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"GENETICAL CONTROL OF BLOOD GROUP SUBSTANCES IN BODY SECRETIONS."

by

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B. Boettcher

21st March, 1966.

GENETICAL CONTROL OF BLOOD GROUP SUBSTANCES

IN

BODY SECRETIONS

TABLE OF CONTENTS

Page

SECTION B.

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SUMMARY

A conceptual scheme for some cases of infertility in humans, a reaction between ABO blood group antigens on spermatozoa and the appropriate antibodies in the mucous secretion of the uterine cervix, was proposed by Behrman *et al.* (1960). The premises upon which this scheme was based have been investigated, and couples, infertile with no known physiological reason, have been studied.

ABO antigens in saliva were studied since Behrman *et al.* (1960) reported a significantly high proportion of "aberrant secretors" among couples, infertile with no known cause for their infertility, studied by them.

The results of inhibition tests on secretor salivas, using human "non-immune" anti-A and anti-B sera, and seed extracts from *Ulex europaeus* (anti-H) and *Dolichos biflorus* (anti-A_1), show that, on the average, salivas from A_1 secretors have a greater capacity to inhibit anti-A and *Dolichos* fluids and a lesser capacity to inhibit *Ulex* extract than do those from A_2 secretors. The inhibition titres of A_1, A_2 and B secretor salivas with anti-H (*Ulex*) are uncorrelated with the respective titres with anti-A, anti-A_1 (*Dolichos*) and anti-B testing fluids. These observations are interpreted as demonstrating that the rates at which

genes at the H locus and the ABO locus operate are independent. Aberrant secretors are explained as individuals in whom the genes at these two loci are functioning at greatly different rates.

The decreasing order for reaction of salivas with Ulex has been shown to be O, A_2, A_1, B, whereas the order for red cells has been shown to be O, A_2, B, A_1. It is suggested that this difference is due to almost all H-substance molecules being converted to A- or B-substance on red cells, but not in the saliva, and that the I^B gene is less efficient than the I^{A_1} gene in converting H-substance in the saliva.

Precipitation of A-substance from A_1 and A_2 secretor salivas with Dolichos extract, and measurement of the anti-A_1 (Dolichos) and anti-H (Ulex) inhibition titres of the salivas before and after precipitation, showed that the individual molecules from A_1 salivas have a higher A:H ratio than do those from A_2 salivas. This conclusion has led to a model for the molecular difference between the A_1 and A_2 antigens.

The occurrence of ABO agglutinins in saliva has been investigated because of the difficulties in obtaining samples

of cervical mucus. Agglutinins were detected in a significantly higher proportion of salivas from O individuals than in those from A or B individuals. Anti-B was detected in a higher proportion of salivas from A_1 individuals than in those from A_2 individuals. No simple genetical control for the presence of ABO agglutinins in saliva could explain the data, which seem to agree with Prokop's (1961) suggestion that their presence is due to the passage of small molecular weight molecules into the saliva. The presence of ABO agglutinins in cervical mucus is attributed to the same cause.

The ABO antigens and agglutinins present in the two specimens of cervical mucus studied were the same as those present in the salivas of the respective donors.

ABO antigens on spermatozoa were studied using the agglutination-inhibition technique. The results of tests on spermatozoa from non-secretors were not different from those of the controls, whereas the inhibition titres of spermatozoa from secretors were correlated with the inhibition titres of the respective seminal plasmas, suggesting that spermatozoa can adsorb ABO antigens from the seminal plasma. Incubation of spermatozoa from O non-secretors in seminal plasma from A individuals, and in purified blood

group substance confirmed this.

The investigation of ABO antigens on spermatozoa using fluorescent antisera was found to be unsatisfactory due to non-specific uptake of antisera.

The number of ABO-incompatibly mated couples among the infertile couples studied was similar to the number expected with random mating using the blood group frequencies obtained from Australian-born university students.

It seems that the conceptual scheme for infertility proposed by Behrman et al. (1960) does not operate since, contrary to their assumption, ABO antigens are adsorbed to spermatozoa from the seminal plasma and, consequently, there cannot be a segregation of antigens of sperm. No evidence of a similar interaction between ABO antigens on spermatozoa and the appropriate antibodies in cervical mucus, but involving antigens adsorbed to sperm, has been obtained, since an excess of ABO-incompatible couples was not found among either the infertile couples studied or those where the husband is a secretor. However, some of the wives in the couples showed anti-spermatozoal antibodies which were not ABO-specific, so that some cases of infertility might be due to an antigen-antibody reaction between spermatozoa and

cervical mucus but, if so, ABO-incompatibility is not the basis for the reaction.

The nature of the ABO locus has been considered in the light of modern genetical concepts. It is proposed that crossing-over within the locus can occur, to give an $\underline{I^{A_2B}}$ gene. This concept permits explanation of four pedigrees reported in the literature in which A_2B individuals appear to be, genotypically, $\underline{I^{A_2B}I^{o}}$, and other unusual pedigrees.

Since ABO antigens can be present on red cells without influence from the secretor gene, and since no physical or chemical effect due to the secretor gene, except the presence of ABO antigens in aqueous body secretions, has been shown, it is proposed that the secretor gene acts prior to the $\underline{H}$ gene in those cells producing water-soluble antigen, but not directly on the precursor molecule. Within this concept the Le^b antigen is considered to be formed when the H antigen is added to preformed Le^a substance.

ABO and secretor gene frequencies determined from results obtained in these studies have been included in an Appendix.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

B. Boettcher

November, 1965.

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INTRODUCTION

In 1960 Behrman, Buettner-Janusch, Heglar, Gershowitz and Tew proposed a conceptual scheme for infertility in some humans based on ABO blood group incompatibility. The scheme envisaged a reaction between ABO blood group antigens on spermatozoa and the appropriate antibodies in the mucous secretion of the uterine cervix.

To investigate whether this conceptual scheme operates, it was planned to determine the ABO blood group constitutions of couples infertile for a period of years in whom no reproductive physiological abnormalities had been found. An excess of ABO incompatible matings among such couples when compared with the expected number, based on population blood group frequencies and an assumption of random mating, would give weight to the proposition that the interaction, outlined above, could be a cause of infertility. If it could be a cause of infertility, it would be important to be able to recognize those couples who might experience such a reaction, and so the genetical control of the compounds which would participate in the reaction were to be investigated, too.

Behrman *et al.* (1960) had based their scheme on the experimental results of Gullbring (1957) who demonstrated ABO antigens on spermatozoa and claimed that, in an AB individual, there is a segregation such that some sperm possess the A antigen whereas others possess the B, and on the observation that ABO isoagglutinins occur in the secretion from the uterine cervix of some, but not all, women (Gershowitz *et al.*, 1958). If there is a segregation of antigens on spermatozoa, and if the proposed antigen-antibody reaction occurs then, in a mating of the type O x A, where the male is heterozygous, decreased fertility only might be expected, since only half of the spermatozoa could participate in the reaction whereas, if the presence of the antigens on spermatozoa is zygotically determined, complete infertility might result, since all of the spermatozoa could participate in the reaction. Therefore, it was considered desirable to repeat and to attempt to verify Gullbring's work and his conclusions.

$\underset{\text{♀}}{\text{O}} \times \underset{\text{♂}}{\text{A}}$

It was hoped to verify and to extend the observations of Gershowitz *et al.* (1958) on the presence of ABO isoagglutinins in the mucus from the uterine cervix. However, it was realized that specimens of this secretion are not easily

obtainable, and that only small quantities can be collected in any case, so that it was proposed to examine another mucous secretion, saliva, for the presence of ABO isoagglutinins. It was argued that, since ABO antigens are present in most body fluids of secretors, and the presence of antigens in one body secretion can be inferred, with a high degree of accuracy, from detecting them in another, if ABO isoagglutinins are present in the saliva then, quite reasonably, they could be expected in the mucous secretion of the uterine cervix. Comparison of the results of studies made on the two secretions in the same women would give some indication of the reliability of such deductions.

Since aberrant secretors, individuals lacking, or having very small quantities of an expected A, B or H antigen in their saliva, had been claimed to be more frequent among infertile couples than in the population in general (Behrman et al., 1960), the relative quantities of the A, B and H antigens in salivas from infertile couples and in individuals unselected for their fertility status were to be determined.

The results and significance of the studies outlined above are presented in this thesis.

The results of determinations of the relative quantities of A, B and H antigens in salivas are presented, as are those pertaining to the individual aspects of the proposed mechanism for a possible cause of infertility. The former are discussed with reference to the aspects on which they provide information, viz. the control of the rate of production of H and A/B substances, aberrant secretors, a difference between the A_1 and A_2 antigen molecules, and a difference between the ratios of H and A or B antigens on red cells and in secretions. The latter are discussed in relation to the original proposition of a possible scheme for some cases of infertility. Evidence of an antigen-antibody reaction of the type envisaged leading to infertility, though independent of the ABO blood group system, is presented.

The structural genes responsible for the specificity of blood group antigens, in particular the ABO antigens, and the role of the secretor gene, which determines the presence of the appropriate ABO antigens in the saliva and most other aqueous body fluids, are considered.

Throughout the thesis the following symbols have been used.

The H-h blood group system (Watkins and Morgan, 1955).

Genes: H, h

Antigens: H

Phenotype: Oh (Levine et al., 1955)

The ABO blood group system.

Genes: I^{A_1}, I^{A_2}, I^B, I^o (Xth International Congress of Genetics, 1958)

Antigens: A_1, A_2, B,

The Lewis blood group system.

Genes: Le, le (Race and Sanger, 1962)

Antigens: Le^a, Le^b

Phenotypes: Le(a+), Le(a-) (when considering results using only anti-Le^a); Le(a+b-), Le(a-b+), Le(a-b-) (when considering results using both anti-Le^a and anti-Le^b).

Secretor Status

Genes: Se, se (Levine et al., 1955)

SECTION A

CHAPTER I

LITERATURE REVIEW

A. BLOOD GROUP ANTIGENS IN SECRETIONS

The blood group antigens which can be present in soluble form in the saliva, and other aqueous body secretions, are those of the H-h, the ABO and the Lewis blood group systems. The presence of the H and the A and B antigens in most body fluids is controlled by the secretor gene, which also influences the type of Lewis antigens present in body fluids and their detection on the red blood cells.

Although most of the biochemical work performed in elucidating the genetical pathways leading to the A, B and H antigens has been carried out on substances isolated from aqueous body fluids, the conclusions have been applied to red cell antigens also. Kabat (1956), on the first page of his classic monograph, refers to blood group substances "on or in the surface of erythrocytes and, in soluble form, in the secretions of secretors". Whilst it might be correct to assume that the antigens in these two sites are simply different physical forms of the one substance they might also

be different chemically. Hartmann (1941) has shown that there are two distinct forms of the antigens: (1) a water soluble form, present in the body secretions of secretors but not in the red cells; (2) an alcohol soluble form, present in all the tissues (except the brain) and in the red cells, irrespective of the person's secretor status. Furthermore, there is a difference in the quantitative relationship of the H antigen in the salivas of secretors and on the red cells of persons of groups A_1 and B. On the average, salivas from secretors of blood group A_1 have more H activity than salivas from group B secretors, whereas this order is reversed when the H activity of the red cells is considered (see pp. 68-69).

However, with these reservations in mind, conclusions arrived at from studies on antigens in aqueous secretions will be applied, in this discussion, to antigens on red cells, and vice versa.

The accepted theory for the inheritance of the ABO blood groups is that proposed by Bernstein (1924) who showed that an earlier scheme, proposing their control by two independent loci (Von Dungern and Hirszfeld, 1910), was unacceptable since, in any population where mating is random

with respect to these blood groups and if there is no selection, the product of the frequencies of blood groups O and AB is expected to be equal to the product of the frequencies of blood groups A and B. Bernstein showed that such an expectation was not fulfilled by the data then available, whereas they were consistent with expectations based on a three allelic-gene hypothesis.

The position of the H-h blood group system has been clarified partly by serological and partly by biochemical studies. In 1952 Bhende et al. described an individual whose red blood cells would not react with anti-A, anti-B or anti-H and whose serum contained these three antibodies. The authors (Bhende et al., 1952) suggested that a 'new' allele at the ABO locus had been discovered. However, later pedigree studies showed that, although "Bombay" individuals do not express A or B genes, they can transmit them (Levine et al., 1955; Aloysia et al., 1961). "Bombay" individuals are considered to be homozygous at the H locus for a gene which will not permit the expression of genes at the ABO locus (Levine et al., 1955), the two loci being independent (Aloysia et al., 1961).

A and B soluble blood group substances can be degraded

by bacterial enzymes, liberating simple sugars and giving products having H antigenic activity (Iseki and Masaki, 1953; Iseki and Ikeda, 1956; Watkins, 1956). Molecules with H antigenic activity can be degraded to give a mucopolysaccharide possessing Type XIV pneumococcus activity and fucose (Beeson and Goebel, 1939; Watkins, 1956).

A satisfying scheme for the genetical control of the production of the H, A and B antigenic substances is:

(i) A precursor mucopolysaccharide possessing pneumococcus Type XIV antigenicity gains additional H antigenic specificity due to the action of the H gene.

(ii) This substance is converted to either A or B substance due to the action of the allelic genes $\underline{I}^A$ or $\underline{I}^B$.

The genes $\underline{h}$ (allelic to $\underline{H}$) and $\underline{I}^O$ (allelic to $\underline{I}^A$ and $\underline{I}^B$) are considered to be amorphs.

This scheme is summarized diagrammatically in Figure I.1.

The simple genetical control of the presence of the ABO antigens in saliva was proposed by Schiff and Sasaki

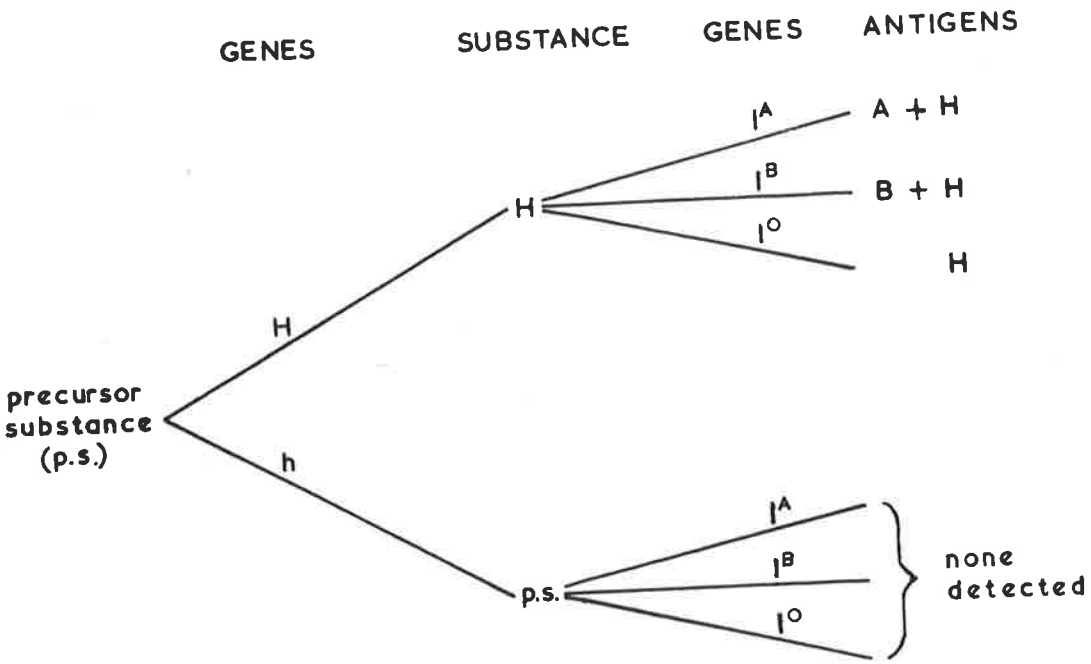

FIGURE 1.1. THE GENETICAL PATHWAYS LEADING TO THE H, A AND B ANTIGENS (modified from Race and Sanger, 1962). The genes h and I^o are considered to be amorphs.

(1932) and has been confirmed by pedigree data (see Race and Sanger, 1962, for a summary). Secretion of the same type of A, B or H antigens present on the red cells is regarded as a dominant Mendelian character controlled by a single autosomal locus unlinked with the ABO locus. When, in this thesis, the unqualified term "secretor" is used, it refers to an individual who secretes in his saliva the appropriate A, B or H antigens.

The presence of the Lewis antigens, Le^a and Le^b, on red blood cells is now considered to be secondary to their appearance in the serum, and the system is regarded as one involving the acqueous body fluids primarily. The presence of the Le^a antigen in saliva is looked upon as a dominant character under the control of a single autosomal locus unlinked with either the ABO or the secretor loci. However, although the secretor and Lewis loci are unlinked (Race and Sanger, 1954), there are interactions between the two characters.

Individuals with the Le(a+b-) red cell phenotype are non-secretors.

Individuals with the Le(a-b+) red cell phenotype are secretors.

Individuals with the Le(a-b-) red cell phenotype are usually secretors.

The red cell phenotype Le(a+b+) does not occur.

Le^a substance cross-reacts with anti-Type XIV pneumococcus serum (Watkins and Morgan, 1959), as do A, B and H substances, and is therefore believed to have the same precursor as the H, A and B antigens. The position of the Le^b antigen in the Lewis system is not quite as clear as that of the Le^a antigen, the difficulties appearing to arise from two leading workers conducting initial studies using different anti-Le^b antisera. The two antisera have slightly different specificities when used in inhibition tests with salivas, though they have the same specificity with red cells, at least of groups O and A_2, the groups most reliable for testing with Lewis antisera. Thus, Grubb (1951) was using anti-Le^{bH}, an antiserum which is inhibited by salivas from all secretors, irrespective of their Lewis genotype, and Ceppellini was using anti-Le^{bL}, an antiserum which is inhibited by secretor salivas which have Le^a present as well, but not by those which lack Le^a substance (Ceppellini, 1959). However, the results with both antisera show that the Le^b antigen is found only in secretors.

Race and Sanger (1962) have published a diagram which summarizes the current concepts of the genetical pathways leading to the presence of the H, A, B and Lewis antigens in saliva and the Lewis phenotypes of the red cells (Figure I.2).

B. THE SECRETOR PHENOTYPE

1. General

For some time after the initial proposal for the genetical control of the secretor phenotype (Schiff and Sasaki, 1932) the secretor classification appeared to be difficult to make, since different European workers reported significantly different frequencies for secretors in the population and different frequencies for secretors within individual ABO groups. Hartmann (1941) studied the division of individuals into secretors and non-secretors and showed that the distribution of the agglutination inhibition titres of 100 random group A_1 and B salivas allowed a clear separation into two classes. It was pointed out that some (6 out of 26) of the non-secretors possessed trace amounts of blood group substance in their saliva, but these amounts were far less than the least of

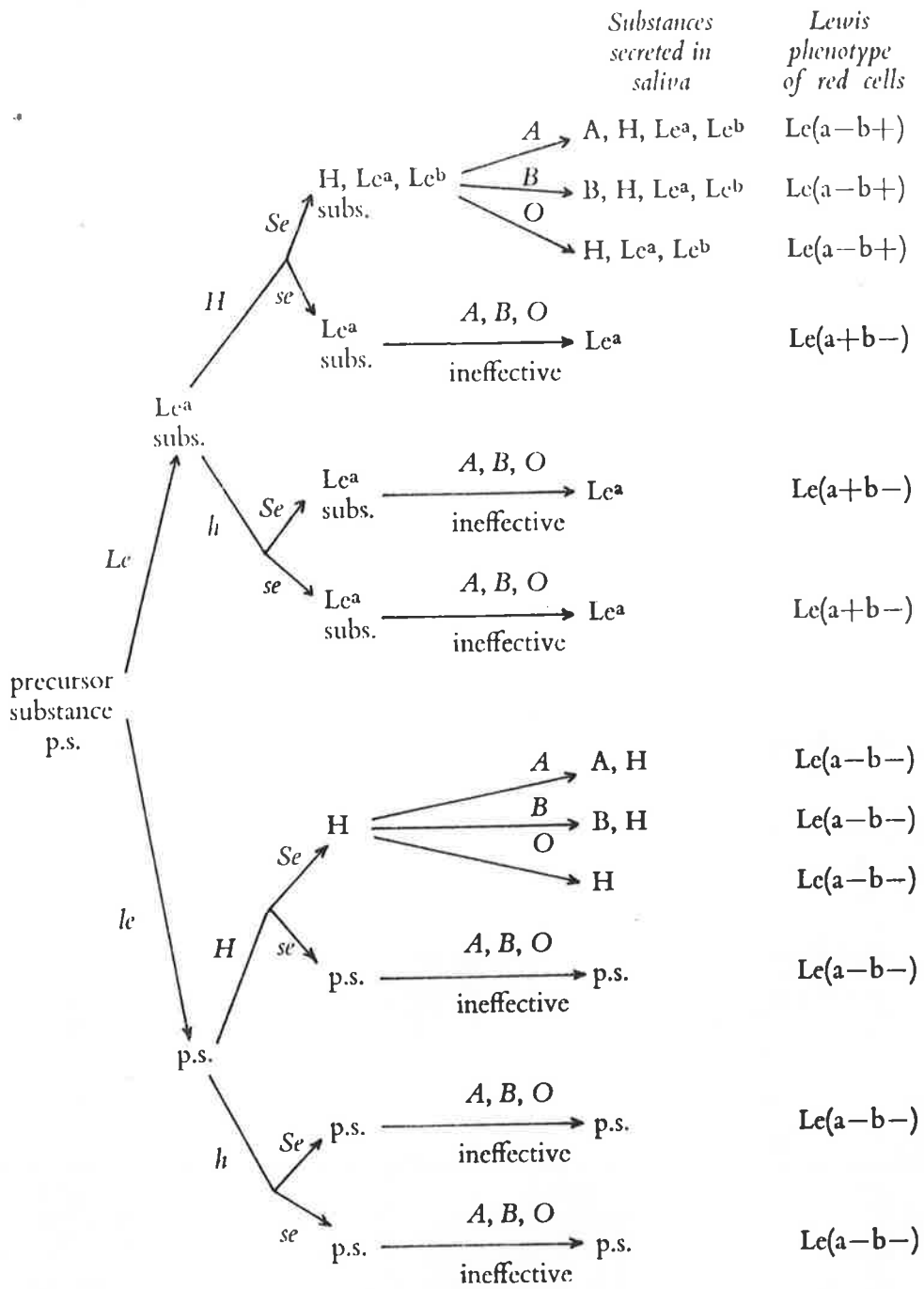

FIGURE I.2. THE GENETICAL PATHWAYS LEADING TO THE PRESENCE OF THE H, A, B AND LEWIS ANTIGENS IN SALIVA AND THE LEWIS PHENOTYPES OF THE RED CELLS (Race and Sanger, 1962).

those in secretor salivas. Race and Sanger (1962) state that Gammelgaard (1942) produced three good histograms for anti-A inhibitions by salivas from 72 A_1 persons, 53 A_2 and 27 A_3 persons. More recently, Morganti et al. (1959) have criticized the secretor/non-secretor distinction since they found a continuous distribution of the individuals secreting more or less blood group substances. This criticism has been discussed by Clarke et al. (1960) who, in their own results, obtained clear distinctions between secretors and non-secretors, and suggested that the absence of such a distinction in the data of Morganti et al. may be due to the use of a titration method which cannot distinguish between the two groups, rather than to the absence of a real distinction between them. In addition, they studied the genetical control of the A, B and H antigens in saliva and obtained data supporting the traditional concept.

