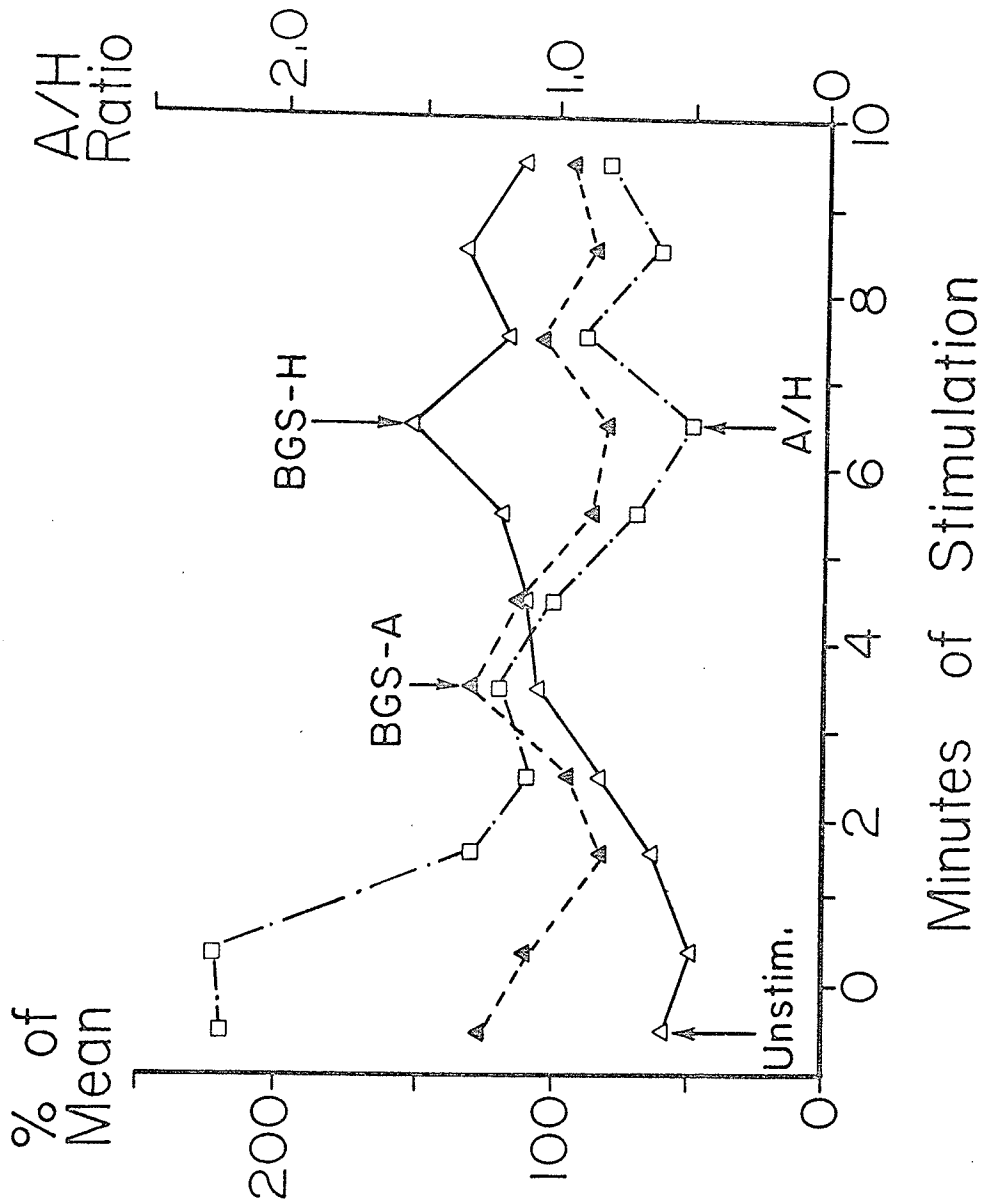
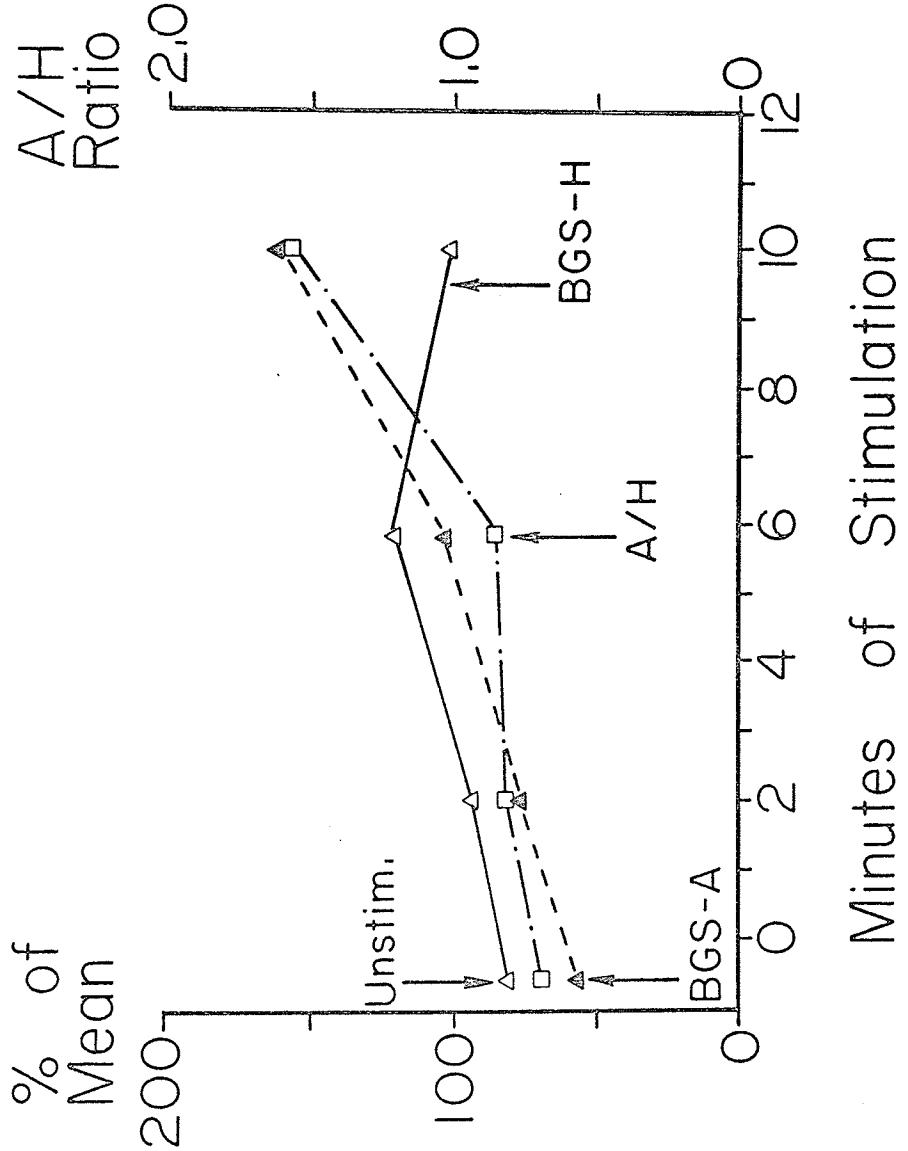