Secretors, irrespective of their ABO blood group, secrete the H antigen in their saliva in varying amounts. Various reagents have been used to detect this antigen, human serum (Sneath and Sneath, 1959), eel serum (Morganti et al., 1959) and saline extracts of the seeds of Ulex europaeus (Cazal and Lalaurie, 1952; Boyd and Shapleigh,

1954; Clarke et al., 1960; Bianco et al., 1960; Plato and Gershowitz, 1961), Lotus tetragonolobus (McNeil et al., 1957a) and Cytisus sessilifolius (Plato and Gershowitz, 1961). The Ulex extract is the most commonly used reagent and is regarded as being the most useful. It is quite probable that the specificities of all of these reagents are not identical, since L-fucose inhibits the activity of eel serum and extracts from Lotus tetragonolobus but not extracts from Cytisus sessilifolius or Laburnum alpinum, whereas this situation is reversed with salicin. The agglutinins of Ulex europaeus are inhibited by both L-fucose and salicin (Bird, 1959a).

2. A antigen in salivas from secretors

When comparing the amounts of A antigen in the salivas of persons with different sub-groups of blood group A, Wiener and Kosofsky (1941), using a "boosted" human anti-A serum from a group B person unintentionally transfused with 150 ml. of group AB blood, and also a test fluid prepared from an immune rabbit serum, were unable to find any statistically significant difference in the inhibiting power of salivas from secretor individuals of blood groups A_1 and A_2, and even a single A_3. However, Race and Sanger

(1962) state that Gammelgaard (1942), using human anti-A, found a distinct fall in antigen secreted from A_1 to A_2 to A_3.

Morganti et al. (1959), in a paper comparing the amount of A, B and O (H) blood group substances in saliva and milk, presented data which showed, although they did not comment on it, that they did not observe any significant difference in the ability of salivas from secretors of groups A_1 and A_2 to inhibit the human anti-A serum which they used.

Baer, Kloepfer and Rasmussen (1961), using pooled antisera from chickens, showed that equal volumes of salivas from secretors of groups A_1, A_2 and A_1B can precipitate approximately the same amount of protein from an A antiserum, though the results of their inhibition tests, upon which they do not comment, show a decrease in the ability of the salivas to inhibit the antiserum from A_1 to A_2 to A_1B which, probably due to the small numbers involved, is not statistically significant.

These results appear to conflict, the inhibition results suggesting that the A_1 salivas possessed more A antigen than the A_2 or A_1B salivas, and the precipitation

results suggesting that the salivas all possessed approximately equal amounts of the A antigen. However, it is possible that the two techniques do not measure only the quantity of antigen present. If the A_2 antigenic site is structurally the same as the A_1 antigenic site, but has a slightly different charge distribution in it due to different adjacent groups, the A_2-anti-A complex might be relatively weaker than the A_1-anti-A complex. i.e. looked at as equilibrium chemical reactions

$$A_2 \text{ antigen} + \text{anti-A} \rightleftharpoons A_2 - \text{anti-A complex}$$
$$A_1 \text{ antigen} + \text{anti-A} \rightleftharpoons A_1 - \text{anti-A complex}$$

Although these complexes might be sufficiently stable to permit their precipitation and, thus, their removal from an equilibrium system, in an agglutination-inhibition reaction red cells would use up the anti-A formed by dissociation of the A_2-anti-A or A_1-anti-A complex until equilibrium is reached between the association and dissociation of the red cells and anti-A.

If the A_1-anti-A complex is more stable than the A_2-anti-A complex, then, in two reaction mixtures with equal quantities of anti-A and of the A_1 and A_2 antigens, there would be more A_1-anti-A complex than A_2-anti-A and, therefore,

in the former reaction mixture, less anti-A to react in agglutination, so that the inhibition titre of the A_1 antigen would be greater than that of the A_2 antigen.

The equilibrium chemical reactions then become,

A_2 antigen + anti-A $\rightleftharpoons$ A_2 - anti-A complex

+

red cells

$\uparrow\downarrow$

red cells - anti-A complex
(agglutinate)

and

A_1 antigen + anti-A $\rightleftharpoons$ A_1 - anti-A complex

+

red cells

$\uparrow\downarrow$

red cells - anti-A complex
(agglutinate)

Such a scheme might explain the apparent conflict between the results of precipitation and agglutination-inhibition tests performed on the same salivas (Baer, Kloepfer and Rasmussen, 1961).

3. H antigen in salivas from secretors

Plato and Gershowitz (1961) studied the inhibition of anti-H reagents from Ulex europaeus and Cytisus sessilifolius seeds by fifty salivas from each of group O, A_1, A_2, B and AB secretors.

Their results show that, on the average, salivas from secretors of different blood groups have different capacities to inhibit the two reagents, in the order $O > A_2 > A_1 \geq B \geq AB$. Their data, when tested statistically, show all of these differences to be significant, except that between B and AB. But, whereas the O and A_2 salivas gave significantly higher inhibition titres with the Ulex extract than with the Cytisus extract, the B and AB salivas behaved in a reverse manner, and the A_1 salivas scored equally with both. The authors considered that their reagents had different types of anti-H specificities and that the ratios of the two antigenic determinants which inhibit the two reagents differ in the different blood groups. In addition, they found that rabbit anti-Cytisus serum inhibited only the agglutination activity of the Cytisus extract with O red cells, whereas rabbit anti-Ulex serum inhibited the activity of both of the extracts.

Morganti et al. (1959), using an eel serum as their anti-H, presented data which show the same order for inhibition activity of secretor salivas as did Plato and Gershowitz, and, although the data of Clarke et al. (1960) do not distinguish between A_1 and A_2 secretors, their order for inhibition activity of salivas with a Ulex extract is O, A, B.

Solomon (1964) studied the agglutination of red blood cells of various groups using a Ulex europaeus extract. By plotting the probit of the percentage of agglutinated cells (ordinate) against the logarithms of the concentration of the Ulex extract (abscissa), regression lines of different slopes and positions were obtained for O, A_2, B and A_1 cells, and these were interpreted as evidence of a qualitative antigenic variability between these cells with regard to the Ulex anti-H reagent. The concentration of the Ulex extract required to produce 50% agglutination of red cells increased in the order O, A_2, B, A_1, the same order of reactivity found by other workers (Race and Sanger, 1962), and one which is different from the inhibition titres of salivas from secretors of these groups.

If the orders O, A_2, B, A_1 for the reactivity of red cells, and O, A_2, A_1, B for the reactivity of salivas are correct, these might indicate some fundamental difference between the antigens on red cells and those in secretions.

4. Aberrant secretors

The term "aberrant secretor" was used by McNeil et al. (1957a) to describe A, B or AB secretors whose saliva lacked, or had very little of, one of the expected antigens H, A or B. Using human anti-A and anti-B from immunized donors and an anti-H derived from the seeds of Lotus tetragonolobus, these authors found six out of 64 selected A or B secretor controls who came into their category of aberrant secretors. A further thirteen were detected in women who had "perinatal pathologic conditions". Of the thirteen, ten were of blood group A, six A_2's and four A_1's, a division commented on by the authors as being probably beyond the possibility of chance selection, though it appears to be close to a $2\frac{1}{2}\%$ one-tailed probability. However, this point is very difficult to assess, since the relative frequencies of the A_1 and A_2 phenotypes in the population studied is not given.

The only comment made by them on a possible explanation for "aberrant secretors" was that individuals lacking one antigen might have this suppressed by a gene during one generation.

A later report (McNeil *et al.*, 1957b) suggested a relationship between aberrant secretion and spontaneous abortion since, of the 47 couples reported who had had at least two spontaneous abortions, in 20 of them one of the partners was classified as an aberrant secretor, in comparison with none of the members of 23 couples who had had at least three children and no abortions.

"Aberrant secretors" have been described also by other workers (Behrman *et al.*, 1960; Roy and Chatterjea, 1962; Race and Sanger, 1962). Race and Sanger, however, using *Ulex* anti-H and human "non-immune" anti-A and anti-B have not yet found an "aberrant secretor" of blood group A or B who secretes H, but not A or B, as described by McNeil *et al.* (1957a).

From data obtained when investigating salivary secretion in sib pairs, Clarke, McConnell and Sheppard (1960) found that when the ratio of the $\log_2$ inhibition titres of

A salivas with Ulex anti-H and human anti-A was plotted, a unimodal distribution which was almost Gaussian was obtained. They suggested that "aberrant secretors" are the extremes chosen from a continuous frequency distribution.

A possible basis for some cases of aberrant secretion is given in observations reported by Kabat (1956). Some human anti-B sera are completely absorbable by rabbit red cells, whereas others are not; in these, some residual activity against human red cells remains. Absorption of anti-B sera with guinea pig cells leaves residual activity against rabbit cells. It is proposed that three types of B receptors occur on human red cells, B_i, B_{ii} and B_{iii}, two on rabbit cells, B_{ii} and B_{iii}, and only B_{iii} on guinea pig cells. It is suggested that human anti-B sera generally contain anti-B_{ii} and anti-B_{iii} and that, therefore, an individual with only B_i in the saliva might be classified as not secreting B substance. One such case has been investigated by Dahr and Lindau (1937), and Kauerz has reported 13 in 50 cases studied (Kauerz, 1938).

With this concept of three types of B receptors on

human red cells in mind, it would be of interest if the cells of those A_2B individuals possessing anti-B in their sera (Moullec and Le Chevrel, 1957; Seyfried et al., 1964; Yamaguchi et al., 1965) were investigated to determine whether they lack B_i, B_{ii} or B_{iii}, and also the receptor at which their anti-B is directed.

"Aberrant secretion" has also been used to describe the situation where an individual fails to secrete one of the A, B or H antigens present in his saliva in another aqueous body fluid, but, since the term has been used already in the sense discussed above, this usage is confusing. It is suggested that, to describe individuals whose secretions differ qualitatively with regard to the A, B or H antigens they possess, the term "inconsistent secretor" be used. Morganti et al. (1959) studied the ABO antigens in saliva and milk, and found some women, particularly of group B, who showed an antigen only in the saliva or only in the milk. If the secretor gene influences the presence of the antigens in all aqueous secretions, the same antigens are expected in both secretions.

Lawler (1959) also compared the antigens in saliva

and milk and found that, although there was a high titre of H-substance in the milk of secretors but not of non-secretors, A and B secretors showed very little of these antigens; in most of the women they were barely detectable. However, on testing the colostrum of three A_1 secretors, large amounts of A antigen were detected. Lawler suggested that, with H-substance being the precursor for the A antigen and colostrum accumulating during pregnancy, there is time for A-substance to concentrate, whereas milk, once lactation is established, does not accumulate for more than a few hours. But, on testing colostrum from one group B mother, she was unable to detect B-substance although there was a high titre of H-substance.

Szulman (1962), using fluorescent-labelled antisera to stain sections of breast tissue, found that the secretion of antigens by the breast is irregular, with some ductules or acini within the one lobule containing antigen side by side with similar structures not containing antigen. Using the same technique, he observed anomalous behaviour with specimens of endocervix, endometrium and three non-lactating mammary glands from group B individuals. Although the H antigen could be demonstrated in these specimens, the B

antigen could not, whereas similar specimens from other individuals possessed both.

It seems, therefore, that there is not always complete agreement between the antigens detectable in all of the aqueous secretions of an individual though, in general, the agreement appears to be good. It is worthy of note that pancreatic secretion of both secretors and non-secretors contains large amounts of antigen (Szulman, 1960). As pointed out by Clarke et al. (1960), there does not seem to be any theoretical reason why there should always be a complete correspondence between saliva and other secretions.

C. INFERTILITY DUE TO ABO BLOOD GROUP INCOMPATIBILITY

1. The proposed scheme

In addition to the report of a high incidence of "aberrant secretors" among couples who have had spontaneous abortions (McNeil et al., 1957b), a high incidence of "aberrant secretors" has been reported among infertile couples in whom no physiological cause of infertility had been determined (Behrman et al., 1960). Of 54 infertile

individuals (presumably women) tested, 21 were considered to be "aberrant secretors", compared with only 2 out of 171 fertile individuals (again, presumably women) tested. These data were obtained when a theoretically possible mechanism for infertility in some couples was being considered.

Behrman et al. (1960) proposed that infertility may, in some cases, arise from an antigen-antibody interaction involving ABO antigens on spermatozoa and the appropriate antibodies in the mucous secretion of the uterine cervix, a model based on the conclusions of Gullbring (1957) and Gershowitz et al. (1958).

Gullbring (1957) investigated the presence of ABO antigens on spermatozoa using the mixed agglutination technique (Coombs, Bedford and Rouillard, 1956) since he found that direct, specific agglutination of spermatozoa was not successful, as had been reported previously (Sjovall, 1949). For the mixed agglutination test, spermatozoa were incubated with antibody, and then were washed and mixed with red blood cells having the antigen recognized by the antibody. As controls the same procedure was followed, but red cells lacking the appropriate antigen were used. With this technique Gullbring examined the

antigens on spermatozoa from AB males and reported that, after incubation of the sperm with a mixture of anti-A and anti-B, and then washing until the washing medium contained no antibody, when A red blood cells were added, some spermatozoa participated in mixed agglutination and were precipitated by low speed centrifugation. Free spermatozoa from the supernatant were removed and were shown to participate in mixed agglutination with B red cells, though not with A cells. He claimed that this experiment identified two populations of spermatozoa in the semen from group AB individuals, the one possessing the A antigen, the other possessing the B antigen. He also reported detecting the D antigen of the Rh series.

Gershowitz et al. (1958) investigated the occurrence of ABO agglutinins in specimens of cervical mucus collected in a menstrual cup or by direct aspiration from the cervical os. Agglutinins were detected in 15 of the specimens from 35 group O women, in 2 of the specimens from 30 group A women, and in none of the specimens from 5 group B and 7 group AB women. In all, ABO agglutinins were detected in 17 of the 77 specimens tested. A later report (Solish, Gershowitz and Behrman, 1961) established that agglutinins

are detectable in the secretions from a significantly higher proportion of group O women than of groups A or B.

This report indicated, also, that a significantly higher proportion of women recorded positive results when two or more specimens taken at separate times were tested than when only one specimen was examined. Antibodies were detected on at least one occasion from 26 women of the 41 who were tested more than once (63%), whereas this proportion was 32 out of the 141 women who were tested once only (24%).

Behrman et al. (1960) presented three sets of data on couples infertile for no known reason as indirect evidence in support of the proposed interaction between sperm and cervical mucus. First, it was claimed that the blood groups (presumably of women) in 100 infertile matings were significantly different from those (again, presumably of women) in 171 fertile matings investigated by the authors and from 794 fertile women published elsewhere (Pearse, 1957), and involved a statistically significant excess of group O.

Secondly, it was claimed that the proportion of ABO

incompatible matings* among their 102 infertile couples (87.3%) was significantly higher than among their 171 fertile couples (38.6%).

Thirdly, 7 of the couples studied by them who had been infertile for at least ten years, five of the mating ♀ O x ♂ A and two of the mating ♀ O x ♂ B, had, between them, produced nine children, all of group O. It was proposed that, in all of these matings, the cervical secretions contained anti-A or anti-B, thus blocking the A or B sperm, but permitting the O sperm to pass and fertilize.

Whilst these three sets of data appear to be in agreement with expectations based on the assumption that, in ABO incompatible matings, the proposed ABO antigen-antibody interaction sometimes can occur, there are some aspects which appear to be not entirely satisfactory. First, the authors chose the initial couples in their fertile group by ascertaining couples where the wife was of blood group O. This would lead to different methods of ascertainment for the fertile and infertile couples, and therefore the comparison of compatible and incompatible matings and the blood group distributions in the two groups are not meaningful. If

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* Here, and elsewhere in this thesis, the term "ABO-incompatible mating" is used to describe a mating in which the wife possesses an ABO blood group antibody which can react with an antigen on her husband's red cells.

the initial couples in both the fertile and infertile groups were ascertained through group O wives (and the text is not completely unambiguous on this point) the criticism would still apply. However, irrespective of how the infertile women were ascertained, it seems that significantly fewer than 89 would be expected to be ABO incompatibly mated.

Secondly, if the presence of anti-A or anti-B in cervical mucus had been the only factor responsible for the infertility of the seven couples who had been infertile for more than ten years, it seems reasonable to expect that O-bearing sperm should have been able to effect fertilization much earlier.

Nevertheless, the proposed interaction seems feasible and is worthy of further investigation and evaluation.

2. ABO antigens on spermatozoa

ABO antigens on seminal spermatozoa were detected by Landsteiner and Levine (1926) using the agglutination-inhibition technique before the secretor/non-secretor distinction had been recognized. This work was extended by Levine and Celano (1961) who reported that appropriate

antibody can be absorbed by, and eluted from, the spermatozoa of both secretors and non-secretors. Popivanov and Vulchanov (1962) supported Gullbring's claim that some sperm from AB males have the A antigen, whereas others have the B, on the basis of their ability to agglutinate some sperm, but not others, from an AB male with an immune rabbit anti-A serum.

Shahani and Southam (1962) published a report which also appeared to support Gullbring's conclusions. Using human anti-A labelled with fluorescein isothiocyanate (FITC) and human anti-B labelled with rhodamine isothiocyanate (RITC) these authors, unlike Holborow et al. (1960) who had reported previously that they were unable to observe fluorescence of A spermatozoa when using fluorescein isocyanate (FIC), claimed to be able to demonstrate specific fluorescence of sperm. They reported that sperm from A donors fluoresced bright green with the FITC-labelled anti-A, whereas very pale fluorescence was observed with sperm from B donors, and that sperm from B donors fluoresced bright orange with the RITC-labelled anti-B, whereas sperm from A donors showed very little fluorescence. Then on incubating sperm from an AB donor with both of the labelled sera, they reported that some sperm fluoresced green,

whereas others fluoresced orange. Coloured plates showing these observations were also published. The reported results, however, do not appear to be perfectly consistent.

It is expected that some of the 16 sperm donors would have been A or B heterozygotes since no technique designed to select homozygous individuals was mentioned, and therefore, in some cases, only half the sperm would be expected to fluoresce. No mention is made of this. Further, the coloured plates are sufficient in themselves to show this point. In the plate showing sperm from an AB male treated with FITC-labelled anti-A (green) and RITC-labelled anti-B (orange), the numbers of sperm of the two colours are (approx.) 20 and 50. With an hypothesis of equal numbers of sperm of the two colours, $\chi^2_1 = 12.9$, $P < .001$. And, even more striking, are the two plates showing sperm from an A male treated with FITC-labelled anti-A and sperm from a B male treated with RITC-labelled anti-B. In both plates all sperm show fluorescence of equal intensity. Under the conclusion of the authors, this would indicate that both semen specimens came from homozygous donors, the probability of which, assuming random selection of sperm donors, is less than 1%.

Recently, Edwards et al. (1964) reported that they were able to get spermatozoa from secretors to participate in mixed agglutinations whereas those from non-secretors and those from a spermatocoele would not, though, in specimens from the non-secretors, non-spermatozoal cells reacted strongly. They concluded that ABO antigens on seminal spermatozoa are derived from the seminal plasma and that, therefore, the antigens on spermatozoa are dependent on the secretor status of the donor.

3. ABO agglutinins in saliva

No investigations of the control of the presence of ABO agglutinins in cervical mucus, other than those referred to above, have been reported, but several authors have studied the presence of ABO agglutinins in saliva and the possibility that their presence is under genetical control.

Using simple agglutination tests, Putkonen (1932) detected ABO blood group agglutinins in saliva from 103 of 728 donors (14%). Agglutinins were detected in 53 of 172 salivas from donors of group O (31%), 33 of 317 salivas from donors of group A (9.5%), and 17 of 136 salivas from donors of group B (12.5%).

Following the work of Miyakoshi (1951), who proposed that the presence of agglutinins in saliva is under the control of a single gene, with absence of agglutinins dominant to their presence, Furuhata et al. (1959) recorded histograms for the titres of anti-A in B and O salivas, and anti-B in A and O salivas and drew up tables comparing the observed numbers of children and their phenotypes from matings in which the parents were classified as to whether or not their salivas possessed antibodies. The data were considered to be consistent with the expectations based on Miyakoshi's model. However, the data did not differentiate between the frequencies of anti-A in B and O salivas or anti-B in A and O salivas, and this inadequacy was criticized by Prokop (1961) who found, as had Putkonen, that agglutinins are detectable in salivas of O persons more frequently than in salivas from A_1, A_2 or B persons. Also, anti-B appears to be detectable in salivas from A_2 individuals more frequently than in those from A_1 individuals.

Prokop (1961) considered that, due to the significantly different frequencies of individuals in the different blood groups possessing detectable salivary agglutinins, the simple mode of inheritance proposed by Miyakoshi (1951) is

inadequate to explain the occurrence of ABO agglutinins in saliva, and suggested that the higher proportion of O salivas with anti-B than A salivas is a reflection of the lower molecular weight of the anti-B agglutinins in O sera.

Wilson and Green (1964), too, found agglutinins in a higher proportion of salivas from group O persons than in those from A or B individuals, and recorded pedigrees which show that, if secretion of ABO isoagglutinins is under direct genetical control, it can not be either a simple autosomal dominant or a sex-linked recessive character.

Other antibodies have been detected in saliva. Thus, when studying the antibodies present in salivas from individuals of group O, Sondermeier and Flatow (1962) were able to detect cross-reacting antibody, Prokop and Kerde (1963), following the personal communication of Thomas, looked for, and detected, anti-C and anti-D of the Rh system, and Scheibe and Gibb (1962) found anti-P, though Prokop (1961) was unable to demonstrate either anti-P or anti-M in the salivas he studied.

D. INFERTILITY DUE TO SPERM AGGLUTININS

Interactions between antigens on spermatozoa and antibodies in the secretions of the female reproductive tract, other than those of the ABO blood group system, have been postulated as a cause of infertility also (Tyler, 1961). Nakabayashi, Tyler and Tyler (1961) reported that 17% of the infertile women studied by them possessed sperm agglutinins in their sera. Recently, the results of a study, carried out to investigate the occurrence of anti-spermatozoal antibodies in the sera of women classified according to their secretor status, have been reported (Franklin and Dukes, 1964a, 1964b). Sperm-agglutinating antibody was detected in 31 of 43 women investigated who had no demonstrable organic cause of infertility (of the 31, only 12 were incompatibly mated with regard to the ABO blood groups); in 7 of 83 women who had known organic causes for infertility; in 2 of 35 women known to be fertile; in 2 of 35 women of unknown fertility and in 1 of 18 women with secondary sterility.

In an effort to determine the role of repeated vaginal deposition of semen in the maintenance of sperm-agglutinating antibody titres, 13 of the 31 infertile

couples who had no demonstrable organic cause of infertility, and where the wife had sperm-agglutinating antibody, were persuaded to restrict intercourse to the use of condoms for two to six months. The antibody titres dropped in all of the 13 women, being undetectable in ten. Of these ten, nine became pregnant on resuming unrestricted intercourse at the expected time of fertilization.

Gel diffusion tests showed two distinct precipitation bands when the serum of one woman, who possessed high titre antibody, was tested for reaction against her husband's seminal plasma (Franklin and Dukes, 1964a).

These results are consistent with the concepts that antigenic substances from seminal plasma can be adsorbed by spermatozoa and that seminal spermatozoa can induce the formation of sperm-agglutinating antibody which may contribute to infertility.

It is of interest that Searcy, Craig and Bergquist (1964), using electrophoresis, were able to remove materials from human seminal spermatozoa with mobilities similar to serum albumins and globulins, though these could not be removed by repeated washings in saline. They concluded that the antigenicity of spermatozoa stems from seminal

plasma, a conclusion supported by other workers who have found that testicular spermatozoa lack antigens present on seminal spermatozoa (Weil and Rodenburg, 1960; Otani, Iino and Kagami, 1964), and who have been unable to differentiate between the antigenicity of seminal plasma and seminal spermatozoa (Weil, Kotsevalov and Wilson, 1956; Otani, Iino and Kagami, 1964).

Reid (1964), when studying the fate of spermatozoa lying on the human cervix, reported that spermatozoa can penetrate cervical tissue to those regions where large subepithelial collections of plasma-cells producing gamma globulin are found, and Straus (1965) claimed that trans-vaginal immunization of women with an extract obtained from pooled seminal spermatozoa can reduce the mobility of sperm in later coitus through the action of a postulated "muco-antibody".

From these reports, it seems possible that spermatozoa can adsorb seminal plasma antigens and they can penetrate tissues to regions where they can be antigenically active, with the consequent formation of antibody which, in some couples, may lead to infertility. The data from recent studies using rabbits, however, appear to be conflicting. Weil and Roberts (1965) report that, although circulating

antibodies were produced in 70% of female rabbits immunized with rabbit seminal plasma in Freund's adjuvant; there was no impairment of fertility, whereas Behrman and Nakayama (1965) found that no pregnancies occurred while, or even for some time after, female rabbits possessed high antibody levels arising from intradermal or intravaginal immunization with homologous testis homogenate.

It is clear that further work is needed in this field.

CHAPTER II

MATERIALS AND METHODS

The materials and methods used in the work described and discussed in the following sections are described here. This has been done to avoid repetition of their description where the same materials and/or techniques have been used in different sections, and so that experimental results can be discussed and logical arguments can be developed without interruption.

Reagents and techniques used commonly are described first, followed by specific details relevant to particular procedures used in work described in various sections.

Unless stated otherwise, it is to be assumed that an experimental procedure named in the text has been performed by the general technique, described here, using the standard testing fluids.

Standard testing fluids (antisera and lectins)

Except for the reagents used in the sections, Reaction between human anti-A and salivas from A_1 and A_2 secretors, and Reaction between Ulex extract and salivas from A_1, A_2,

B and O secretors (pp. 63-66), all work reported was performed using the testing fluids described below. This enables meaningful quantitative comparisons to be made between results obtained at different times, and results reported in different sections.

The results reported in the above-named sections are from experiments performed using other "non-immune" human anti-A and anti-B sera, a Ulex europaeus seed extract, and a Dolichos biflorus seed extract diluted so that it would agglutinate A_1 red cells, but not A_2 red cells, within one minute.

Anti-A

Four sera from group B individuals were chosen for their low haemolytic activity and lack of any suggestion of "boosting", and were pooled.

The pooled solution had an agglutination titre of 256 with A_1 cells and 128 with A_2 cells.

Anti-B

Three sera from group A individuals were chosen for their low haemolytic activity and lack of any suggestion of "boosting", and were pooled.

The pooled solution had an agglutination titre of 256 with B cells.

Ulex europaeus seed extract (anti-H)

Seeds from the gorse, Ulex europaeus, were collected from bushes growing in the wild in the Adelaide hills region. Due to the explosive seed dispersal of this plant, shed, and therefore mature, seed is difficult to collect. However, tests were unable to show any difference in the haemagglutinating capacity or specificity of extracts from immature, green, seeds or mature, black, seeds.

Seed pods containing (mainly) mature seeds were picked and were kept in a warm room (37°C.), in covered containers, for three days. Twice daily the shed seeds were removed and put into storage at room temperature.

The shed seeds were winnowed to remove dust and debris and were then crushed to a fine powder in a porcelain ball mill. The powder was mixed with eight volumes (w/w) of .87% sodium chloride solution and shaken in the cold (5°C.) for 24 hours. On settling, the supernatant was removed, filtered and stored. The sediment was mixed with two volumes of .87% sodium chloride and shaken for a further

24 hours. On settling, the supernatant was removed, filtered and added to the previous one.

The filtered solution was centrifuged in the cold (5°C.) for 30 minutes at 12,840 ^{x}g (10,000 r.p.m.). The clear, pale yellow supernatant was kept as the anti-H extract.

The extract had agglutination titres of 128 with O, 64 with A_2, 16 with B and 8 with A_1 red cells.

Dolichos biflorus seed extract (anti-A_1)

Dolichos biflorus seeds were obtained from the C.S.I.R.O. Division of Plant Industry (seed lot CPI8342). A saline extract was prepared in a manner similar to that used for the Ulex extract.

The specificity of the extract was determined by testing against the panel of "test" cells at the South Australian Red Cross Blood Transfusion Service. The extract had an agglutination titre of 256 with A_1 cells and 16 with A_2 cells.

The large volumes (200-400 ml.) of the four testing fluids were divided into 20 ml. samples for storing in deep-

freeze. When a new 20 ml. sample was to be used, it was thawed completely and divided into 2 ml. lots, which were then used. This procedure was used to minimize thawing and re-freezing.

Agglutination tests

All agglutination tests were performed with 2% suspensions of the appropriate red cells in physiological (.87%) saline on blood spotting tiles incubated in humid boxes. Before suspension, the red cells were washed three times in physiological saline.

The tiles are of white, opaque glass with the 1" x 1" segments separated by a $\frac{1}{8}$" wide and $\frac{1}{32}$" deep "gutter". The humid boxes are of clear plastic with overhanging lids and are effectively airtight. A large piece of wet foam-plastic covers the bottom of each box and glass rods on top of this prevent contact between it and the blood tile.

Red cell suspensions and the testing fluids were mixed by "swirling" the blood spotting tile.

Titration results

The reciprocal of the greatest dilution of a fluid which would just agglutinate a 2% cell suspension was recorded

as its titre. To ascertain this dilution, all agglutinations, apparently negative macroscopically, were examined microscopically (25^{x}) using a binocular dissecting microscope. When looking for agglutination microscopically, the blood spotting tile was tilted and returned quickly to horizontal to give a flow of the blood cell suspension across the tile. This technique was found to be very sensitive and permitted identification of very weak agglutinations since it is easy to detect a few clumps, of even two red cells joined together, in a field consisting, otherwise, of single cells moving independently.

Agglutination inhibition tests

Soluble blood group antigens in aqueous solutions were detected by the agglutination inhibition technique. In these tests a testing fluid of sufficient strength to agglutinate appropriate red cells was mixed with the solution to be tested for the presence of antigen, and incubated. The appropriate cells were then added to the mixture.

If the solution under test possessed the appropriate soluble blood group antigen, some of the testing fluid would be used in reacting with it, thus decreasing the capacity of the testing fluid to agglutinate the red cells, whereas, if

the solution did not possess the antigen, the agglutinating capacity of the testing fluid would not be altered.

Quantitative comparisons of the agglutination inhibition capacity of different solutions against the one testing fluid were carried out by comparing the testing fluid titres which could just be inhibited by different solutions. This technique has been used and discussed at length by Hartmann (1941). Drops of the first solution under test for the presence of antigen were put onto each of the squares in one row across the 6x10 blood spotting tile, using a square-tipped Pasteur pipette held vertically. The same pipette, rinsed in water, saline and the second test solution, was used to spot the second solution across the second row, and so on. The last (sixth) row on the tile was used as a control and drops of saline were used instead of a test solution.

Using the same Pasteur pipette, the testing fluid, neat and in doubling dilutions, was spotted onto the drops of the solutions under test in rows down the tile in a similar manner. The drops were mixed by "swirling" the tile. This gave rows across the tile of the solutions under test mixed with decreasing concentrations of testing fluid from neat to 1 in 256.

The tile was then incubated at room temperature for half an hour, after which time one drop of a 2% red cell suspension was added, using the same Pasteur pipette, and, after mixing, the tile was incubated at room temperature for a further hour (or more).

The agglutination reactions involving the solutions under test were compared with those of the control and the lowest dilution of the testing fluid inhibited completely by the test solution was recorded as its inhibition titre against that testing fluid.

The above procedure is not satisfactory for solutions which can inhibit a volume of testing fluid equal to, or greater than, their own (i.e. a titre $\leqslant 1$) since all agglutination reactions would be negative. With such solutions agglutination inhibition tests involving one drop of neat testing fluid mixed with one drop of increasing dilutions of the solution under test were used. The reciprocal of the greatest dilution of the solution which would inhibit completely the agglutination was recorded as its inhibition titre, a value strictly comparable with that described above.

Although, commonly, doubling dilutions of testing fluids were used in such tests, some of the work involved dilutions other than these but, irrespective of the actual dilutions

used, all were made by mixing a given volume of neat testing fluid with a given volume of physiological saline to give a final volume of about 10 ml.

This agglutination inhibition procedure has proved very satisfactory and the titres recorded are highly reproducible, to less than one doubling dilution, as has been found by Hartmann (1941). With care, identical titres can be obtained from duplicate determinations, even when consecutive testing fluid dilutions differ by a factor of less than two.

In much of the work to be described, the inhibition titres of many solutions have been converted to $\log_2$. Since this converts the distribution of the titres of blood group substances in salivas to approximately normal distribution (Clarke, McConnell and Sheppard, 1960), tests of significance are then facilitated.

Collection of specimens

Most of the saliva specimens studied came from university students. These specimens were obtained in 1 oz. wide-mouthed McCartney bottles. At the same time, each student provided a finger-prick of blood in a 3" test-tube containing about 2 c.c. of physiological saline. Both

specimens from each student were numbered identically. The donors of the specimens were known since each student recorded his name, sex, age and country of birth on a sheet next to the number of his specimens. Since Asians cannot become Australian citizens, and since the blood group gene frequencies of Asians are known to be different from those of Caucasians, where gene frequencies are calculated in this work the data pertaining to Asian students have been excluded.

Although some of the gene frequencies for Australian- and European-born students have been shown to be significantly different, the data have been pooled to give gene frequencies for Australian-domiciled students.

Blood specimens were kept at 5°C. until they were grouped, usually about 6 hours after collection. For blood-grouping, the specimens were centrifuged, the supernatants were discarded, and the cells were resuspended to give an approximately 2% suspension, as judged by the eye, by comparison with an accurately-formed 2% suspension. Four drops of each specimen were put in a row across a blood spotting tile and each was mixed with a drop of a testing fluid in the order, anti-A, anti-B, Ulex extract, Dolichos extract. The sub-groups A_1 and A_2 could be determined by the reactions with the Ulex and Dolichos reagents. Cells of known groups were used as controls.

The saliva specimens were divided into two as soon as possible after collection, and each was labelled appropriately. One portion, to be used for agglutinin determinations, was deep-frozen. The other portion, to be used for antigen determinations, was boiled within one hour of collection. This portion, after cooling to room temperature, was then deep-frozen.

On removing saliva specimens, either boiled or unboiled, from deep-freeze it was found that, on thawing, the materials responsible for the viscosity of saliva had aggregated and were readily precipitated by centrifugation. The supernatants were clear, non-viscous and were easy to handle, unlike the neat saliva. Another technique satisfactory for removing the viscosity of saliva without affecting ABO antigens or agglutinins in it was suggested by Mr. G. Vos. Equal volumes of saliva and ether are shaken for 10 minutes and the mixture is centrifuged for 5 minutes at 3,000 r.p.m. This produces three layers, a top layer of ether, a semi-solid middle layer, and a lower layer of clear saliva which is non-viscous and is easily handled. Although tests showed that this technique did not affect antigen or agglutinin titres, it was not used in any of the work reported here.

ABO antigens were detected and titred using the agglutination-inhibition technique, and ABO agglutinins were detected and titred by agglutination.

Agglutination of A_1, A_2, B and O red cells by Ulex extract

The 20 specimens of A_1 and B cells, and the 10 specimens of A_2 and O cells were chosen at random from the laboratory pilot samples from blood donors of the appropriate groups reporting to the Red Cross Blood Transfusion Service on the one day, and were tested on the following day. The given numbers of specimens were chosen since it was known that they could be handled simultaneously, so that all tests were carried out under identical conditions.

Accurate 2% suspensions of all cells were prepared.

The scoring technique of Race and Sanger (1958) was used, viz.

	Agglutination reaction	Score
+++	large agglutinates	10
++	clear agglutination	8
+	weak agglutination	5
(+)	clear agglutination seen microscopically	3
w	very weak agglutination seen microscopically	2
	negative	0

The total score for each specimen was determined by adding the individual scores for the reactions with doubling dilutions of the Ulex extract.

Precipitation of A substance from salivas of A_1 and A_2 secretors using a Dolichos extract

Nineteen salivas from group A_1, and twenty from group A_2, secretors were chosen at random from specimens kept in deep-freeze and known to have come from secretors. (Twenty salivas of each type had been chosen but one proved to be from a group A_1 non-secretor. The error had occurred when checking result sheets for the appropriate serial numbers for A_1 secretor salivas. The number of a saliva specimen had been read inadvertently with the result for the specimen below it on the list.)

Six specimens from group O secretors were selected for use as controls, and a specimen from an A_x secretor was obtained.

Procedure

(i) The inhibition titres of the salivas with Ulex and Dolichos extracts were determined.

(ii) 1 ml. of each saliva was mixed with 1 ml. of the Dolichos biflorus extract, and incubated for 24 hours at 5°C. A visible precipitate occurred in each tube.

(iii) The tubes were centrifuged at 12,840^{x}g (10,000 r.p.m.) in a refrigerated centrifuge for 20 minutes.

(iv) The supernatants were removed and boiled to denature and precipitate the remaining protein from the Dolichos extract in solution. (Preliminary tests had shown that boiling will release any A antigen bound to the Dolichos extract.) These were then centrifuged at 12,840^{x}g (10,000 r.p.m.) for 20 minutes. The inhibition titres of the supernatants with Ulex and Dolichos extracts were determined.

(v) The amounts of protein in the precipitates were determined relative to each other using the colourimetric method of Lowry et al. (1951).

(N.B. No attempt was made to determine the mass of protein in the precipitates. All that was desired was to determine whether one precipitate had twice or three times, etc. the amount of protein in another.)

The reagents used were:

(i) Alkaline copper reagent.

50 ml. of 2% Na_2CO_3 in 0.10 NaOH and 1 ml. of 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate.

(ii) Folin reagent.

Proprietary product (Univar) diluted to make it IN in acid.

The precipitates were resuspended in 1 ml. of physiological saline and dissolved by adding 1 ml. of 2% Na_2CO_3 in 0.10 N NaOH. 0.1 ml. of each of the dissolved precipitates was mixed with .5 ml. saline, 3 ml. of alkaline copper reagent and .3 ml. of Folin reagent.

Optical densities (O.D.'s) of these solutions were determined using a Unicam spectrophotometer with $\lambda = 750$ mμ. (Preliminary tests had shown linearity of the O.D./Dolichos protein comparison from O.D. of 0 to .7.)

ABO agglutinins in saliva

All saliva specimens, irrespective of the blood group of the donor, were tested against 2% suspensions of A_1, A_2, B and O cells.

The anti-A titres reported are those for reactions with A_1 cells.

ABO antigens and agglutinins in cervical mucus

The two specimens of cervical mucus tested were provided by Dr. F. Welch.

Each specimen was collected on a sterile, cotton-wool swab. Identical swabs had been tested and shown not to possess blood group specificity.

Since mucus could not be expressed from the swabs with Spencer-Wells forceps, eight drops of saline were added to each, and they were then allowed to stand for 15 minutes.

Clear, non-viscous liquid was obtained from the swabs by putting the tip of a Pasteur pipette against them, and sucking. This liquid was used as the cervical mucus specimen for testing.

ABO antigens on spermatozoa

Semen specimens from unknown donors were obtained from the South Australian Institute of Medical and Veterinary Science. The specimens were obtained by the Institute for testing in connection with infertility. Consequently, a

number of specimens with very few spermatozoa were encountered, and discarded without examination of the antigens they possessed.

Since it was not permissible to obtain the semen donors' names and/or their blood groups, the ABO blood groups of secretor donors were determined from the seminal plasmas. The blood groups of non-secretors could not be determined.

The mixed agglutination technique was that used by Gullbring (1957).

Spermatozoa were obtained from whole semen collected in a dry jar by allowing the semen to stand for about one hour, to enable precipitation of non-motile spermatozoa and non-spermatozoal cells, and removing the supernatant. Spermatozoa were precipitated from this by centrifugation.

The precipitated spermatozoa were washed three times in physiological saline, and were then resuspended in saline.

The spermatozoa were incubated for 30 minutes with the appropriate antiserum. Different temperatures, 5°C., 20°C. and 37°C. were used in different experiments. They were then washed three times in physiological saline, the last washing solution was tested for its ability to inhibit an

anti-human globulin serum, which was found to be negative. The spermatozoa were then resuspended, divided into different tubes and appropriate red blood cells were added. The tubes were then incubated for 15 minutes, at temperatures of 5°C., 20°C. and 37°C. in different experiments, and centrifuged at approximately 1,000 r.p.m. The precipitates were broken up by tapping. Samples of the specimens were examined microscopically for agglutination.

Antibody elution was performed by incubating spermatozoa specimens, treated as above, and red cells at 56°C. for 5 minutes. Agglutination of red cells was examined without centrifugation.

Fluorescent antisera were prepared by a method similar to that described by Nairn (1962).

Carbonate-bicarbonate buffer pH 9.0, 0.5 M, was prepared by dissolving 3.7 gm. $NaHCO_3$ and 0.6 gm. anhydrous Na_2CO_3 in distilled water to give 100 ml. of solution.

Physiological saline buffered at pH 7.1 by 0.01 M phosphate (phosphate buffered saline) was prepared by dissolving 8.5 gm. NaCl, 1.07 gm. Na_2HPO_4 (anhydrous) and 0.39 gm. $NaH_2PO_4.2H_2O$ per litre of distilled water.

Fluorescein isothiocyanate (FITC) was obtained from Fluka, Switzerland. This was used dry at the rate of 3 mgm./1 ml. of serum.

Rhodamine isothiocyanate (RITC) was obtained from Gurr's, England. This was used dry at the rate of 3 mgm./ 1 ml. of serum.

Rhodamine sulphonyl chloride was prepared by grinding 0.5 gm. Lissamine rhodamine B200 (I.C.I.) in 1 gm. of PCl_5 in a mortar and extracting the sulphonyl chloride as a clear dark solution by dissolving in 5 ml. acetone and filtering. This solution was used at the rate of 0.1 ml./1 ml. of serum.

Conjugation of the sera was carried out by mixing one volume of serum with two volumes of carbonate-bicarbonate buffer and adding slowly the appropriate amount of fluorochrome to this mixture whilst stirring in a cold-room. Stirring was continued for about 20 hours.

The conjugates were purified by gel filtration using Sephadex G-25 and phosphate-buffered saline. Passage of the protein conjugates through the chromatography column was easily observed using a portable U.V. lamp, the conjugates were the first fluorescent fractions to leave the column.