A/H Ratio
2.0
1.0
0
% of Mean
200
100
0
BGS-H
BGS-A
A/H
Unstim.
0
2
4
6
8
10
Minutes of Stimulation

Figure 4 - 13. The effect of the duration of stimulation on the blood group A and H activities in the submandibular saliva of the subject K.K. A sample of unstimulated submandibular saliva was collected, followed by a 12 minute collection of SLD-stimulated submandibular saliva. Four consecutive one minute samples of stimulated saliva was pooled prior to the assays. The ratio of A activity to H activity is also shown.


A/H Ratio
2.0
1.0
0
% of Mean
200
100
0
Unstim.
BGS-H
A/H
BGS-A
0
2
4
6
8
10
12
Minutes of Stimulation

doses of pilocarpine (Dische et al., 1962, 1969). Boettcher (1967) demonstrated that precipitation of A molecules from A_1 and A_2 salivas with Dolichos biflorus extract removed all detectable A activity and some of the H activity. The author concluded that some molecules possessed both A and H specificities while others possessed only H specificity. If prolonged stimulation produced an increase or decrease in the rate of synthesis and secretion of molecules of either population, there would be a change in the A/H ratio of the saliva.

A second possible explanation would involve shifts within a single population of BGS molecules, heterogeneous with respect to the A/H ratio. The carbohydrate chains of glycoproteins are present in varying degrees of completeness. This so-called microheterogeneity has been demonstrated for a number of glycoproteins including a porcine submaxillary mucin which possessed blood group A specificity (Carlson, 1968). The co-presence of A and H specificities on the molecule of human BGS is also an example of microheterogeneity. Those carbohydrate chains which possess the H structure lack the terminal immunodominant sugar of the A structure. Spiro (1970) has suggested that the degree of microheterogeneity of glycoproteins might be affected by the rapidity with which the precursor protein core passes through the channels formed by the membranes of the endoplasmic reticulum and the Golgi apparatus which contain the glycosyl transferases. Ericson (1968) has demonstrated that pilocarpine-stimulation caused an increased secretion of preformed glycoprotein by the

rat submandibular gland, followed by an increased rate of glycoprotein biosynthesis. Levine et al. (1972) have demonstrated carbohydrate free protein core in human parotid saliva which was collected using a prolonged gustatory stimulus. Presumably, the rate of secretion was too great to permit the attachment of the sugar units. The synthesis of a structure with A specificity requires the addition of an N-acetyl-galactosamine on to a substrate having the H structure. If upon increased or prolonged stimulation the rate of biosynthesis of BGS increased, one might expect an increase in the microheterogeneity and a fall in the A/H ratio.

One subject (R.K.) did show a large fall in the A/H ratio with duration of stimulation. The A activity remained constant over the ten minutes, whereas H activity increased. It would appear that the limiting step was the rate of addition of the N-acetyl-galactosamine. It may be relevant to note that R.K. was the only subject of the sub-group A_2 in the 4 subjects studied. Watkins and Morgan (1956b) and Boettcher (1967) have suggested that the product of the A_2 gene is less efficient than the product of the A_1 gene in converting the H precursor into A substance.

It would be difficult, however, to reconcile this theory with the increase in the A/H ratio in the subject K.K. during the eighth to the twelfth minutes of stimulation.

A third possibility for changes in the A/H ratio would involve the action of glycosidases within the submandibular gland which may cleave sugars involved in blood group specificity from preformed BGS. Hartmann (1941) has demonstrated that under

sterile conditions, pieces of submandibular gland can cause the destruction of BGS. Menguy (1970) has shown α-L-fucosidase activity in submandibular-sublingual saliva. If these enzymes are active within the gland, the relative activities of the various specific glycosidases may influence the A/H ratio of the preformed BGS molecules. When the rate of secretion is increased, the time that the BGS molecules would be subjected to the action of these enzymes would be decreased and the A/H ratio would depend more on biosynthesis.

III Relative Contributions of Individual Salivary Glands to the Blood Group Activity of the Whole Saliva

Most investigators who have made quantitative studies of salivary blood group substances have used whole saliva. An exception to this was the work of Wolf and Taylor (1964) on the respective blood group activities of lemon-juice-stimulated sublingual, submandibular and parotid salivas. They observed high activities in sublingual saliva, with inhibition titres ranging from 64 to 16,384. The inhibition titres of the submandibular saliva samples were between 16 and 1,024. Slight blood group activity was found in the parotid saliva of 5 of the 16 secretor individuals who were studied. The authors suggested that the concentration of blood group substance in the secretion was related to the proportion of mucous-secreting cells in the gland.

We have employed the previously described quantitative

hemagglutination-inhibition technique to examine the blood group A activity of a number of salivary secretions. We have included in this study, unstimulated and wax-stimulated whole saliva, unstimulated and sour lemon drop stimulated-submandibular saliva and lip-mucous-secretion and citric acid-stimulated sublingual saliva and palatal gland secretion. The study included 10 group A secretors and 2 group AB secretors.

The results for the 12 subjects are given in Table IV - 5 and the mean values with standard errors are shown in Figure 4 - 14.

A wide variation amongst individuals was found for all of the secretions. In spite of the rather large standard errors it is obvious that the blood group activity of the lip-mucous and palatal secretions and the sublingual saliva was much higher than that of whole or submandibular saliva. The whole saliva had higher activity than did submandibular saliva.