Fluorescent-labelled gamma globulin was precipitated by making solutions 40% saturated with ammonium sulphate. The precipitates were re-dissolved in phosphate-buffered saline and dialyzed against it to remove ammonium sulphate.

After reaction, spermatozoa were mounted in 90% glycerol, 10% phosphate-buffered saline. A Leitz Ortholux microscope fitted for fluorescence microscopy with an HBO 200 W high-pressure mercury lamp was used for microscopy.

Electrophoretic separation of FITC-labelled antisera was performed using a Beckman continuous flow paper electrophoresis instrument, model CP, with veronal buffer, pH 8.6, made from 5.52 gm. diethyl barbituric acid and 30.90 gm. sodium diethyl barbiturate dissolved in 7.5 l. of distilled water to give a final ionic strength of 0.02.

Studies on couples infertile with no recognized physiological cause

Some couples were ascertained by being made aware of these studies when enquiring from the Children's Welfare and Public Relief Department about adoption. Such couples were given a circular at the Department stating the details of the research programme, and, if they wished to participate,

were asked to contact the Genetics Department. Other couples were referred by infertility specialists, Drs. Mary Walker and F. Welch.

Before including a couple in the study, a statement from their doctor that there was no known reason for infertility, despite extensive tests, was necessary.

Tests for spermatozoal agglutinins were performed using the technique of Franklin and Dukes (1964a).

Semen specimens were adjusted to a concentration of 50×10^6 sperm/ml. as determined using a Petroff-Hauser bacteria counter with phase-contrast microscopy. 0.05 ml. of the sperm sample and 0.5 ml. of the wife's serum, undiluted and diluted 1:5 with physiological saline, were mixed and incubated at 37°C. for four hours. Samples were removed at ½, 1, 2, 3 and 4 hours and examined under phase contrast for agglutination.

CHAPTER III

EXPERIMENTAL RESULTS

A. ABO ANTIGENS IN SALIVA

1. Agglutination inhibition capacities of salivas from individuals of different ABO blood groups

(a) Reaction between human anti-A and salivas from A_1 and A_2 secretors

Of 171 saliva specimens obtained from university students, 55 proved to be from group A secretors and 16 from group A non-secretors. The capacity of 51 of the A secretor salivas to inhibit a human anti-A serum was determined - there was insufficient saliva in four of the specimens for the determination to be carried out - and the distribution of this capacity was found to be bimodal (Figure III.1).

Fifty four of the fifty five group A secretors were able to be recalled and their red blood cells were typed for their A_1 or A_2 subgroup. There were 43 group A_1 secretors (of whom four supplied insufficient specimens for titration) and 11 group A_2 secretors. The results of this sub-grouping showed that, in the above distribution,

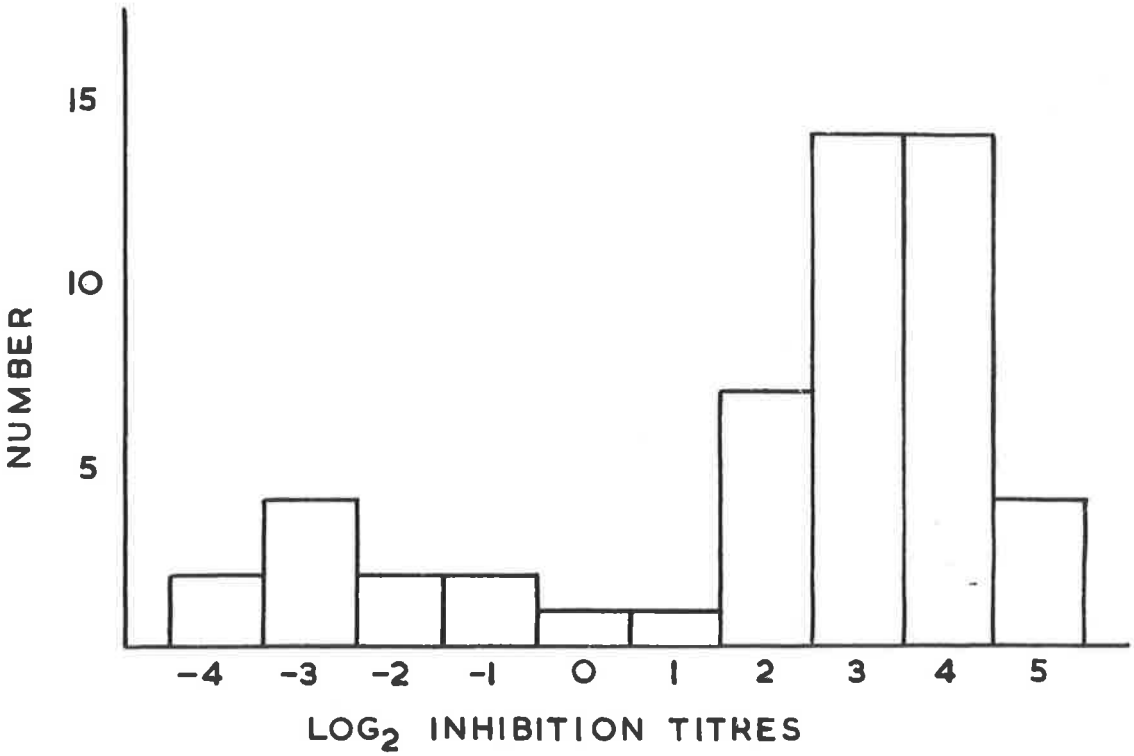


FIGURE III.1. THE DISTRIBUTION OF THE LOG_2 INHIBITION TITRES OF 51 SALIVAS FROM GROUP A SECRETORS WITH A HUMAN ANTI-A SERUM.

the class with the smaller mode was associated with A substance in the salivas of A_2 secretors, the other one with A substance in the salivas of A_1 secretors.

In this sample the titres of the A_1 secretors were all >1 and the titres of the A_2 secretors were <1. To enable the data to be presented conveniently as a histogram, and to facilitate the calculation of the test of significance, all titres were transformed to $\log_2$. The t test to determine whether the average $\log_2$ inhibition titres of A substance in the salivas of A_1 and A_2 secretors were significantly different gave $t_{49} = 16.02$ with $P < .001$.

To confirm the significance of the greater capacity of salivas from A_1 secretors to inhibit human anti-A than salivas from A_2 secretors, stored salivas from different donors were tested. Salivas from 17 people classified as group A_1, and six classified as group A_2 were tested with Ulex extract (to determine the secretor status) and with the B anti-serum referred to above. Fourteen of the 17 A_1 salivas, and five of the six A_2 salivas, were from secretors. The fourteen A_1 salivas gave titres >1 and the five A_2 salivas gave titres <1 in inhibition tests with the B antiserum.

The probability of obtaining non-overlapping inhibition titres for salivas from 14 A_1 secretors and five A_2 secretors, with the hypothesis that there is no difference, is .00016.

Consequently, it can be concluded that, on the average, salivas from A_1 secretors have a greater capacity to inhibit human anti-A serum than have salivas from A_2 secretors.

These results have been reported previously (Boettcher, 1964a).

(b) Reaction between Ulex extract and salivas from A_1, A_2, B and O secretors

During the study, reported above, the ability of salivas from secretors, irrespective of their blood group, to inhibit a Ulex extract was determined also (Figure III.2).

The average inhibition titres followed the same order as that determined by Plato and Gershowitz (1961) viz. O, A_2, A_1, B, in descending order, though the differences between average titres for the different blood groups were not statistically significant.

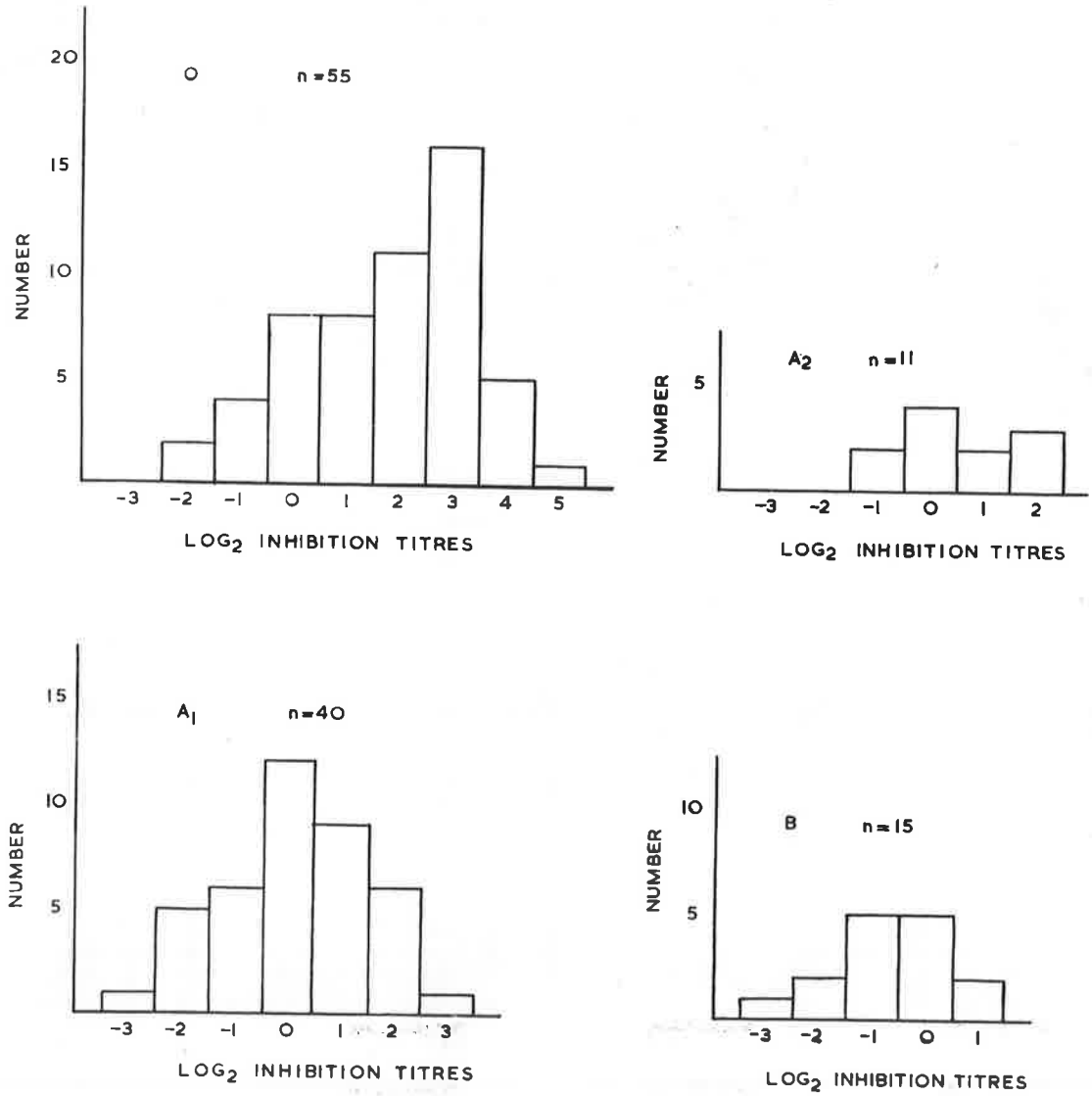


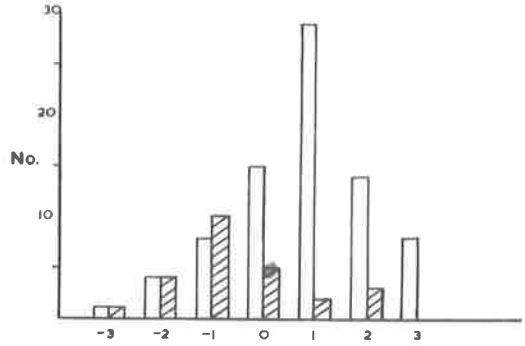
FIGURE III.2. LOG_2 INHIBITION TITRES OF SALIVAS FROM A_1, A_2, B AND O SECRETORS WITH ULEX EXTRACT. The average log_2 inhibition titres for the salivas represented above are: O = 1.72 ; A_2 = 0.56 ; A_1 = 0.13 ; B = -0.67. The differences between consecutive average titres are not significant.

(c) Correlations between titres of salivas from A_1, A_2 and B secretors with pairs of testing fluids

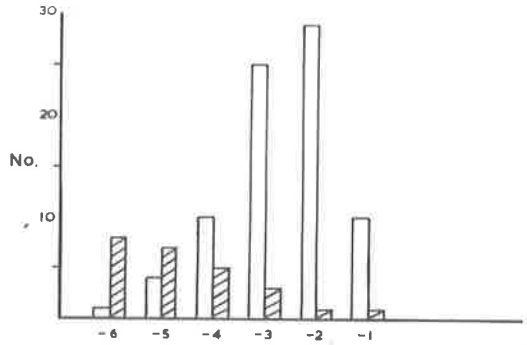
A larger study, independent of that described above, using saliva specimens obtained from university students was undertaken to investigate the relationships between different ABO blood group antigens in the same saliva, to obtain data on the occurrence of "aberrant secretors" (McNeil et al., 1957a) in a group of people unselected for their fertility status, since it has been suggested that, among couples infertile with no apparent physiological cause, there is a higher proportion of "aberrant secretors" (Behrman et al., 1960).

The inhibition titres of the salivas from secretors of various blood groups were determined using four different testing fluids, human anti-A and anti-B, and seed extracts from Ulex europaeus (anti-H) and Dolichos biflorus (anti-A_1). The distribution of the inhibition titres of the A_1, A_2, B and O secretor salivas are presented as histograms in Figure III.3, and the averages are given in Table III.1.

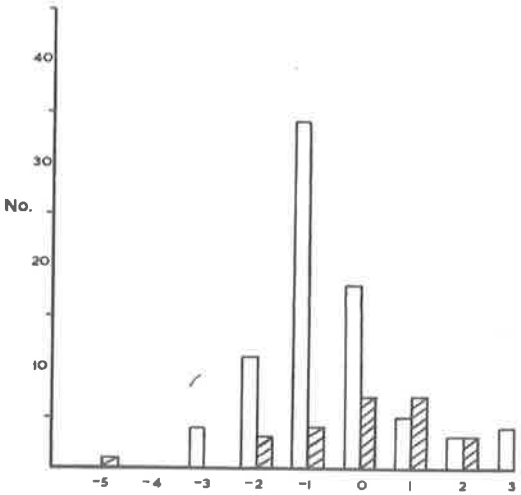
From Table III.1. it can be seen that the ability of salivas from secretor individuals of blood groups O, A_2, A_1 and B to inhibit an extract of Ulex europaeus decreases in



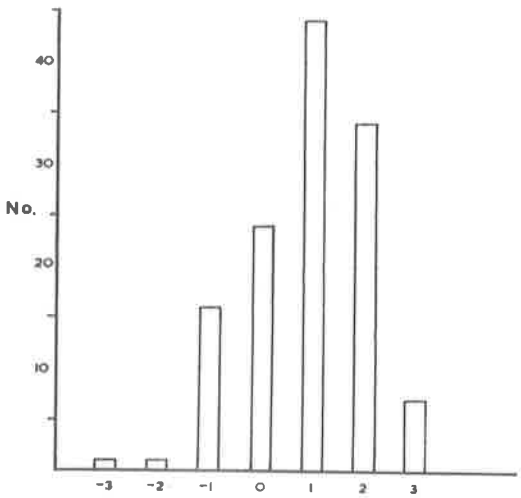
A_1 AND A_2 SALIVAS WITH ANTI-A



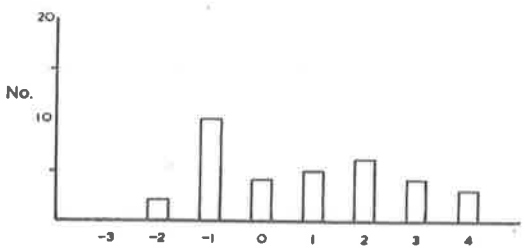
A_1 AND A_2 SALIVAS WITH DOLICHOS



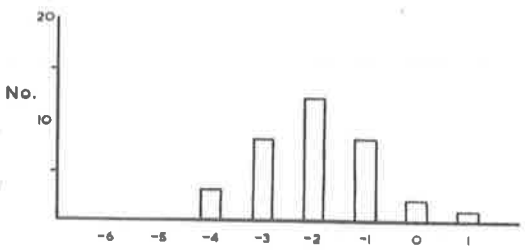
A_1 AND A_2 SALIVAS WITH ULEX



O SALIVAS WITH ULEX



B SALIVAS WITH ANTI-B



B SALIVAS WITH ULEX

FIGURE III.3. LOG_2 INHIBITION TITRES OF A_1, A_2, B AND O SALIVAS WITH TESTING FLUIDS. (The log_2 inhibition titres of A_2 salivas are indicated by hatched blocks, those of other groups are indicated by unfilled blocks.)

TABLE III.1. THE AVERAGE LOG_2 INHIBITION TITRES OF SALIVAS FROM A_1, A_2, B AND O SECRETORS, AND THE RESULTS OF TESTING THE STATISTICAL SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE PAIRS OF AVERAGES INDICATED

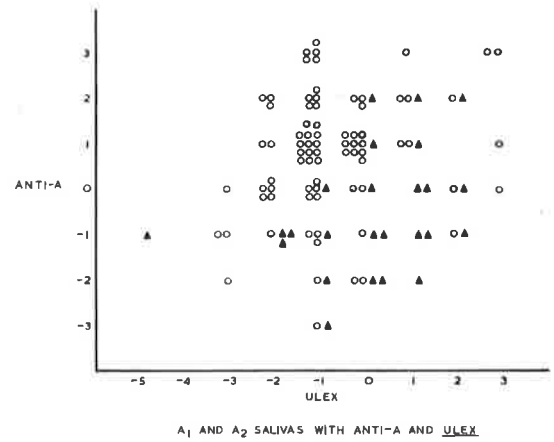
TESTING FLUID	BLOOD GROUP	NUMBER	AVERAGE LOG_2 INHIBITION TITRES AND STANDARD ERRORS	STATISTICAL SIGNIFICANCE OF COMPARISONS
ULEX EXTRACT	O	127	0.88 ± 0.032	S.N.D. $= 4.14$ $P < .001$
	A_2	25	-0.08 ± 0.316	$t_{102} = 1.53$ $.10 > P > .05$
	A_1	79	-0.57 ± 0.151	$t_{112} = 4.38$ $P < .001$
	B	35	-1.91 ± 0.194	
HUMAN ANTI-A	A_1	79	0.78 ± 0.151	$t_{102} = 3.36$ $P < .001$
	A_2	25	-0.52 ± 0.265	
DOLICHOS EXTRACT	A_1	79	-2.65 ± 0.124	$t_{102} = 7.27$ $P < .001$
	A_2	25	-4.60 ± 0.277	
HUMAN ANTI-B	B	35	0.71 ± 0.308	

that order. This order is the same as that determined in the study reported earlier (p. 65) and is the same as that found by Plato and Gershowitz (1961). Although, in the above data, the difference between the average $\log_2$ inhibition titres for A_2 and A_1 salivas with the Ulex extract is not statistically significant, that in Plato and Gershowitz's data is significant at the 1% level and, therefore, the order O, A_2, A_1, B appears to be correct.

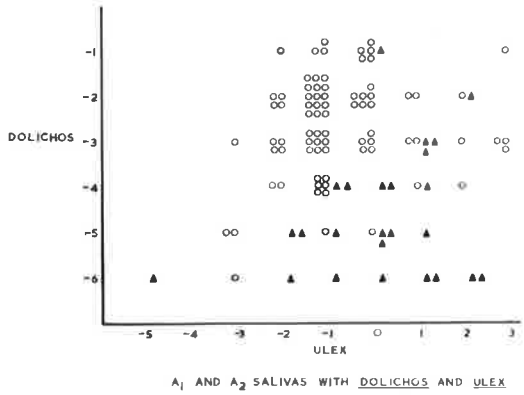
The data show, also, that the differences between the average $\log_2$ inhibition scores for salivas from A_1 and A_2 secretors with both human anti-A and Dolichos biflorus extract are significantly different, with A_1 secretor salivas, on the average, having greater inhibition scores with both of these testing fluids.

The $\log_2$ inhibition scores of individual salivas within the various blood groups with pairs of testing fluids have been plotted graphically (Figure III.4), the correlation coefficients for each of the pairwise comparisons have been calculated, and the statistical significance of each of these correlation coefficients has been determined (Table III.2).

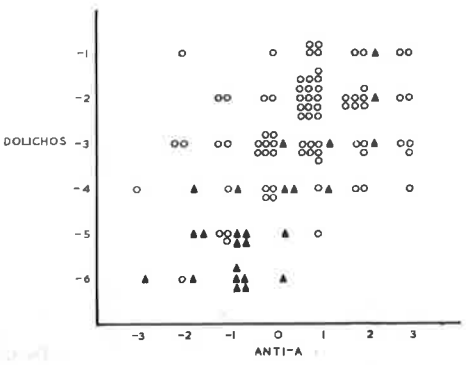
This analysis suggests that there is no correlation between the titres of both A_1 and A_2 salivas with human



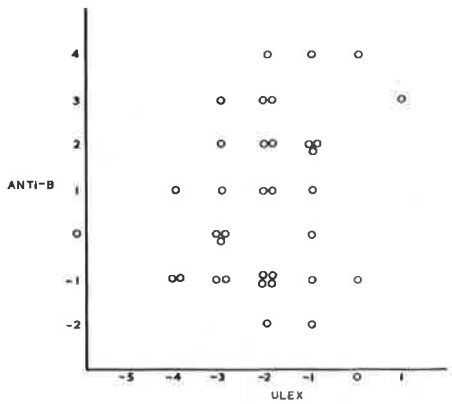
A_1 AND A_2 SALIVAS WITH ANTI-A AND ULEX



A_1 AND A_2 SALIVAS WITH DOLICHOS AND ULEX



A_1 AND A_2 SALIVAS WITH DOLICHOS AND ANTI-A



B SALIVAS WITH ANTI-B AND ULEX

FIGURE III.4. PAIRWISE COMPARISONS OF LOG_2 INHIBITION TITRES OF A_1, A_2 AND B SALIVAS WITH TESTING FLUIDS.