There appeared to be more group A substance in unstimulated than in stimulated lip-mucous secretion. Unstimulated lip-mucous secretion could not be collected from 3 of the individuals. If in the calculation of the mean group activity for lip-mucous secretion only those values were included for which both stimulated and unstimulated samples were assayed, this difference between stimulated and unstimulated was still observed.

Blood group activity was determined in the parotid saliva of 3 of the individuals and the values were found to be very low. This part of the study was not pursued and the results are not shown.

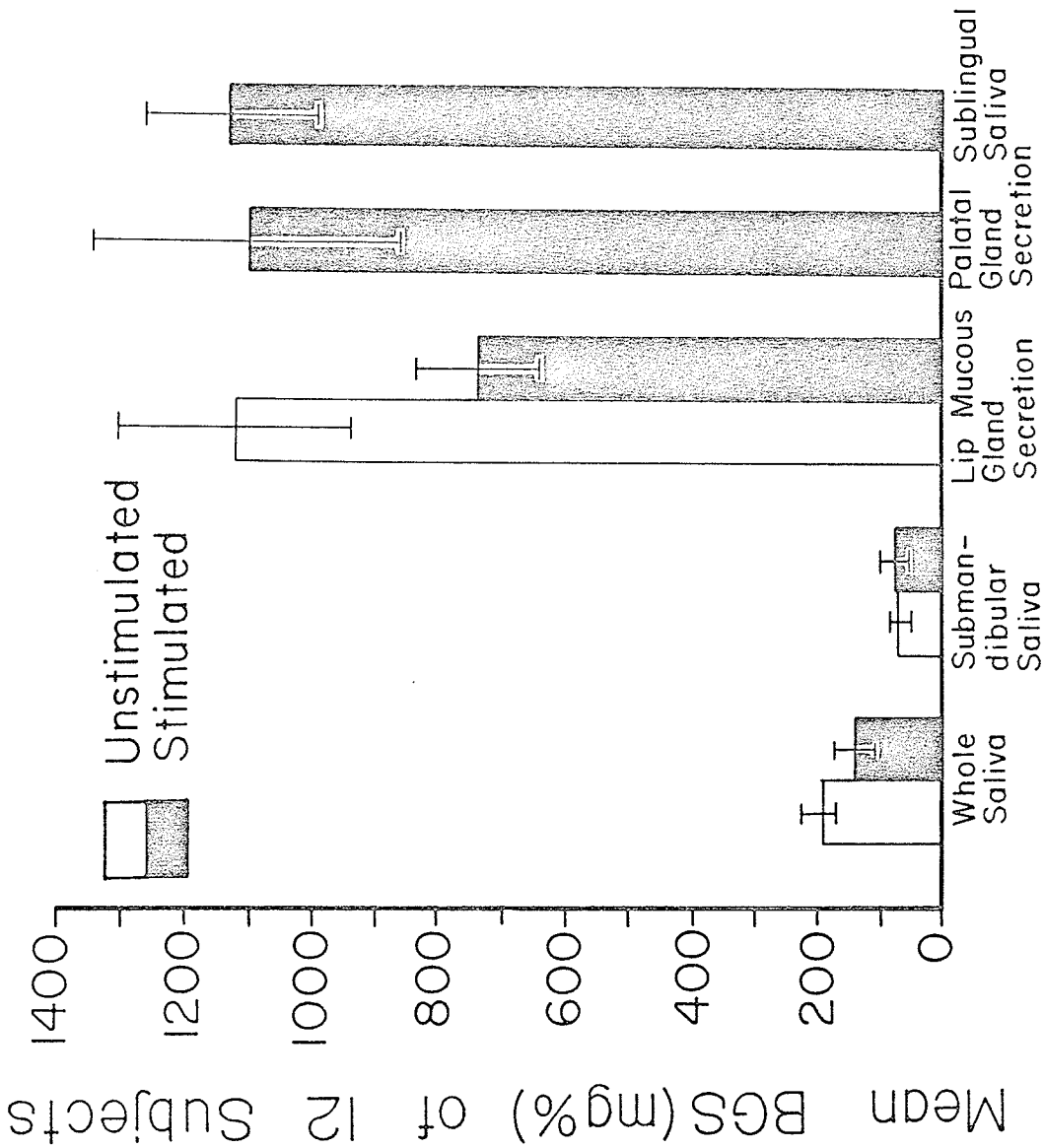
Table IV - 5

The Blood Group A Activity* in Various Salivary Secretions of Twelve Subjects

Sample Subject	Unstim. Whole Saliva	Stim. Whole Saliva	Unstim. S.M.S.	Stim. S.M.S.	Unstim. L.M.S.	Stim. L.M.S.	Stim. Palatal Secretion	Stim. S.L.S.
R.K.	79	66	10	51	794	1000	741	1290
D.S.	114	48	81	16	n.t.	209	408	310
S.B.	200	129	54.9	37.2	977	1070	1120	1780
A.W.	116	457	25.7	n.t.	1050	379	3180	576
H.C.	224	126	162	159	1450	1100	1180	1450
S.C.	159	72.4	110	87.1	1480	1050	1990	1170
M.F.	354	200	61.7	47.8	n.t.	645	490	870
K.K.	239	138	100	235	2180	1230	n.t.	1380
R.Y.	214	52.5	10.2	46.8	324	407	693	602
H.U.	182	97.6	19.9	38.9	1290	632	468	1230
B.O.	126	117	97.5	23.4	602	446	1175	1910
J.P.	282	166	53.7	36.3	n.t.	776	795	1100
Mean	191	139	65.6	70.8	1130	745	1110	1140
± SE	±22	±32	±13	±20	±183	±98	±248	±139

S.M.S. - Submandibular saliva
L.M.S. - Lip-mucous secretion
S.L.S. - Sublingual saliva
n.t. - Not tested
* - The activity is expressed as mg% with respect to the hog gastric mucosal standard

Figure 4 - 14. A histogram representation of the mean values with standard errors of the data presented in Table IV - 5.


Mean BGS (mg%) of 12 Subjects
1400
1200
1000
800
600
400
200
0
Unstimulated
Stimulated
Whole Saliva
Subman-dibular Saliva
Lip Mucous Gland Secretion
Palatal Gland Secretion
Sublingual Saliva

In light of the findings of Wolf and Taylor (1964) the high activities of lip-mucous and palatal secretions and sublingual saliva are not surprising. All three secretions are of a mucous nature.

With the aid of this data and with several assumptions, it is possible to speculate on the source of the BGS in the whole saliva. Schneyer and Levin (1955a) estimated that the submandibular glands contributed about 70% of the volume of the resting whole saliva. In our calculations we have used this figure and have assumed that the sublingual and minor mucous glands made a combined contribution of about 10% of the whole saliva (Dawes and Wood, 1973a). As unstimulated palatal gland secretion or sublingual secretion was not collected, we further assumed that the value obtained for the unstimulated lip-mucous gland secretion was representative of all 3 secretions. The blood group activity which was contributed by the parotid saliva was considered negligible.