In the comparisons involving A_1 and A_2 salivas:

- ● denotes a point representing titres of an A_1 saliva.
- ▲ denotes a point representing titres of an A_2 saliva.

TABLE III.2. CORRELATION COEFFICIENTS OF THE LOG_2 INHIBITION TITRES OF A_1, A_2 AND B SALIVAS WITH PAIRS OF TESTING FLUIDS

TESTING FLUIDS	BLOOD GROUPS OF INDIVIDUALS TESTED	NUMBER TESTED	CORRELATION COEFFICIENTS (r)	SIGNIFICANCE OF CORRELATION COEFFICIENTS
HUMAN ANTI-A AND ULEX EUROPAEUS EXTRACT	A_1	79	.243	$.05 > P > .02$ (Testing r = 0)
	A_2	25	.337	$.10 > P > .05$ (" ")
DOLICHOS BIFLORUS AND ULEX EUROPAEUS EXTRACTS	A_1	79	.199	$.10 > P > .05$ (" ")
	A_2	25	.263	$.20 > P > .10$ (" ")
HUMAN ANTI-A AND DOLICHOS BIFLORUS EXTRACT	A_1	79	.364	$P < .001$ (" ")
	A_2	25	.753	$P < .001$ (" ")

anti-A and Ulex extract, and with Dolichos and Ulex extracts, but there is a positive correlation between titres from tests with human anti-A and Dolichos extract (Table III.2).

Although the correlation coefficient for the $\log_2$ inhibition titres of the 79 A_1 salivas with human anti-A and Ulex extract is significantly different from O ($.05 > P > .02$), it seems reasonable to accept that the statistical significance of this value is a chance result since, first, it is not significantly different from the comparable correlation coefficient obtained from the data on A_2 salivas (S.N.D. = 0.465, P = 0.64), which is not significantly different from O. Secondly, the $\log_2$ inhibition titres of the A_1 salivas with human anti-A and Dolichos extract are correlated, and the correlation coefficient of the $\log_2$ inhibition titres of the salivas with Dolichos and Ulex extracts is not significantly different from O.

2. Relationship between ABO antigens in saliva and on red blood cells

Although the decreasing order for the inhibition of a saline extract of the seeds of Ulex europaeus by secretor salivas has been established as O, A_2, A_1, B (see pp. 65, 67)

the order for agglutination of red blood cells by Ulex extracts has been reported as O, A_2, B, A_1 (Race and Sanger, 1962; Solomon, 1964).

The agglutinating ability of the Ulex europaeus extract, used in the inhibition tests with salivas reported in the previous section, was tested with 20 specimens of A_1 and B cells, and 10 specimens of A_2 and O cells.

The agglutination reactions of 2% suspensions of each of the specimens of red cells were scored according to the method suggested by Race and Sanger (1958). The average scores for specimens of red cells of the four groups were determined and the tests of significance of the differences between these average scores were calculated (Table III.3).

From these results it can be concluded that the ability of the Ulex extract to agglutinate red blood cells of different ABO blood groups decreases in the order O, A_2, B, A_1.

3. Relationship between A_1 and A_2 antigens in saliva

The agglutination inhibition capacity of a saliva often has been taken as some quantitative estimate of the amount of the particular blood group antigen present. However, as was pointed out earlier in this thesis (pp. 18, 19).

TABLE III.3. THE AVERAGE SCORES OF RED BLOOD CELLS OF GROUPS A_1, A_2, B AND O WHEN REACTED WITH A ULEX EUROPAEUS EXTRACT, AND THE RESULTS OF TESTING THE STATISTICAL SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE AVERAGE SCORES INDICATED

BLOOD GROUP OF RED CELLS	NUMBER OF SAMPLES	AVERAGE SCORE	STATISTICAL SIGNIFICANCE OF COMPARISONS
O	10	52.50	$t_{18}=17.71$ $P < .001$
A_2	10	43.00	$t_{28}= 9.18$ $P < .001$
B	20	21.55	$t_{38}= 2.53$ $.02 > P > .01$
A_1	20	17.95	

it is possible that the significantly different inhibition capacities of salivas from group A_1 and A_2 secretors might not be due to a quantitative difference in the amount of antigen present, but to a qualitative difference of the type of antigen present.

Two techniques which have been used to determine the relative amounts of soluble antigen present in salivas are those of agglutination inhibition and antibody precipitation. It has been assumed that each of these techniques provides some measure of the amount of a particular antigen present. If it does, there should be proportionality between the decrease in the inhibition titre of a particular blood group substance in a saliva, and the amount of precipitate formed on reacting the saliva with the appropriate precipitin. Therefore, if two salivas are used, one of which has an initial inhibition titre twice that of the other, and all of the blood group substance is precipitated from each, the amount of precipitate formed by the one saliva should be twice that formed by the other.

To test whether this proportionality exists, a Dolichos biflorus extract was used with salivas from A_1 and A_2 secretors. The Dolichos biflorus extract was chosen first, because

it could be obtained in large quantities. Secondly, because it had been found to be satisfactory for use in agglutination inhibition tests. Thirdly, because it will precipitate with salivas from A_1 and A_2 secretors, and appears to recognize only one identical antigen in each (Bird, 1959b). And, fourthly, because there is no possibility of interference due to anti-species antibodies which might occur if an immune antiserum, prepared in an animal of a different species, was used.

Since it has been shown that individual molecules of blood group substances from an A person possess both H and A antigenicity (Watkins and Morgan, 1956/57), it would be expected that, on precipitation of A substance from salivas of A_1 and A_2 secretors with a Dolichos extract, the inhibition titres of the salivas with a Ulex extract should decrease due to the simultaneous removal of H antigen.

The inhibition titres of salivas from 19 A_1 and 20 A_2 secretors with Ulex europaeus and Dolichos biflorus seed extracts were determined (initial Ulex and Dolichos titres). After reaction of the salivas with an equal volume of Dolichos extract to precipitate A antigen and, consequently, H antigen, the relative amounts of protein in the precipitates were

determined using the colourimetric Folin-Ciocalteau estimation of Lowry *et al.* (1951). After removal of excess *Dolichos* extract in the supernatants by boiling and centrifugation, the *Ulex* and *Dolichos* inhibition titres were determined and multiplied by 2 to allow for the dilution due to addition of the *Dolichos* extract, and to make these titres (final *Ulex* and *Dolichos* titres) strictly comparable with the initial *Ulex* and *Dolichos* titres.

As controls, six salivas from group O secretors were included. These gave the amount of the *Dolichos* extract which precipitated on standing without reacting with A antigen, and therefore served as the "blank" for the determination of the relative amounts of protein in the *Dolichos* extract precipitated by A antigen in the various salivas. During the experiment the inhibition titres of these salivas with the *Ulex* extract remained constant apart from the decrease due to alteration in volume.

Saliva from a group A_x secretor (Mrs. M., Jakobowicz *et al.*, 1960), supplied by Dr. Rachel Jakobowicz, was included. Since such salivas do not inhibit anti-A reagents it was of interest to determine whether it would precipitate some of the *Dolichos* extract. Duplicate tests showed that this saliva did not precipitate any more of the *Dolichos* extract than the O controls.

The reduction in the inhibition titres of the salivas from 19 A_1 and 20 A_2 secretors due to the treatment with the Dolichos extract, and the optical densities (O.D.'s) after treatment of aliquots of the precipitates formed during the treatment have been determined, and are presented graphically (Figure III.5); the exact values can be obtained from Table III.4.

The correlation coefficients for the reductions in the inhibition titres of the salivas and the O.D.'s of the precipitates have been determined for the 19 A_1 salivas, the 20 A_2 salivas and for the 39 salivas taken together.

For the 19 salivas from A_1 secretors:

$r = 0.887 \quad t_{17} = 7.93$ (Testing for $r = 0$) $\quad P < .001$

i.e. the correlation coefficient is significantly different from 0. However, it is not significantly different from 1 ($t_{17} = 0.4973$; $.6 > P > .5$).

Similarly, for the 20 salivas from A_2 secretors:

$r = 0.781 \quad t_{18} = 5.31$ (Testing for $r = 0$) $\quad P < .001$

$t_{18} = 0.9504$ (Testing for $r = 1$) $\quad .4 > P > .3$

The two correlation coefficients are not significantly different from each other (S.N.D. = 0.9118), nor is the

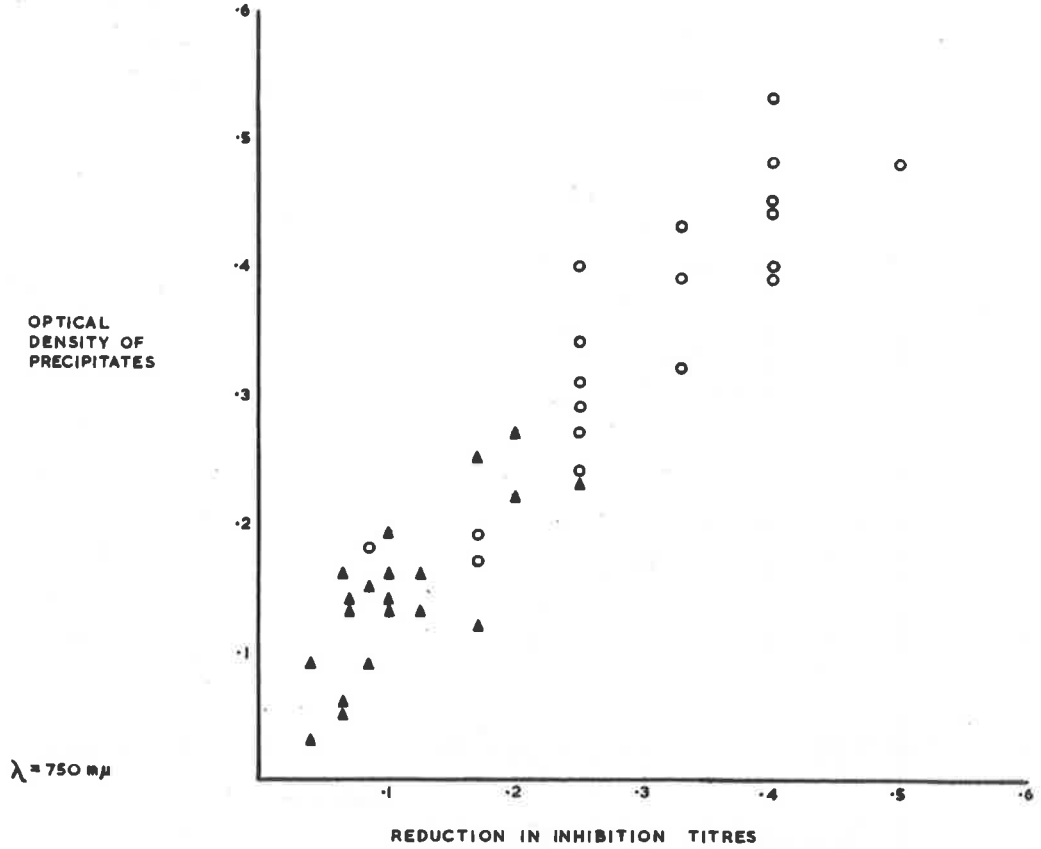


FIGURE III.5. COMPARISON OF THE OPTICAL DENSITIES (O.D.'S) OF PRECIPITATES FORMED, AND THE REDUCTIONS IN INHIBITION TITRES WHICH OCCURRED DURING REACTION OF SALIVAS FROM 19 A_1 SECRETORS (OPEN CIRCLES) AND 20 A_2 SECRETORS (FILLED TRIANGLES) WITH A DOLICHOS BIFLORUS EXTRACT.

The O.D.'s were determined on aliquots of the precipitates. Protein was estimated by the method of Lowry et al. (1951).

The inhibition titres were determined with the Dolichos biflorus extract.

TABLE III.4. INHIBITION TITRES OF A_1 AND A_2 SALIVAS WITH ULEX AND DOLICHOS EXTRACTS BEFORE AND AFTER TREATMENT AND THE OPTICAL DENSITIES OF PRECIPITATES

A_1 salivas number	Initial Dolichos titre	Final Dolichos titre	Reduction in Dolichos titre (Dol)	Initial Ulex titre	Final Ulex titre	Reduction in Ulex titre (Ulex)	Ulex / Dol	O.D. of PPTS.
805	.25	0	.25	.50	.20	.30	1.20	.40
806	.25	0	.25	.75	.33	.42	1.68	.34
810	.33	0	.33	.50	.25	.25	.76	.32
813	.085	0	.085	.40	.33	.07	.82	.13
823	.25	0	.25	.20	.10	.10	.40	.31
826	.33	0	.33	.33	.125	.205	.62	.43
830	.40	.004	.40	.50	.20	.30	.75	.44
835	.25	0	.25	1.25	.33	.92	3.68	.29
837	.33	0	.33	.60	.17	.43	1.30	.39
855	.17	0	.17	.20	.08	.12	.71	.19
861	.17	0	.17	.40	.25	.15	.88	.17
867	.40	0	.40	1.00	.50	.50	1.25	.40
872	.25	0	.25	.25	.20	.05	.20	.27
944	.40	.005	.40	.60	.10	.50	1.25	.45
1015	.40	0	.40	.40	.25	.15	.38	.48
1016	.50	0	.50	.60	.25	.35	.70	.48
1160	.25	0	.25	.50	.40	.10	.40	.24
1245	.40	0	.40	.20	.10	.10	.50	.39
1206	.40	.004	.40	.75	.25	.50	1.25	.53
A_2								
812	.07	0	.07	.40	.17	.23	3.29	.13
818	.10	0	.10	.60	.17	.43	4.30	.19
832	.20	0	.20	.40	.17	.23	1.15	.27
839	.20	0	.20	.90	.40	.50	2.50	.22
886	.065	0	.065	.90	.50	.40	6.15	.06
912	.085	0	.085	1.25	.25	1.00	11.76	.15
918	.10	0	.10	2.00	.33	1.67	16.70	.14
927	.17	0	.17	1.25	.50	.75	4.41	.25
975	.07	0	.07	.80	.25	.55	7.86	.14
982	.125	0	.125	.90	.25	.65	5.20	.16
1043	.25	0	.25	1.00	.50	.50	2.00	.23
1096	.04	0	.04	1.00	.20	.80	20.00	.09
1131	.065	0	.065	.50	.33	.17	2.62	.05
1140	.10	.004	.096	1.00	.33	.67	6.98	.16
1147	.04	0	.04	2.50	1.50	1.00	25.00	.03
1148	.065	0	.065	1.50	.50	1.00	15.38	.16
1158	.125	0	.125	2.00	.40	1.60	12.80	.13
1190	.085	.004	.081	1.50	.40	1.10	13.58	.09
1244	.17	.004	.166	1.75	.33	1.42	8.49	.12
1271	.10	0	.10	1.50	.80	.70	7.00	.13

pooled correlation coefficient (0.8373) significantly different from 1.

Therefore, the amount of protein precipitated from a Dolichos biflorus extract by a saliva appears to be proportional to the reduction in the inhibition titre of the saliva with the Dolichos extract during the treatment.

The initial and final Ulex and Dolichos titres, and the reductions in these titres have been recorded. The value of the reduction in the Ulex titre (Δ ULEX) divided by the reduction in the Dolichos titre (Δ DOL) has been calculated for each saliva and is recorded also (Table III.4).

It can be seen that the value of $\frac{\Delta \text{ ULEX}}{\Delta \text{ DOL}}$ for each of the 19 A_1 salivas is less than 4, whereas only 5 of the 20 A_2 salivas gave values of less than 4. Arranged in a 2x2 contingency table, the results give:

	$\frac{\Delta \text{ ULEX}}{\Delta \text{ DOL}} > 4$	$\frac{\Delta \text{ ULEX}}{\Delta \text{ DOL}} < 4$	
A_1 SALIVAS	0	19	19
A_2 SALIVAS	15	5	20
	15	24	39

$\chi^2_1 = 20.1$ $\quad P < .001$

Thus, it can be concluded that, when salivas from group A_1 and A_2 secretors are reacted with a Dolichos biflorus extract, the decrease in the ability of the salivas from A_2 secretors to inhibit a Ulex europaeus extract per unit decrease in their ability to inhibit a Dolichos biflorus extract, is greater, on the average, than that of salivas from group A_1 secretors.

B. ABO AGGLUTININS IN SALIVA

Using saliva specimens taken at irregular intervals over a period of two years from members of the laboratory staff and of my own family, and testing for the presence of ABO agglutinins, it has been found that all specimens from the one individual recorded the same result throughout, i.e. those persons whose salivas contained agglutinins showed them at each testing, and those persons whose salivas did not contain agglutinins did not show them at any of the testings. Further, the titre of agglutinins in saliva specimens taken at the same time daily from four individuals possessing them (one A_1, one B and two O's), proved to be stationary for the period of a month over which the salivas were tested. All salivas were deep-frozen immediately after collection and were tested on the same day with the same 2%

cell suspensions. The titres varied, at most, one doubling dilution from the respective modes.

These results indicate that the secretion of ABO agglutinins in saliva is a stable condition and are in agreement with the results of Prokop (1961), who found that, though immunization increased serum titres markedly, there was little effect on salivary agglutinins, and are contrary to those of Sondermeier and Flatow (1962), who reported variation in salivary agglutinin titre with time, and even daily variation.

Saliva specimens from university students were collected and tested during three separate periods approximately six months apart. To be able to test for heterogeneity between the results recorded for salivas in the separate periods, these results were initially kept separate. The results were grouped according to blood group, sex, period during which the saliva was tested and birthplace.

Tests of heterogeneity established that the blood group frequencies of European-born and Australian-born students were significantly different (Table A.1), but that, within individual blood groups, there was no significant heterogeneity between those who recorded salivary agglutinins

and those who did not. (The numbers of Asian-born students were too few to test statistically.) Accordingly, the data within individual blood groups were pooled. Further tests showed that, within individual blood groups, there was no significant heterogeneity of the data between sexes, so the data for both sexes were pooled.

The grouped data are presented in Table III.5.

These show that there is no significant heterogeneity between the results recorded during the three separate periods during which the saliva specimens were collected and tested and, therefore, it seems that the method of testing is reliable and that over the whole period the sensitivity of testing did not alter.

The frequency of anti-A in O salivas is significantly greater than in B salivas. ($\chi^2_1 = 27.73$ $P < .001$) as is the frequency of anti-B in O salivas when compared with A_2 ($\chi^2_1 = 18.42$ $P < .001$) and A_1 salivas ($\chi^2_1 = 115.6$ $P < .001$). Of special interest is the comparison of the frequency of anti-B in A_1 and A_2 salivas.

TABLE III.5. THE PRESENCE OF ABO AGGLUTININS IN SALIVAS

PERIOD OF TESTING	O SALIVAS				A_2 SALIVAS		A_1 SALIVAS		B SALIVAS		TOTALS
	ANTI-A ONLY	ANTI-B ONLY	ANTI-A AND ANTI-B	NONE	ANTI-B	NONE	ANTI-B	NONE	ANTI-A	NONE	
1	10	4	46	16	9	6	23	34	12	6	166
2	12	20	112	26	24	11	53	60	27	18	363
3	24	12	115	21	24	17	62	83	27	20	405
TOTALS	46	36	273	63	57	34	138	177	66	44	934

	anti-B detected	anti-B not detected	
A_1 salivas	138	177	315
A_2 salivas	57	34	91
	195	211	406

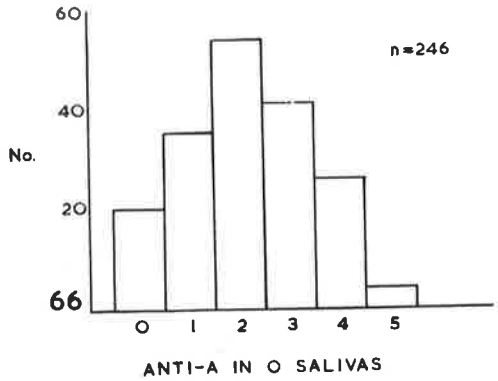
$\chi_1^2 = 10.03$ $\quad .01 > P > .001$

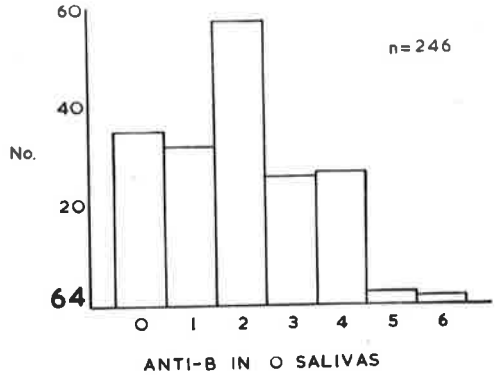
These data show that anti-B is detectable in a higher proportion of group A_2 individuals than of group A_1 individuals.

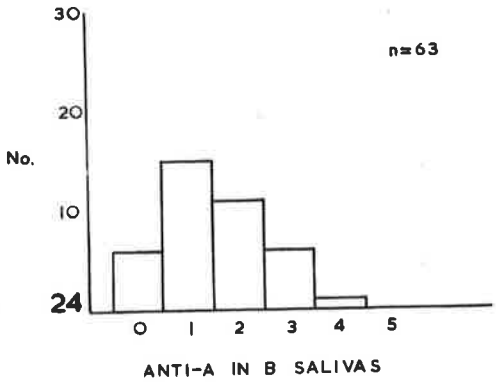
The titres of agglutinins in 529 of the salivas were determined and are presented in Figure III.6.

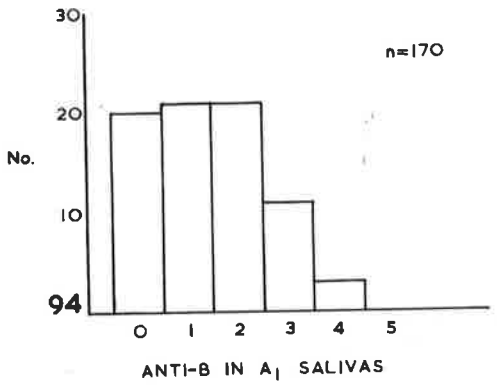
It is noted that anti-A_1 was detected in the saliva of two individuals, one A_2 and one A_2B. Subsequent testing showed that these individuals possessed anti-A_1 in the serum also.

During this study pedigrees were collected which contradict salivary agglutinin secretion being either a dominant or recessive character under the simple control of a single autosomal locus (Figure III.7) and, since the population data do not show a significant difference between the









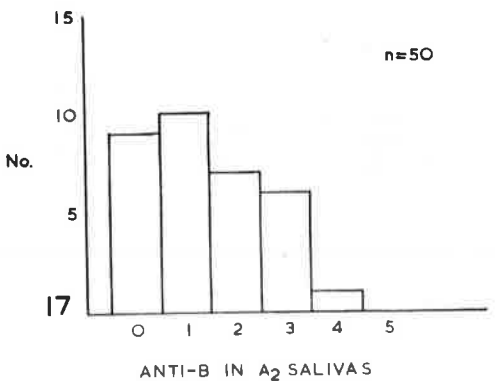


FIGURE III.6. LOG_2 TITRES OF ABO AGGLUTININS IN SALIVAS FROM A_1, A_2, B AND O INDIVIDUALS.

The numbers of salivas from individuals of the respective blood groups in which agglutinins were not detected are indicated to the left of the y-axis.

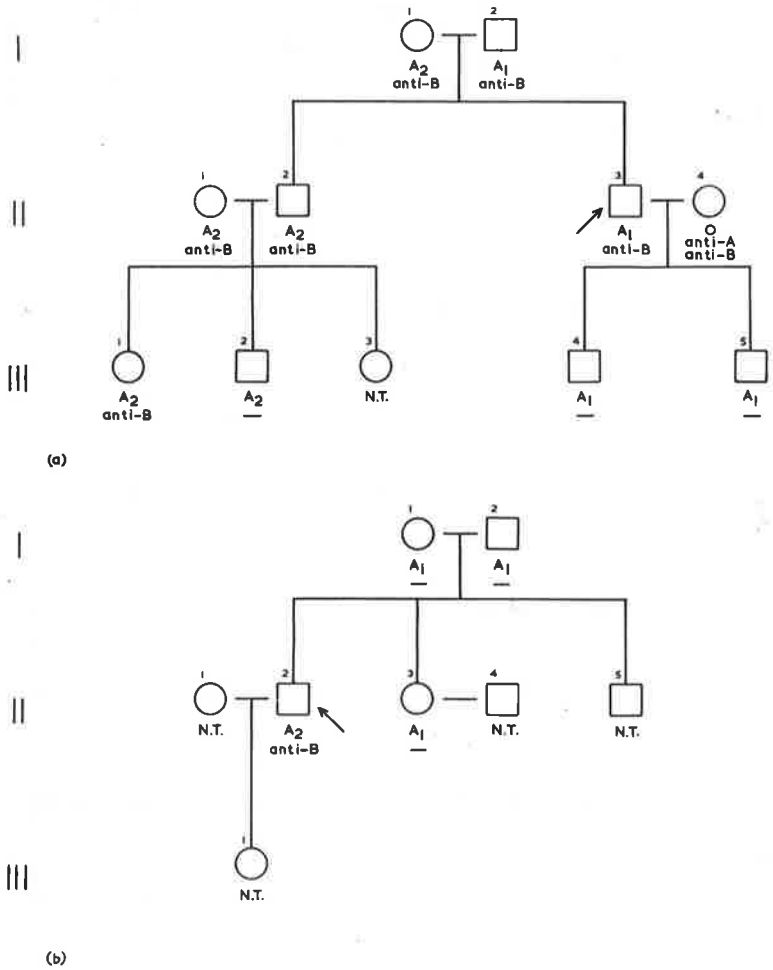


FIGURE III.7. PEDIGREES INCONSISTENT WITH THE PRESENCE OF ABO AGGLUTININS IN SALIVA BEING EITHER A DOMINANT OR A RECESSIVE CHARACTER UNDER THE CONTROL OF A SINGLE LOCUS.

Blood groups are shown with, beneath them, the agglutinins detectable in the saliva. - means no agglutinins detectable, N.T. means not tested.

(a) The B pedigree. This pedigree shows that the presence of (anti-B) isoagglutinins in saliva is not a recessive character under the simple control of a single locus.

(b) The L pedigree. This pedigree shows that the presence of (anti-B) isoagglutinins in saliva is not a dominant character under the simple control of a single locus.

frequencies of the phenotypes in males and females, control by a sex-linked locus is ruled out.

C. ABO ANTIGENS AND AGGLUTININS IN CERVICAL MUCUS

Only two specimens of cervical mucus from different donors have been obtained for study. When testing the samples, saliva specimens, collected within half an hour of the time of collection of the cervical mucus, were also tested.

Donor 1. Blood group A_2B

Antigens in saliva and their titres: A($\frac{1}{4}$), B($\frac{1}{2}$), H($\frac{1}{16}$).

Agglutinins in saliva, and their titres: anti-A_1, active at 18°C. (4). anti-A_1 in serum, active at 18°C. Titre=16.

Antigens in cervical mucus: A, B and H (Titres not determined.)

Agglutinins in cervical mucus: anti-A_1 active at 18°C. (Titres not determined.)

Donor 2. Blood group A_1.

Antigens in saliva: none.

Agglutinins in saliva: none.

Antigens in cervical mucus: none.

Agglutinins in cervical mucus: none.

It can be seen that, in these two donors, ABO blood group antigens and agglutinins detected in the saliva have been detected in the cervical mucus, also.

D. ABO ANTIGENS ON SPERMATOZOA

Attempts have been made to repeat Gullbring's experiments on the detection of ABO antigens on spermatozoa using the mixed agglutination technique he described (Gullbring, 1957). When using this technique, some samples of semen appeared to give good positive results, though not all of the spermatozoa in any samples which were expected to participate in the agglutination did so. Thus, only about 10% of the sperm from a known heterozygous A male participated in mixed agglutination, whereas half of them were expected to, on the assumption that a spermatozoon possesses the ABO antigen determined by the gene it carries, as had been concluded by Gullbring (1957). Also, the spermatozoa in two specimens from the same group A non-secretor would not participate in mixed agglutination, nor could detectable anti-A be eluted from them though, in parallel tests conducted on spermatozoa from a group A secretor, the sperm participated in mixed agglutinations and anti-A could be eluted from them

by heating at 56°C after incubating them in anti-A serum and then washing three times in saline.

Since the technique of mixed cell agglutination, as used by me, seemed to be unsatisfactory for grouping spermatozoa, further tests for the presence of A and B antigens were made using the antibody inhibition and the fluorescent antibody techniques.

The inhibition titres of both seminal plasma and washed spermatozoa. at a concentration of 10^8 per ml., were determined. Those specimens with a high titre in the plasma recorded higher titres in the sperm than those with a lower titre in the plasma. Sperm from non-secretors were not significantly different from the saline controls (Figure III.8).

It should be noted that preliminary work was carried out on semen specimens from donors of known blood group and secretor status, whereas the figure (Figure III.8) is based on data from semen specimens obtained from unknown donors. The secretor status and blood group of these were inferred from the results of tests carried out on the seminal plasma. Since the earlier tests, and also published data, indicated that the antigens present in the seminal plasma are the same as those in the saliva, this appears to be a valid procedure.

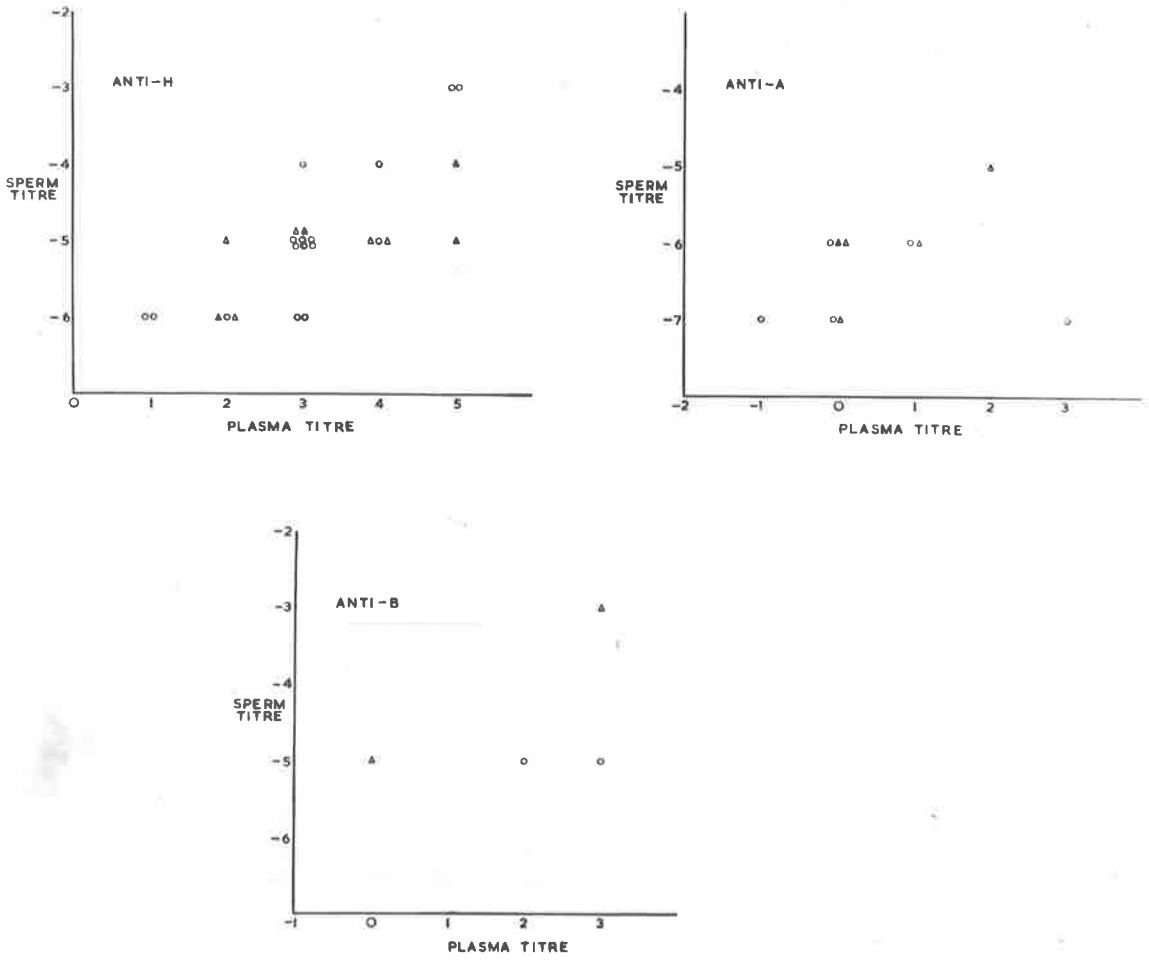


FIGURE III.6. LOG_2 INHIBITION TITRES OF SEMINAL PLASMA AND SPERMATOZOA.

o - tests carried out on spermatozoa and seminal plasma on the day of collection.

△ - tests carried out on the same day on specimens collected over a period three weeks and stored deep-frozen.

Eleven specimens where the seminal plasma showed no inhibition are not included.

Of 36 semen samples tested, 11 did not have detectable blood group substance. From the figure it can be seen that the titres of antigens in seminal plasma are significantly greater than the titres of antigens in saliva (cf. Figure III.3).

Of the 36 semen samples tested (Figure III.8), 20 were tested on the days they were collected with the same set of testing solutions which were kept deep-frozen when not in use, whereas 16 of the specimens were deep-frozen immediately on collection over a period of three weeks and were thawed and tested on the one day using the same set of testing solutions as for the other semen specimens.

The two sets of results are indicated separately in the figure, but there does not appear to be any difference between the results obtained using the two techniques.

The correlation coefficient of the $\log_2$ inhibition titres of the seminal plasma and spermatozoa in the 25 semen specimens which inhibited the Ulex extract is .75, and is significantly different from o at the 0.001 level ($t_{23} = 5.3$). However, it is not significantly different from 1.

When these results were obtained it appeared that,

contrary to earlier conclusions, ABO antigens are adsorbed onto spermatozoa from the seminal plasma and that ABO antigens are not present on spermatozoa from non-secretors.

Accordingly, spermatozoa from a previously tested O non-secretor were washed three times in physiological saline and incubated for 24 hours at 5°C in seminal plasma from a group A_1 secretor containing 1 ppm of Merthiolate as preservative. The spermatozoa were again washed three times in physiological saline and were then shown to have the capacity to inhibit anti-A serum and an extract from the seeds of Ulex europaeus, a capacity lacking in the untreated spermatozoa (Boettcher, 1965). The titres, however, could not be determined, since there was insufficient material left after the treatment. This procedure has been repeated several times and also sperm have been incubated in purified A and B substances (Commonwealth Serum Laboratories), with similar results.

When spermatozoa from an A male were observed to fluoresce with human anti-A serum labelled with fluorescein isothiocyanate (FITC) it seemed that, in spite of the earlier failur of Holborow et al. (1960), who used sera labelled with fluorescein isocyanate (FIC) and were unable to cause sperm to fluoresce. fluorescent antibodies were the

appropriate tool for examining the ABO antigens on spermatozoa. However, when O and B sperm became available and were used as controls, it was found that they fluoresced with the labelled antisera with an intensity indistinguishable from that of A sperm, and this has been the experience throughout the work with fluorescent-labelled antisera, i.e. no difference has been detected between the test sperm and the controls. Under conditions in which sperm under test fluoresced, so did the controls, and where test sperm would not fluoresce neither would the controls.

The critical test aimed for was to obtain a fluorescent-labelled anti-A serum which would cause sperm from an A donor to fluoresce, but not the sperm from an O donor, and then to use it to examine the proportion of sperm which would fluoresce in a mixture of sperm from A and O donors and in those from a known heterozygous A donor. However, satisfactory results have not yet been obtained in spite of examining the effects of many alterations in technique, such as

(a) altering the time of incubation of sperm in labelled antiserum,

(b) using labelled γ-globulin instead of whole serum,

(c) altering the temperature of incubation of sperm with antibody from 5°C. through 37°C.,

(d) heat eluting the antibody after incubation,

(e) pre-treating the spermatozoa with papain,

(f) using rhodamine sulphonyl chloride and rhodamine isothiocyanate (RITC) as fluorochromes,

(g) using the indirect staining technique with unlabelled human anti-A and labelled goat anti-human globulin (Baltimore Biologicals) and

(h) using electrophoretically-separated, FITC-labelled antibody molecules with least nett negative charge.

The aim of all of the variations in procedure, listed above, was to obtain a difference between the uptake, or the retention, of labelled sera by spermatozoa having and not having the appropriate antigen. The last variation was tried since it has been reported that fluorescent-labelled antibody molecules with greatest nett negative charge are those which are most readily adsorbed non-specifically to tissues (Nairn, 1962).

E. INFERTILITY DUE TO AN ANTIGEN-ANTIBODY INTERACTION

The ABO blood groups of thirty five infertile couples who have been married for at least three years, and who have no recognized physiological reason for their infertility, in spite of extensive medical investigation, have been

determined. If, as suggested by Behrman et al. (1960), an ABO antigen-antibody reaction involving sperm and cervical mucus is responsible for infertility in a high proportion of such couples a greater number of ABO incompatible matings would be expected among these couples than would be expected by chance.

The secretor status of the couples and the presence of ABO agglutinins in the women's salivas have also been tested. Fourteen of the thirty five couples have been examined to see whether the women possess serum spermatozoal agglutinins since, whilst this investigation was in progress, Franklin and Dukes (1964a, b) reported finding sperm-agglutinating antibodies in the sera of a high proportion of women for whom no reason had been found previously for their infertility, and suggested that anti-spermatozoal antibodies can cause infertility.

Of the 35 couples, 24 were compatibly mated and 11 were incompatibly mated with regard to their ABO blood groups. These numbers do not differ significantly from the numbers expected on the basis of the ABO blood group frequencies given earlier, assuming random mating. i.e. in this group of infertile couples, there is not an excess of ABO incompatibly-mated couples.

TABLE III.6. RESULTS OF TESTS ON COUPLES INFERTILE WITH NO RECOGNIZED PHYSIOLOGICAL CAUSE

NUMBER	ABO BLOOD GROUPS ♀	♂	COMPATIBLE OR INCOMPATIBLE	SECRETOR STATUS ♀	♂	ABO AGGLUTININS IN WIFE'S SALIVA	SPERMATOZOA AGGLUTININS IN WIFE'S SERUM
1	O	O	COMP.	SEC	SEC	ANTI-A + ANTI-B	NONE DETECTED
2	O	A_1	INC.	NON	NON	ANTI-A + ANTI-B	YES
3	O	A_1	INC.	NON	NON	ANTI-A + ANTI-B	YES
4	A_1	O	COMP.	SEC	SEC	NONE DETECTED	NONE DETECTED
5	A_1	A_1	COMP.	SEC	SEC	ANTI-B	NONE DETECTED
6	A_1	A_1	COMP.	SEC	SEC	NONE DETECTED	NONE DETECTED
7	A_1	B	INC.	SEC	SEC	ANTI-B	YES
8	A_1	B	INC.	SEC	SEC	NONE DETECTED	NONE DETECTED
9	A_2	O	COMP.	NON	SEC	ANTI-B	NONE DETECTED
10	A_2	O	COMP.	SEC	NON	ANTI-B	NONE DETECTED
11	B^2	O	COMP.	N.T.	N.T.	N.T.	NONE DETECTED
12	B	A_1	INC.	NON	SEC	NONE DETECTED	YES
13	A_1B	O	COMP.	SEC	SEC	NONE DETECTED	NONE DETECTED
14	A_2B	O	COMP.	SEC	SEC	ANTI-A_1	YES
15	O	O	COMP.	SEC	SEC	ANTI-B	NOT TESTED
16	O	O	COMP.	SEC	SEC	ANTI-A + ANTI-B	" "
17	O	O	COMP.	SEC	SEC	ANTI-A + ANTI-B	" "
18	O	O	COMP.	SEC	NON	ANTI-A + ANTI-B	" "
19	O	A_1	INC.	SEC	SEC	ANTI-A	" "
20	O	A_2	INC.	NON	NON	ANTI-A + ANTI-B	" "
21	O	B	INC.	SEC	SEC	ANTI-A	" "
22	O	B	INC.	SEC	NON	ANTI-A + ANTI-B	" "
23	A_1	O	COMP.	SEC	SEC	NONE DETECTED	" "
24	A_1	A_1	COMP.	SEC	SEC	NONE DETECTED	" "
25	A_1	A_1	COMP.	SEC	SEC	NONE DETECTED	" "
26	A_1	A_1	COMP.	NON	SEC	ANTI-B	" "
27	A_1	A_2	COMP.	SEC	NON	NONE DETECTED	" "
28	A_1	B^2	INC.	NON	SEC	NONE DETECTED	" "
29	A_1	B	INC.	NON	SEC	NONE DETECTED	" "
30	A_2	O	COMP.	SEC	NON	ANTI-B	" "
31	A_2	O	COMP.	NON	SEC	ANTI-B	" "
32	B^2	O	COMP.	SEC	NON	ANTI-A	" "
33	B	B	COMP.	NON	SEC	NONE DETECTED	" "
34	A_1B	O	COMP.	N.T.	N.T.		" "
35	A_1B	B	COMP.	SEC	SEC		

COMP. = Compatibly mated with regard to ABO blood groups.
INC. = Incompatibly mated with regard to ABO blood groups.
To be incompatibly mated, the wife must possess an antibody against one of the agglutinogens on the husband's
SEC = Secretor. NON = Non-secretor. N.T. = Not tested.
Tests for spermatozoal agglutinins were carried out using the technique of Franklin and Dukes (1964a).

When the ABO blood group combinations of couples where the wife possesses spermatozoal agglutinins are considered:

	ABO compatible couples	ABO incompatible couples	
Wife possesses agglutinins	1	4	5
Wife does not possess agglutinins	8	1	9
	9	5	14

the probability of random association in this group between ABO incompatibility status and sperm agglutinins in the wife's serum is 0.023 (Finney et al., 1963), which is regarded as being statistically significant, i.e. there appears to be, in these data, some association between the possession of sperm agglutinins and ABO-incompatible matings.

The woman who possesses sperm agglutinins and is ABO compatibly mated is the one whose secretions have been examined and reported on p. 79, Donor 1. This woman is now married for the second time. In her first marriage she became pregnant three times, pregnancy being surgically

terminated on each occasion for economic reasons.

One couple who have co-operated fully in the investigation (couple number 7, Table III.6) appear to be of interest. It is the husband's second marriage, and his wife's first. In his first marriage the husband produced three children without difficulty, but his second marriage has been childless for 3½ years. When first examined, the second wife possessed very strong sperm agglutinins which agglutinated her husband's sperm within five minutes, producing clumps of 5-20 sperm which still had freely-moving flagella.

A sample of the wife's serum maintained in deep-freeze has been tested against 34 specimens of spermatozoa from different donors and has agglutinated them, irrespective of the blood group of the donor. The sperm agglutinins are not ABO specific since spermatozoa from individuals of groups O, A_1, A_2, B and A_1B have been tested.

The couple have restricted intercourse to the use of condoms and it has been observed that the sperm-agglutinating activity has decreased, though it has not completely disappeared, in a period of four months. Further observations are to be carried out with this couple.

During the course of the investigations two couples have conceived. One couple (number 18) has a child of group O. The other pregnancy (couple number 25) terminated in miscarriage.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

A. ABO ANTIGENS IN SALIVA

1. Agglutination inhibition capacities of salivas from individuals of different ABO blood groups

(a) Reaction between human anti-A and salivas from A_1 and A_2 secretors

In two separate studies of the inhibition of "non-immune" human anti-A by salivas from group A_1 and A_2 secretors it has been established that, on the average, salivas from A_1 secretors have greater inhibiting capacity than salivas from A_2 secretors. These results agree with those of Gammelgaard (1942) but not with those of Wiener and Kosofsky (1941), nor those of Morganti et al. (1959).