If our assumptions were correct and all the sources of salivary BGS have been tested, the BGS concentration of the individual secretions multiplied by their respective volume contribution to the whole saliva should approximate the BGS concentration of the whole saliva.

mg% BGS of whole saliva = (0.7) mg% BGS of submandibular saliva + (0.1) mg% BGS of lip-mucous gland secretion + (.2)% BGS of parotid saliva

= (0.7) (66) + (0.1) (1130) + (.2) (0)

= 159 mg%

The value obtained by calculation is somewhat less than the value obtained in the assay of unstimulated whole saliva. This would suggest that not all of the assumptions were justified. The concentration of BGS in stimulated lip-mucous secretion was considerably less than that in stimulated palatal gland secretion or sublingual saliva. If the blood group activity of the two latter secretions decreased upon stimulation such as occurred with lip-mucous secretion, the use of the value for the unstimulated lip-mucous secretion as being representative may have underestimated the mean blood group activity of the 3 secretions.

Upon stimulation, the percentage contribution by the parotid glands to the whole saliva volume is increased. To calculate the relative glandular contribution to the blood group activity of the stimulated whole saliva we have assumed that the relative volume contribution of the submandibular glands drops to 50% (Shannon, 1967) while the sublingual and minor mucous glands continue to contribute 10% of the whole saliva volume (Dawes and Wood, 1973a). The mean value for the BGS concentrations of the stimulated lip-mucous and palatal gland secretions and sublingual saliva was considered representative of the three secretions.

mg% BGS in stimulated whole saliva = (0.5) (70.8) + (0.1) (1000) + (0.4) (0) = 135 mg%

The value of 135 mg% obtained by calculation is very close to that which was found in the assay of the stimulated whole saliva. This finding could be subject to the criticism that the nature of the stimulus was not the same for each of the secretions. However, it is unlikely that the nature of the stimulus would affect the blood group activity to any great extent.

It would appear that the majority of the blood group substance in the whole saliva originated from the sublingual and minor mucous glands.

Amongst individuals, the proportional contribution to the whole saliva volume by the various salivary secretions shows considerable variability. Schneyer and Levin (1955a) measured the unstimulated flow rates of whole, parotid, submandibular and sublingual saliva in 23 individuals. The combined volume of the secretion from the 3 pairs of glands per minute was considerably less than the whole saliva flow rate. This left an average of 41% (range: 0 to 90%) of the whole saliva volume "unaccounted for". The "unaccounted for" volume was subsequently shown to be a result of undue stimulation during the collection of the total mixed saliva (Schneyer, 1956). They calculated the proportional contribution by the individual secretions as a percentage of the combined volume secreted by the submandibular, sublingual and parotid glands per unit time. They found a range of 44 to 94%

(mean: 60.1) for the submandibular secretion, 0 to 18.8% (mean: 4.7%) for the sublingual secretion and 0 to 52.7% (mean: 26%) for the parotid secretion. In a study on 7 subjects, Ong and Dawes (personal communication) found a range of 15.5% to 42.2% (mean: 28.4%) in the contribution of unstimulated parotid saliva when expressed as a percentage of unstimulated whole saliva. The proportional contribution of stimulated parotid saliva was between 37.4% and 45.4% (mean: 43.0%). Dawes and Wood (1973a) reported a range of 3% to 23% (mean: 8%) and 2% to 15% (mean: 7%) in the percentage contribution of unstimulated and stimulated minor mucous secretions, respectively.

The great range in the proportional contributions of the individual secretions combined with large differences in their relative concentrations of BGS may account for much of the variability amongst individuals in the blood group activity of the whole saliva. For instance, a secretor individual in whom the minor mucous glands showed a high rate of secretion and the parotid gland a low rate of secretion, would have a high concentration of BGS in the whole saliva. Furthermore, the proportional contribution of parotid saliva shows a circadian rhythm of low amplitude (3.5%) (Ong and Dawes, personal communication). It is not known whether the relative contribution made by the minor mucous glands also displays a rhythm. If this did occur, it would result in another source of variance in the concentration of BGS in whole saliva, unless the time of day of

the saliva collection was standardized.

A method of eliminating these difficulties would be to study BGS in the secretions of individual glands. This has previously been suggested by Wolf and Taylor (1964). As it is relatively easy to collect large volumes of submandibular saliva this would seem to be the best choice.

CHAPTER V

GENERAL DISCUSSION

CHAPTER V

GENERAL DISCUSSION

In most previous studies which have involved hemagglutination or hemagglutination-inhibition, twofold serial dilutions of the antiserum or the inhibiting substance were used. The titre was determined by visual inspection. This required a subjective judgement on the part of the investigator. Solomon et al. (1965a) state that the agreement of titres from different laboratories is generally poor and, that at best, the titre is conceded to have an error of one doubling dilution in either direction. It is thus advisable that the same individual read all titrations which are to be compared.

Methods which have used microscopic cell counting to determine the end point of the titration have eliminated the necessity of a judgement of titre by the investigator, which, as a result, makes the method more objective. A further improvement of this technique was the use of probit analysis (Wilkie and Becker, 1955a). However, to obtain reproducible results it is necessary to count a large number of cells. This is a tedious procedure and, if a large number of samples must be analysed, the time required may become restrictive. Promising results have been reported by Gibbs (1965) when the cells are counted by an electronic particle counter.

Dybkjaer (1966) devised a method to separate free cells from agglutinated cells by their respective sedimentation

properties. It did not require complicated equipment and he obtained results comparable to other quantitative procedures. We have adapted this technique to quantitative hemagglutination-inhibition.