The opposing results of Wiener and Kosofsky might be due to the use of a "boosted" serum by these authors, since immune sera are difficult to inhibit by salivas and, for this reason, are avoided by most British workers.

Since Morganti et al. (1959) did not obtain a clear difference between the inhibition capacities of salivas from

secretors and non-secretors, their work has been criticized by Clarke et al. (1960), who suggested that the titration method used was not adequate. An inadequate technique might also be the reason why they did not detect a significant difference in the inhibiting capacities of the A_1 and A_2 secretor salivas that they tested.

The results of precipitating A substance in salivas from 19 A_1 and 20 A_2 secretors by a Dolichos biflorus extract have shown that those salivas with greatest inhibition titres have also the greatest capacity to precipitate protein from the extract. Inhibition titres, therefore, appear to be a valid measure for comparing the amounts of a particular antigen in salivas. In view of these results, those of Baer, Kloepfer and Rasmussen (1961) seem difficult to reconcile. The precipitation abilities of the A_1, A_2 and A_1B salivas with pooled chicken antisera were equal, whereas their inhibition capacities decreased markedly in the order given. Perhaps, due to the preparation of immune sera in chickens, antigens other than A were involved in the precipitation tests, but did not participate in the agglutination inhibition reactions.

(b) Reaction between Ulex extract and salivas from A_1, A_2, B and O secretors.

The observations reported by Plato and Gershowitz (1961) that salivas from secretors of different ABO blood groups have different capacities to inhibit a Ulex europaeus extract, decreasing in the order O, A_2, A_1, B, have been confirmed in the two series of salivas examined here.

In view of these consistent results, and those when other anti-H reagents have been used, Cytisus sessilifolius extract (Plato and Gershowitz, 1961) and eel serum (Morganti et al., 1959), it seems that this order is correct, and differs from the order of reactivity of red cells with Ulex europaeus extracts, which has been shown to be O, A_2, B, A_1.

(c) Correlations between titres of salivas from A_1, A_2 and B secretors with pairs of testing fluids

The correlation coefficients of the inhibition titres of salivas from A_1 and A_2 secretors with a Ulex extract and a human anti-A serum, and with a Ulex and a Dolichos extract, have been determined and are not significantly

different from O. Neither is the correlation coefficient of the inhibition titres of salivas from B secretors with the Ulex extract and a human anti-B serum.

Consequently, if the genetical pathways leading to the A, B and H antigens as given in Figure I.1 are accepted, the amount of H-substance formed by an individual does not influence the amount of A- or B- substance to be formed and secreted in the saliva, unless its amount is limiting. i.e. an individual can form large amounts of H- substance and convert a little, or much, of it to A- or B- substance. But, an individual who forms only a little H- substance cannot produce large quantities of A- or B- substance.

These results, and the concept that the rates of operation of the H-h and ABO genes are independent, are consistent with the observations of Clarke et al. (1960), who found that the distribution of the ratio of the $\log_2$ inhibition titres of A- secretor salivas with a Ulex extract and human anti-A is unimodal and almost Gaussian. "Aberrant secretors" (McNeil et al., 1957a) are explained as individuals in whom the genes at the H and ABO loci are functioning at greatly different rates. This explanation is similar to that of Clarke et al. (1960) who suggested that the category is only

composed of individuals whose ratios of A- substance to H in group A, or B substance to H in group B, fall outside the normal range because it is composed of the extreme drawn from a continuous frequency distribution.

There will tend to be some correlation between the anti-H and anti-A or anti-B inhibition titres in those individuals who form only small amounts of H- substance and which, therefore, limit the amount of A- or B- substance able to be formed. It is suggested that the positive correlation coefficients determined in all of the comparisons are, in part, due to this.

2. Relationship between ABO antigens in saliva and on red blood cells

If, as is assumed, the antigens in the saliva are the same as those on the red cells, the reversed order of H-activity of B and A_1 red cells and salivas, which has been shown, might be due to a difference in the proportion of unconverted H- substance in these two locations.

Since attempts to differentiate homozygous $\underline{I^A I^A}$, and heterozygous, $\underline{I^A I^O}$, blood group A individuals on the basis of the agglutinability of their red cells with Ulex europaeus extracts have not proved successful, it seems that $\underline{I^A I^O}$

individuals do not have more H antigenic sites exposed on their red cells than have $\underline{I}^A\underline{I}^A$ individuals. For this to occur, it seems that almost all H- substance on the red blood cells is converted to A- substance in $\underline{I}^A\underline{I}^O$ individuals, and that the reactivity of A red cells with Ulex extracts is due to some H activity still being detectable after additional specificity has been added to H antigen to convert it to A antigen. The greater activity of B red cells than of A_1 red cells with Ulex extracts, therefore, might be due to greater H activity of the B antigen than of the A antigen.

Watkins and Morgan (1956/57) have shown that salivas from A secretors possess some molecules with A+H antigenicity and some with only H antigenicity. To explain the greater H activity of salivas from A_1 secretors than those from B secretors, it is proposed that the former possess more unconverted H- substance than the latter i.e. the gene $\underline{I}^B$ is more active in converting H- substance to B- substance than is the gene $\underline{I}^{A_1}$ in converting H- substance to A- substance.

Such a concept is consistent with the proposal that the dominance of the genes $\underline{I}^A$ and $\underline{I}^B$ over $\underline{I}^O$ is not equal, based on the observations that the expression of the A antigen on

red cells of group AB is diminished, by comparison with the expression of the A antigen on A cells, whereas the expression of the B antigen differs little on cells of groups AB and B (Wiener, 1945).

The hypothesis that salivas from B secretors contain more unconverted H- substance than those from A_1 secretors could be tested by precipitating all of the molecules having A or B antigenicity from A_1 and B secretor salivas and determining the amounts of H antigen remaining.

3. Relationship between A_1 and A_2 antigens in saliva

Since it has been shown that the inhibition titre of an A secretor saliva with a Dolichos extract is a valid measure of the amount of antigen present, from the results of inhibition tests performed with salivas from group A_1 and A_2 secretors it can be argued that, salivas from A_1 secretors possess more A and less H antigen than do salivas from A_2 secretors. Further, since it has been established that the reduction in Ulex inhibition titre, per unit reduction in Dolichos inhibition titre, is greater for A_2 secretor salivas than for A_1 secretor salivas, and since Dolichos biflorus extracts recognize an antigen common to A_1 and A_2 salivas

(Bird, 1959b), it seems that the individual A- substance molecules in salivas from A_1 secretors possess more A antigen and less H antigen than do the A- substance molecules in salivas from A_2 secretors.

From a consideration of these results and of this concept for the individual A- substance molecules in the salivas of A_1 and A_2 secretors, a model for the difference between A_1- and A_2- substance is proposed.

The points taken into consideration in proposing this model are:

1. No immunological or biochemical difference between A_1- and A_2- substance has been established, except the different intensities of the reactions between red cells of groups A_1 and A_2 and the antibodies anti-A and anti-A_1, and the quantities of A and H antigens in salivas from A_1 and A_2 secretors.

2. The extract of Dolichos biflorus seeds, regarded as an anti-A_1 (Bird, 1959b), agglutinates A_2 cells weakly (Bird, 1952), and precipitates with a single component common to A_1- and A_2- substance (Bird, 1959b).

3. The blood group substances are macromolecules, A_1- substance having more A antigen and less H antigen than A_2- substance.

4. Anti-A_1 antibodies are found in the sera of 1-2% of A_2 individuals, but about 26% of A_2B individuals (Juel, 1959).

It is proposed that A_1 antigenicity resides in a number of linked adjacent A antigenic units on one macromolecule and that, whereas the $\underline{I}^{A1}$ blood group gene specifies the addition of a number of linked adjacent A antigenic units to the preformed H substance, the $\underline{I}^{A2}$ gene specifies the addition of only one. This would account for the great immunological and biochemical similarities between the A_1 and the A_2 antigenic substances, and also for the different A:H ratios of the individual molecules from A_1 and A_2 individuals.

Anti-A_1 is thought of as being directed partly against the A antigen, and partly against the link between adjacent A antigenic units.

Immunological similarity to the A_1 structure might then arise in some A_2 individuals due to the proximity of separate A antigenic units in the blood group substance macromolecules, thereby preventing these individuals from forming anti-A_1 antibodies, though not conferring upon them the A_1 antigen, since they lack the union between adjacent A antigenic units.

The probability of immunological similarity to the A_1 antigen arising from the proximity of individual A antigenic units in A_2B individuals would be reduced by the presence of A, B and H antigenic units and this could account for the greater proportion of A_2B than A_2 individuals who possess anti-A_1 antibodies.

If this model is correct, those A_2 individuals with anti-A_1 in their serum would be expected to possess individual blood group substance molecules with a significantly low A:H ratio. This expectation could be tested using the same procedure by which the H:A ratios for the A- substance molecules in salivas from A_1 and A_2 secretors have been determined.

B. ABO AGGLUTININS IN SALIVA

The presence of anti-A and anti-B in a significantly higher proportion of saliva specimens from group O individuals than in those from individuals of blood groups B and A, respectively, and the presence of anti-B in a significantly higher proportion of salivas from group A_2 individuals than in those from A_1 individuals, argue against simple genetical control for the presence of ABO agglutinins in saliva, as do the pedigree studies of Wilson and Green (1964). Their

results are not consistent with it being a sex-linked recessive or an autosomal dominant character, and the pedigrees presented here are not consistent with it being an autosomally-controlled character, either dominant or recessive. The population data presented are not consistent with an hypothesis of sex-linkage, since there is no difference between the male and female phenotypic frequencies as would be expected with a sex-linked character and the order of gene frequencies necessary to explain the data.

It seems, therefore, that the presence of ABO agglutinins in saliva is not a character under simple genetical control.

Prokop's suggestion that low molecular weight isoagglutinins pass more readily into saliva than those of higher molecular weight (Prokop, 1961), based on the finding that individuals of different ABO genotypes possess ABO antibodies of different molecular weights (Filitti-Wurmser et al., 1953/54), can be used to account for the significantly higher frequency of O salivas possessing anti-A or anti-B than B and A salivas, respectively, since O individuals possess higher concentrations of low molecular weight antibodies than A or B individuals. But, if it is, the finding of anti-B in

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a significantly higher proportion of salivas from A_2 individuals than in those from A_1 individuals, should be interpreted as evidence that A_2 individuals possess more low molecular weight anti-B than A_1 individuals. If this is so, other evidence of this should be obtainable.

It is now accepted that anti-A and anti-B from group O women will traverse the placenta more readily than that from B and A women, respectively, due to the higher proportion of antibodies with lower molecular weight in the former (Franklin, 1962; Polley, Adinolfi and Mollison, 1963), and that this is the reason why almost all cases of ABO haemolytic disease of the new-born (H.D.N.) involve an O mother. (See Levene and Rosenfield, 1961, for review.) Unfortunately, insufficient data are available to determine whether B children of A_2 mothers suffer H.D.N. more frequently than those of A_1 mothers, as is to be expected if A_2 individuals possess more low molecular weight anti-B than A_1 individuals. However, Freda and Carter (1962), when studying anti-A and anti-B titres in maternal and foetal (cord) sera found that there was more anti-A and anti-B present in the foetal serum when the mother was group O than when she was either B or A, and that when the mother was of blood group A_2 more antibody was detectable in the foetal serum than when she was A_1.

Since Franklin and Kunkel (1958) have found that sera of new-born infants contain similar concentrations of 7 S globulin but very little 19 S globulin when compared with adults, a conclusion supported by Kochwa et al. (1961) who showed, further that 7 S - γ_2 globulin concentrations were in equilibrium on both sides of the placenta, and since the acacia technique used by Freda and Carter demonstrates "incomplete" antibodies which are generally 7 S globulins, it appears that A_2 individuals possess more low molecular weight anti-A than do A_1 individuals.

Blood group agglutinins have been detected in concentrated human urine (Prager and Bearden, 1965). Anti-A, anti-B and anti-D of the Rh system have been detected in urine from immunized persons, but not in urine from non-immunized persons. The agglutinins have been characterized as γ2-globulins by their stability to mercapto-ethanol and their reactions to specific anti-globulin antisera. These studies lend further support to the concept of the ability of lower molecular weight antibodies to pass across membranes into aqueous fluids.

Consequently, the hypothesis for genetical control of the presence of ABO agglutinins in saliva as proposed by

Miyakoshi (1951) is unacceptable, as are several other simple genetical schemes. However, Prokop's suggestion, that low molecular weight agglutinins can pass into saliva whereas those of higher molecular weight cannot, appears to be satisfactory and is in agreement with observations made on antibodies traversing the placental and the kidney glomerular membranes. Since it seems that the presence of ABO agglutinins in saliva is due to the passage of small molecular weight globulins across membranes, in an analogous manner to their passage across the placenta, it appears reasonable to expect, until further studies clarify this point, that individuals with detectable agglutinins in their saliva have them in their other mucous secretions also, and these, in women, would include the secretion from the uterine cervix.

C. ABO ANTIGENS AND AGGLUTININS IN CERVICAL MUCUS

The observations made on the two specimens of cervical mucus studied parallel those made on specimens of saliva obtained at the same time from the two donors. A further similarity between the properties of these two secretions is that, for each secretion separately, it has been established

that agglutinins are present in a significantly higher proportion of group O individuals than in individuals of other blood groups (Prokop, 1961; Solish et al., 1961).

In attempting to explain the occurrence of haemagglutinins in the cervical secretion, Solish et al. (1961) proposed two possible explanations.

(i) Due to a difference in the molecular sizes of antibodies, O women permit passage of higher concentrations of anti-A and anti-B across membranes than do A or B women and, therefore, the increased incidence of agglutinins detected in secretions from O women compared with A and B women may simply be a reflection of the greater amounts of antibodies able to pass into the mucous secretions.

(ii) Since administration of a local soluble typhoid antigen causes production of a vaginal antibody, it is possible that antigenic stimulation, of perhaps bacterial origin, may stimulate ABO agglutinin titres.

If the latter is the correct explanation, the difference between the frequencies of agglutinins in secretions from O and A or B women is hard to explain. The conclusions presented in the previous section also support the former explanation.

D. ABO ANTIGENS ON SPERMATOZOA

The results obtained by Edwards *et al.* (1964), who were able to get sperm from secretors to participate in mixed agglutination but not those from non-secretors, and those reported here, can be interpreted as demonstrating that testicular spermatozoa do not possess ABO blood group antigens, but can adsorb them, and that this occurs with secretors when sperm are ejaculated in seminal plasma possessing them, but not with non-secretors.

Before such a conclusion can be accepted it seems advisable to re-evaluate earlier reports which have provided contrary conclusions.

Gullbring claimed that, in addition to A and B antigens, his mixed cell agglutination technique detected the D antigen of the Rh series on spermatozoa. Neither Levine and Celano (1961) nor Quinlivan and Masouredis (1962) could confirm the presence of the D antigen, and these findings have been accepted by Edwards *et al.* (1964) since they found it absent from leucocytes. Furthermore, the presence of the D antigen elsewhere than on erythrocytes is seriously questioned (Lawler and Shatwell, 1962). It seems that Gullbring's use of the mixed cell agglutination technique is suspect and,

since it is the technique devised by one of the authors of a paper reporting opposing results when using it (Edwards et al., 1964), it would appear unwise to accept Gullbring's conclusions.

Perhaps the lack of a satisfactory anti-B serum, which would have permitted testing of spermatozoa presumed to possess only the A antigen, led Popivanov and Vulchanov (1962) to conclude that their sperm-agglutinating rabbit anti-A serum was able to agglutinate A sperm in semen from an AB male, leaving B sperm unagglutinated.

Edwards et al. (1964) have shown that, although spermatozoa from non-secretors will not participate in mixed agglutination, non-spermatozoal cells in such semen specimens react strongly. The application of results obtained from antibody absorption or absorption and elution techniques on sedimented cellular deposits from semen to spermatozoa only, therefore, appears to be invalid, and these were the techniques used by Levine and Celano (1961) when they reported finding ABO antigens on spermatozoa from secretors and non-secretors.

The results of the studies by Shahani and Southam (1962), who used fluorescent antisera to examine the ABO antigens on spermatozoa, must be treated with reservation.

First, the absence of an observation that some sperm fluoresced in any of the samples of spermatozoa from A or B donors, presumably unselected in respect of their genotypes, would indicate, by their conclusions, that all donors were homozygotes, a very unlikely event. Secondly, the equal fluorescence of all sperm from A and B donors and the unequal colour segregation of sperm from an AB donor, as shown in the coloured photographs, suggest that the authors experienced non-specific uptake of labelled antisera, similarly to that reported here, though they did not comment upon it.

Holborow et al. (1960) and Allison (1964), also, have been unable to obtain satisfactory results using the fluorescent antibody technique.

It seems reasonable, therefore, now to conclude that ABO antigens are to be found only on seminal spermatozoa from secretors and that these are adsorbed from the seminal plasma.

Adsorption of ABO antigens to cells has been demonstrated also with buccal epithelial cells (Swinburne, Frank and Coombs, 1961) and with platelets (Lewis, Draude and Kuhns, 1960).

The ABO blood group antigens are not the only substances which can be adsorbed to spermatozoa from seminal plasma. Searcy, Craig and Bergquist (1964), using electrophoresis, were able to remove material from human seminal spermatozoa with mobilities similar to serum albumins and globulins, though these could not be removed by repeated washings in saline. They concluded that the antigenicity of ejaculated spermatozoa stems from seminal plasma, in agreement with the observation of Weil and Rodenburg (1960), that spermatozoa collected from spermatocoeles lack powerful antigens associated with seminal spermatozoa. Also, Franklin and Dukes (1964a) have shown that anti-spermatozoal antibodies have anti-seminal plasma specificities. Recent studies appear to show that the antigenicity of seminal spermatozoa is due to substances adsorbed from the seminal plasma since antibodies can be produced in test animals against seminal plasma and seminal spermatozoa though not against testicular spermatozoa. Furthermore, anti-seminal plasma antisera react with seminal spermatozoa, though not with testicular spermatozoa and, in agar precipitation (Ouchterlony) tests, anti-seminal spermatozoa antisera show identical reactions with seminal plasma and seminal spermatozoa homogenates (Otani, Iino and Kagami, 1964; Weil, Kotsevalov and Wilson, 1956).

Not only is the question of ABO antigens on spermatozoa relevant to the present problem, but it is relevant also to the fundamental problem of whether the genes in spermatozoa or, for that matter, in animal gametic cells after the first meiotic division, are functional.

The pollen grain of plants represents a short gametophytic generation during which at least two mitoses occur and at least some genes are functional, since gametophytic characters can be expressed (e.g. carbohydrate in the pollen grain of maize). However, there is no gametophytic generation in animals.

Muller and Settles (1927) after studying the problem, using stocks of Drosophila in which deficiencies and duplications were able to be passed through spermatozoa without any detectable effects, concluded that genes in spermatozoa are in a dormant condition, the cytoplasm of the spermatozoa functioning like a watch running down and utilizing only material produced during the operation of the prematuration diploid nucleus. This approach of recovering unbalanced gametes with large deficiencies and duplications from translocation heterozygotes in Drosophila has been used widely with consistent results, and it is now accepted that, in Drosophila at least, sperm function independently of their

gene content (Novitski and Sandler, 1957). However, situations which seem to indicate selection of sperm on the basis of their gene content have been encountered, but all, on closer examination, have been shown to have their basis elsewhere (Lindsley and Sandler, 1958; Novitski and Sandler, 1957; Sandler, Hiraizumi and Sandler, 1959).

As an example, with segregation distortion (SD) in Drosophila (Sandler, Hiraizumi and Sandler, 1959), heterozygous males ($\underline{SD}/\underline{SD}^{+}$) produce backcross progeny where the the ratio of $SD:SD^{+}$ is 5:1 or 10:1. However, it appears that, during synapsis, the SD chromosome induces breakage in the homologue bearing the wild-type allele leading to cells which cannot complete spermiogenesis.

The t alleles of the mouse provide a very interesting case (Dunn, 1960). At the T locus there is a number of recessive alleles, some of which are lethal in the homozygous condition, e.g. $\underline{t}^{n}/\underline{t}^{n}$. Some male heterozygotes, $\underline{t}^{n}/\underline{+}$, transmit the $\underline{t}^{n}$ allele to significantly more than half of their progeny, though there is no such effect when the female is the heterozygote. Further, some heterozygous males, $\underline{t}^{n}/\underline{t}^{x}$, are sterile whereas others have reduced fertility. It could be suggested that both problems, the unequal transmission by $\underline{t}^{n}/\underline{+}$ male heterozygotes, and the

sterility of $\underline{t}^n/\underline{t}^x$ heterozygotes might be based on mechanisms dependent on the particular $\underline{t}$ allele carried by a sperm, especially since it has been shown (Braden, 1958) that the time of copulation in relation to the time of ovulation has an effect on the transmission ratio of $\underline{t}^n$:$\underline{+}$ from $\underline{t}^n/\underline{+}$ male heterozygotes. If mating occurs before ovulation the $\underline{t}^n$ allele is transmitted to more than half of the progeny, whereas if mating is delayed until the time of ovulation, or later, the proportion becomes a half. Also, the number of sperm from $\underline{t}^n/\underline{t}^x$ heterozygotes able to reach the site of fertilization has been shown to be low (Braden and Gluecksohn-Waelsch, 1958).

If favourable selection of sperm carrying $\underline{t}$ alleles was responsible for their transmission from $\underline{t}^n/\underline{+}$ heterozygote males, it would be difficult to explain the sterility of $\underline{t}^n/\underline{t}^x$ males. If mature $\underline{t}^n$ sperm were able to influence $\underline{+}$ sperm, it would be difficult to explain the equal transmission of $\underline{t}^n$ and $\underline{+}$ alleles from an artificial mixture of these types of sperm (Braden and Gluecksohn-Waelsch, 1958).