In our hands, the precision of the hemagglutination-inhibition did not appear to be as great as Dybkjaer obtained for quantitative hemagglutination. This is not surprising as the inhibition reaction is more complex and consequently has more possible sources of error. Our results compare favourably with those of Gibbs et al. (1961a) who studied quantitative hemagglutination-inhibition using hemocytometer counts to determine the percent agglutination. They found a coefficient of variation of 5.7% for 10 replicate assays of their standard A substance performed on the same day. With our technique, experiments with the same protocol yielded coefficients of variation from 2% to 6%. Solomon et al. (1965b) who studied hemagglutination by cell counting procedures found coefficients of variation of 5.7% in the HD_{50} of 10 replicate assays. They encountered a 3.4% error associated with the hemocytometer counts alone. The analogous procedures in our technique (centrifugation + separation of free cell layer + addition of Drabkin's solution) yielded a coefficient of variation of 2%.

With our technique no exceptional skills nor complicated equipment are required to obtain accurate determination of BGS in saliva. We have routinely analysed

17 samples of saliva in a single experiment with little difficulty. The limiting factor with respect to time is the 20 hour incubation period which was required to ensure equilibrium. This necessitates that experiments be spread over 2 days. We suggest that the technique is somewhat less tedious than those which use microscopic cell counting to estimate the percentage agglutination. A relative advantage which the latter techniques possess with respect to the one which we describe is that lower concentrations of the reactants are used. This would be important if one or more of the reactants were available only in small quantities.

We have found that when submandibular saliva is placed in a boiling water bath for 10 minutes there is a reduction in blood group activity. Once the sample has been boiled, it retains its activity when stored frozen. Samples which were frozen without prior boiling showed a large decrease in activity. We recommend that when submandibular samples cannot be assayed immediately they should be boiled and stored frozen. However, the results of quantitative blood group determinations on samples treated in this manner should not be compared with those of samples which were assayed immediately.

The application of this technique to the examination of the effect of several variables upon the concentration of BGS in submandibular saliva has led to some puzzling results. Prolonged sour lemon drop stimulation in some experiments has resulted in up to a sixfold change in the concentration of BGS.

The pattern differed, however, in different subjects and in the same subject when tested on different days. It is hard to rationalize differences in the response unless it is assumed that variables exist, which influence the secretion of BGS which we have failed to control. As the present results show such great differences in the patterns of response to stimulation we can offer little advice to other workers in the field as to a regimen for the collection of stimulated saliva. We can conclude, however, that changes in BGS concentration due to stimulation could be a major source of variance in experiments.

The concentration of BGS in unstimulated submandibular saliva collected from the same subject on different days shows much less variability than that in stimulated saliva. We did not observe differences greater than twofold in blood group activity between comparable samples. We recommend that in future, comparisons in the concentrations of BGS in saliva be done only on unstimulated samples, at least until the effect of stimulation on salivary blood group activity is clarified.

The experiments to determine the origin of the BGS in the whole saliva have demonstrated that sublingual saliva and the minor mucous gland secretions possess high blood group activity. The ability of forensic scientists to identify, in some cases, BGS in saliva stains found on cigarette paper and drinking glasses may be a result of the high activity in

the lip-mucous gland secretions. Our rather crude calculations suggest that the sublingual and minor mucous glands contribute about 70% of the BGS of the whole saliva while forming only about 10% of the whole saliva volume. This study further underlines the heterogeneity of the salivary secretions.

We have emphasized the fact that changes in the relative flow rates of the different salivary secretions would influence the concentration of BGS in whole saliva. We have also pointed out that individual variation in the concentration of BGS may in part be a result of individual variation in the contributions of the different salivary secretions. On the basis of several population and family studies on the concentration of BGS in whole saliva, it has been suggested that the amount of ABH substance secreted in the saliva is under genetic control (Plato and Gershowitz, 1962; Clarke et al., 1960; Guisti et al., 1972). Another possible explanation for the results which has not been explored is that the relative contribution of individual secretions to the whole saliva is under genetic control rather than the concentration of BGS in the secretions. Ericson (1971) has shown that the most important cause of individual differences in the flow rates of stimulated parotid saliva is differences in the size of the parotid glands. It seems likely that the size of salivary glands will, at least partially, be determined by inheritance. Similarly, it does not seem too unreasonable to suppose that

the density of minor mucous glands in the oral mucosa might also be an inherited character. Thus, before a decision can be taken as to whether the amount of ABH secreted is controlled at a genetic level, it would be necessary to carry out family and population studies on the blood group activities of individual secretions. This in turn would require a standardized procedure for the saliva collection in which all variables which affect salivary BGS concentration are defined and controlled.

CHAPTER VI

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