It seems, therefore, that both these phenomena, the unequal transmission and the sterility, are due to mechanisms operating before the reductional division of meiosis.

There seems to be, therefore, no documented case in animals of dimorphism of spermatozoa due to the action of genes after meiosis.

Consequently, any selective mechanism in animals assuming selection of spermatozoa on the basis of the genes they carry should be viewed exceedingly cautiously. Further than this, it is suggested that the a priori hypothesis should be, until positive evidence to the contrary is obtained, that there is no selection of sperm in animals based on their genotype, and proposals to the contrary (Matsunaga and Hiraizumi, 1962; Murray, Knox and Walker, 1965) should be examined critically in the light of these results.

It may be that, in the cases examined so far, the characters used are those identifiable in the mature organism and that these might not be expected to be expressed in the sperm but that, if characteristics peculiar to spermatozoal cells alone, such as the flagellar antigens, were examined, characters determined by the allele in the particular sperm cell might be detectable.

It can be seen, therefore, that the proposed mechanism for infertility which initiated the research programme (Behrman et al., 1960) cannot operate since there is not a

segregation of blood group antigens on separate spermatozoa. But, it is possible that a similar scheme might operate based on adsorbed ABO antigens on spermatozoa and the appropriate antibody in the secretion of the uterine cervix though, in this case, there could not be differential spermatozoal selection for any one donor.

E. INFERTILITY DUE TO AN ANTIGEN-ANTIBODY INTERACTION

Within the infertile couples studied, the proportion of ABO incompatibly mated couples (31%) is almost exactly that expected under random mating with expectations based on the phenotypic frequencies found among Australian-born university students (35%). Two other recent studies in which the proportion of ABO incompatible matings among apparently normal infertile couples has been determined, show similar results. Thus, of the 43 infertile couples without demonstrable organic reasons for infertility studied by Franklin and Dukes (1964b), 15 (38%) were ABO incompatibly mated and, of 50 apparently normal infertile couples examined by Whitelaw, Grams and Antone (1962), 21 (42%) were incompatibly mated. The compatibility status of the infertile couples in the latter study was compared by the authors with that of 136 couples who had had at least 4 children, and were

found to be not significantly different.

ABO Compatibility Status of Couples

	Incompatible	Compatible	
Infertile Couples	21 (42%)	29	50
Fertile Couples	46 (34%)	90	136
	67	119	186

$$\chi^2_1 = 1.06 \qquad .50 > P > .30$$

In addition, the rates of failure to conceive after artificial insemination by donor were compared for inseminations using ABO incompatible and compatible semen. Of 48 women inseminated with ABO compatible semen, 17 (36%) failed to conceive and, of 100 women inseminated with ABO incompatible semen, 33 (33%) failed to conceive (Whitelaw, Grams and Antone, 1962).

In the light of these results, it seems that the finding of a significantly high proportion of ABO-incompatible matings

among couples infertile for no known reason reported by Behrman et al. (1960) lacks confirmation and should be viewed with reservation.

Since ABO antigens can be detected on seminal spermatozoa from secretors by the antibody-inhibition technique, and since some women possess cervical ABO agglutinins, it seems that an ABO antigen-antibody interaction involving sperm and cervical mucus occurs in some women, but there appears to be no sound evidence that this leads to infertility. However, if sperm agglutinins can produce infertility (Franklin and Dukes, 1964a, b) it seems that the different results might be due to the different actions of the two types of antibodies. Human ABO antibodies have not been found to agglutinate sperm, whereas sperm agglutinins were the specific agents looked for by Franklin and Dukes. Perhaps, to reduce fecundity appreciably, antibodies active with spermatozoa need to immobilize them.

Although an association with ABO compatibility status has been found for the infertile couples studied in which the wife was shown to possess antisperm antibodies, this is regarded at present as a chance result, since no such association has been found in the larger studies of Franklin and Dukes (1964b).

The role of sperm agglutinins in infertility has been suggested but, as yet, there is little confirmatory evidence. It seems that sperm agglutinins need not, necessarily, lead to infertility, since some women who are normally fertile possess them (Franklin and Dukes, 1964b).

SECTION B.

CHAPTER V.

MODIFICATION OF BERNSTEIN'S THEORY FOR THE INHERITANCE OF THE ABO BLOOD GROUPS IN THE LIGHT OF MODERN GENETICAL CONCEPTS

Bernstein's three allelic gene theory for the inheritance of the ABO blood groups in Man (Bernstein, 1924), extended to include a number of sub-groups, has been accepted for forty years. In this period the concept of the gene has received a great deal of attention and modification. Whilst it must be agreed that the theory has proved very satisfactory for four decades, a few observations, inconsistent with it, have been made during this time. It appears desirable, therefore, that the theory should be re-evaluated in the light of modern genetic concepts for, as will be shown, a number of the apparent inconsistencies can then be resolved.

When Bernstein proposed his theory the concept of a gene was a bead on a string, where each gene, or bead was regarded as being an indivisible unit capable of operating as the fundamental unit of function, mutation and recombination. The chemical nature of the genetic material was unknown. (See Morgan, 1926, for discussion.)

Nowadays we know that the genetic material is DNA or, rarely, RNA, and we have a different concept of the gene. The functional gene is envisaged as a length of DNA or RNA which determines the order of the amino acids in a single polypeptide (Strauss, 1960). Recombination, involving only a few nucleotides, possibly only one, can occur within such a gene and mutation can result from a change in a few nucleotides or, again, only one. Independent mutations within one gene need not involve the same sub-genic units. (See Benzer, 1961, for discussion.) Since these are now considered to be properties of structural genes in general, recombination would be expected to occur within genes at the ABO locus and, if it did, some recombination products would be expected to be detectable.

With the ABO blood groups we are aware that the antigenic sites that react with anti-A and anti-B are only part of the structures specified by the genes I^A and I^B, and which are added to preformed H substance since, in addition to these sites, there is at least the antigenic site which combines with cross-reacting anti-A and anti-B from O serum (Race and Sanger, 1962). Furthermore, it is generally believed that A_1 substance has two antigens, A and A_1, whereas A_2 has only one, A (Race and Sanger, 1962).

If we accept that A_1 substance is specified by the gene $\underline{I}^{A_1}$ within which the A and A_1 sub-units specify the A and A_1 antigenic sites then, in the gene $\underline{I}^{A_2}$, the sub-unit homologous with the A_1 sub-unit must be silent (Figure V.1). What of the sub-unit within the gene $\underline{I}^{B}$ which specifies the B antigenic site? It is possible that it is (a) homologous with either the A_1 or the A sub-unit, or (b) homologous with neither, or (c) of such dimensions that it has parts homologous with both the A_1 and A sub-units (Figure V.1). If the first possibility is correct, recombination sometimes might occur between the B and the non-homologous sub-unit, say A, to give a gene containing the A and B sub-units, leading to the possibility of a gene which, if inherited with an $\underline{I}^{o}$ gene, would give rise to an A_2B phenotype. If the second possibility is correct, both an $\underline{I}^{A_2B}$ and an $\underline{I}^{A_1B}$ gene could be formed by recombination. If the last possibility is correct, only $\underline{I}^{A_1}$, $\underline{I}^{A_2}$, $\underline{I}^{B}$ or $\underline{I}^{o}$ genes could occur.

Following publication of Bernstein's hypothesis for the inheritance of the ABO blood groups (Bernstein, 1924), a large number of pedigrees was examined in attempts to obtain further evidence in support of it. (See Wiener, 1945, for a summary of many of the results of investigations.)

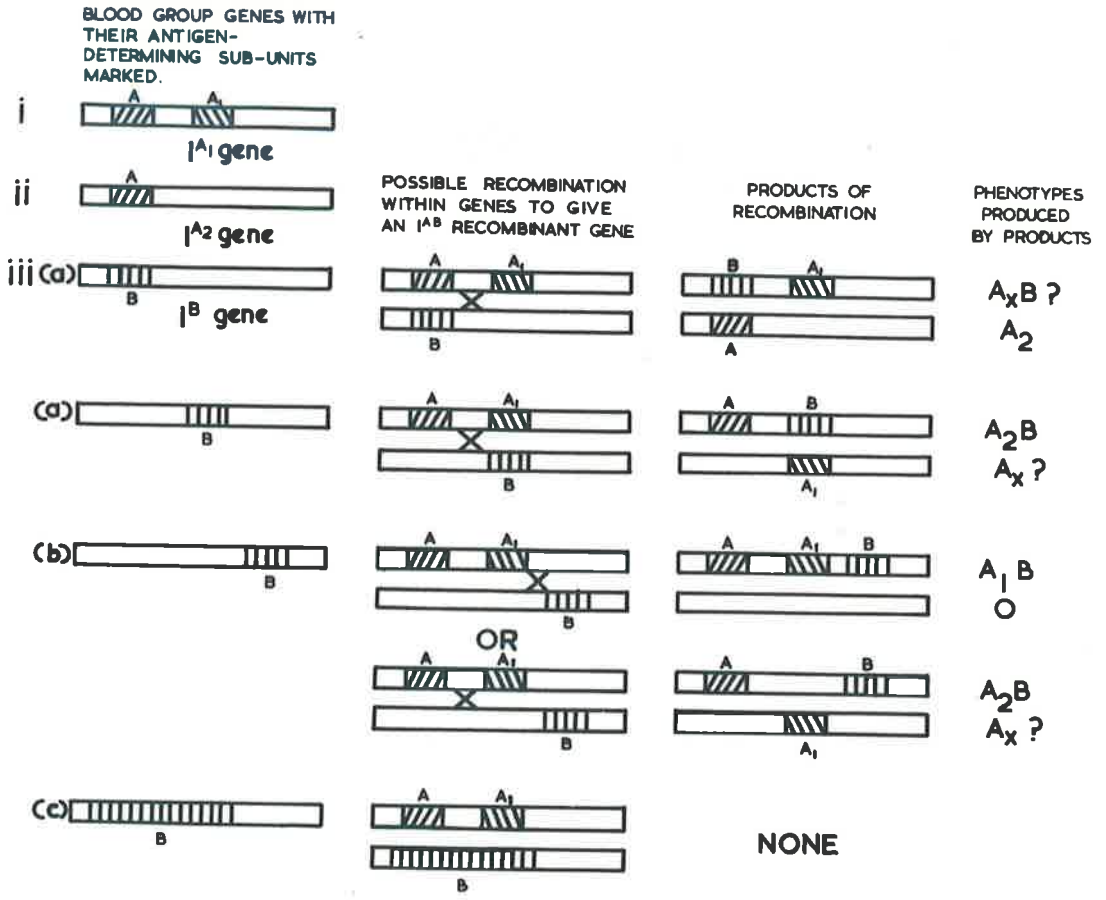


FIGURE V.1. THE POSSIBILITY OF AN $\underline{I}^{AB}$ GENE BEING FORMED BY RECOMBINATION WITHIN THE ABO LOCUS.

(i) and (ii) The blood group genes, $\underline{I}^{A_1}$ and $\underline{I}^{A_2}$, showing the sub-units specifying the A and A_1 antigenic sites.

(iii) The gene, $\underline{I}^{B}$, and possible relationships between the A and A_1 sub-units with the B sub-unit,

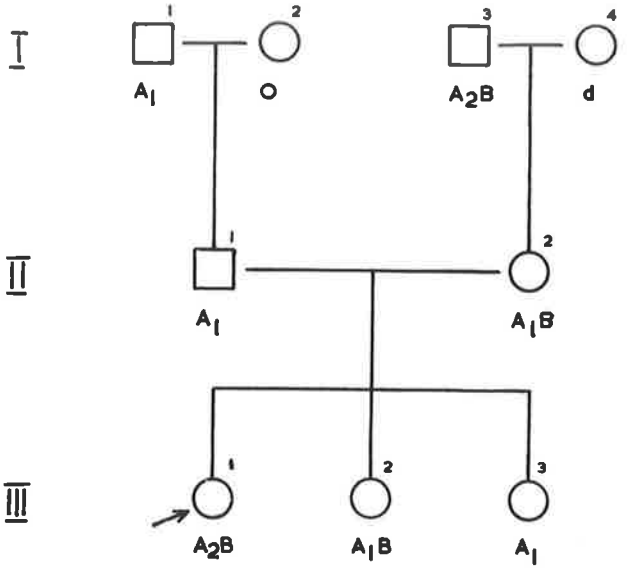
(a) The B sub-unit is homologous with either the A or the A_1 sub-unit.

(b) the B sub-unit is not homologous with either and

(c) the B sub-unit has parts homologous with both A and A_1 sub-units.

Under Bernstein's hypothesis an AB individual can not produce a group O offspring, whereas if A and B antigens are controlled by non-allelic genes this is possible. Thousands of pedigrees showing blood groups consistent with expectations under Bernstein's hypothesis were recorded, but a few anomalies were detected. Most of the anomalies could be ascribed either to poor technique or non-paternity, but two pedigrees which could not be explained on either of these grounds were published. Thus, Haselhorst and Lauer (1930, 1931) and Kossovitch (1929) each reported an A_2B woman having an O child. However, in spite of such rare apparent exceptions, Bernstein's hypothesis has proved very successful and, with its extension to four alleles to include the subgroups A_1 and A_2 (Thomsen, Friedenreich and Worsaae, 1930), appears to be universally accepted.

Recently, two pedigrees subjected to careful study have provided similar apparent exceptions to Bernstein's theory (Figure V.2). Seyfried, Walewska and Werblińska (1964) described the first, a family in which an O woman had an A_2B daughter who, by an O husband, produced two daughters with A_2B phenotypes. The A_2B phenotypes involved unusual variants of the B antigen and anti-B antibody since, in the sera of the three A_2B individuals, there was an anti-B antibody active



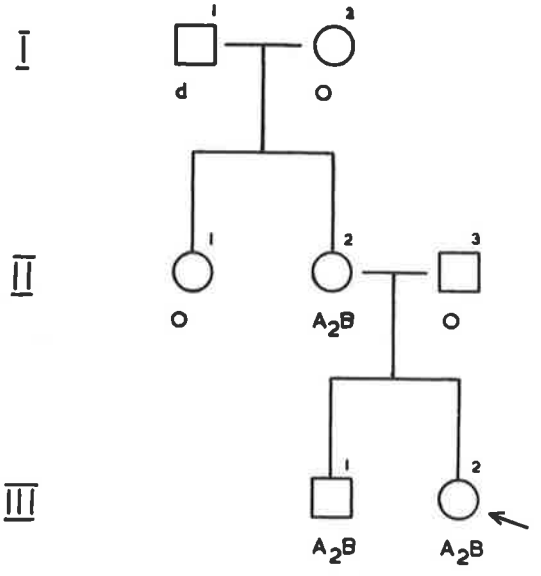


FIGURE V.2. TWO RECENT PEDIGREES WHICH PROVIDE APPARENT EXCEPTIONS TO BERNSTEIN'S THEORY FOR THE INHERITANCE OF THE ABO BLOOD GROUPS.

(a) The pedigree provided by Yamaguchi, Okubo and Hazama (1965).

(b) The pedigree provided by Seyfried, Walewska and Werblińska (1964).

against all B cells tested other than those in the same pedigree. The second, involving a similar B variant, showed that an A_2B male produced an A_1B daughter who, with a known heterozygous A_1 husband, produced three daughters, one A_2B, one A_1B and the other, A_1. All four individuals in the pedigree possessed a variant B antigen and anti-B antibody similar to those described above (Yamaguchi, Okubo and Hazama, 1965). Seyfried *et al.* and Yamaguchi *et al.* proposed explanations for their pedigrees which are similar to the one proposed here.

Therefore, it appears that, although Bernstein's theory is widely accepted, the few well-documented apparent exceptions warrant its reconsideration in the light of contemporary genetical concepts.

These four reports are consistent with the hypothesis that recombination can occur very rarely between the A and B sub-units to produce an $\underline{I}^{A_2B}$ gene. One would expect such recombination to be rare, and, in fact, the frequency of the postulated $\underline{I}^{A_2B}$ gene is far less than the product of the $\underline{I}^{A_2}$ and $\underline{I}^{B}$ gene frequencies.

If recombination occurred within an $\underline{I}^{A_1}/\underline{I}^{B}$ heterozygote to give an $\underline{I}^{A_2B}$ gene, the reciprocal product formed would be

a gene having only the A_1 sub-unit. Such a gene may have no phenotypic effect, e.g. if A_1 cannot be expressed independently of A, in which case it would be classified as $\underline{I}^{O}$, or it may have a phenotype which causes it to be classified as $\underline{I}^{A_x}$, lacking the anti-A_1 customary with this group.

Other unusual pedigrees which might involve an $\underline{I}^{A_2B}$ gene have been reported. Moullec and Le Chevrel (1959) have published a pedigree in which a variant B antigen has been inherited through four generations, with each person possessing it also having the A_2 antigen. The probability of the five persons involved inheriting the same variant B antigen from one parent and having it partnered on each occasion by an A_2 antigen inherited from the other, presumably unrelated, parent is less than 2 in 100,000. (See Race and Sanger, 1962, for discussion.) Since these five A_2B individuals possess anti-B antibody active against all B cells tested except those in the same pedigree, it seems that the A_2B phenotype is similar to those described by Seyfried *et al.* (1964) and by Yamaguchi *et al.* (1965), and that the pedigree involves the inheritance of an $\underline{I}^{A_2B}$ gene.

Andersen (1960) has reported several cases of AB persons possessing a B antigen on the red cells and an anti-B in the serum. Most of these cases might be ascribed to the category

of acquired B-like antigens, such as those described by Cameron (1960), especially since the B antigenic character of the cells was shown to be transitory in one case subjected to careful study, but this character within one particular family (the Pe family) appeared to be inherited and was stable for over two years. The variant AB phenotypes (one A_1B and three A_2B) perhaps involve an $\underline{I}^{A_2B}$ gene, with the A_1B phenotype being due to the $\underline{I}^{A_2B}$ gene being partnered by an $\underline{I}^{A_1}$. However, one puzzling feature is that B substance could not be detected in the saliva of the one A_2B secretor tested.

A pedigree described by Cahan et al. (1957) and Fisher and Cahan (1962) might also be accounted for on the basis of an $\underline{I}^{A_2B}$ gene (Figure V.3). In this pedigree an A_x man had an A_2B son who, partnered by an A_1 wife, produced an A_x and an A_2B child. It is proposed that the son's genotype is $\underline{I}^{A_2B}/\underline{I}^{A_x}$ and his phenotype, consequently, A_2B.

Although this discussion has assumed that A and B substances are under the control of genes at a single locus, biochemical and serological evidence indicates that the antigenic specificity of the blood group substances resides in the carbohydrate moiety. Since the product of a structural gene is considered to be a polypeptide it is perhaps unlikely that the blood group substance is a primary gene

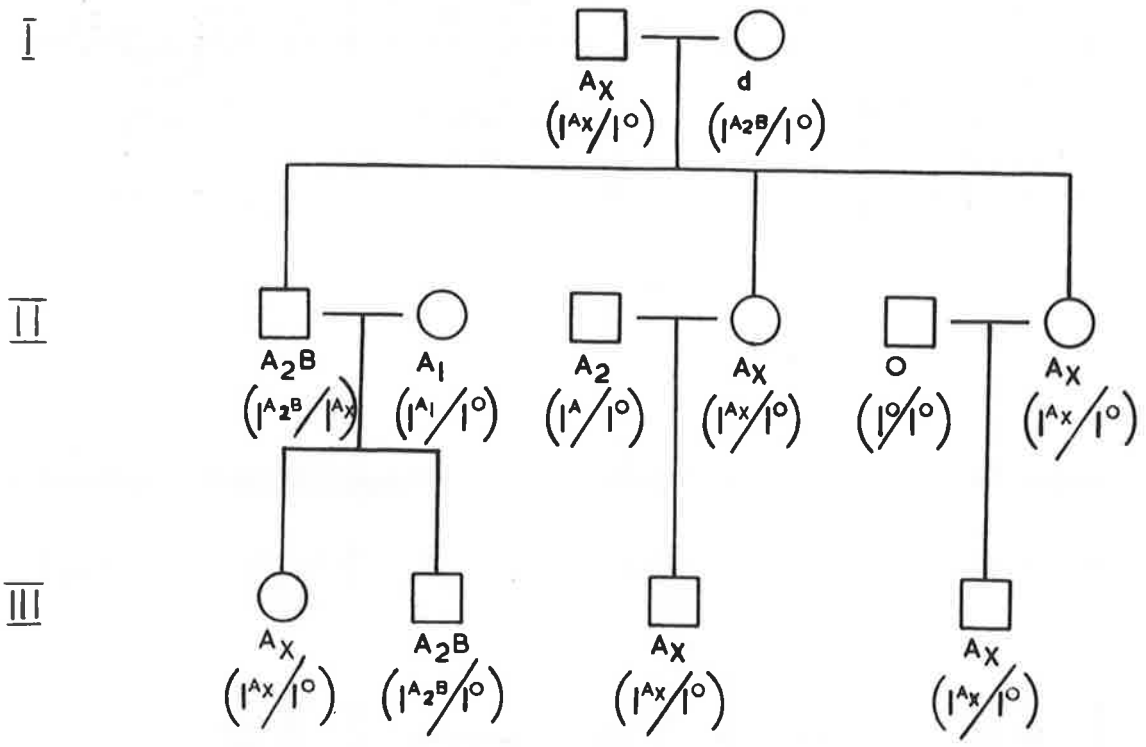


FIGURE V.3. THE PEDIGREE OF THE FAMILY IN WHICH A_x AND A_2B ARE INHERITED THROUGH A PERSON OF BLOOD GROUP A_2B (Cahan et al., 1957; Fisher and Cahan, 1962). Phenotypes are given. Suggested genotypes are indicated in brackets.

product, but possibly the product of some reaction catalyzed by a gene-determined enzyme. Since A substance contains the disaccharide N-acetylgalactosaminoyl-galactose, B substance contains galactosyl-galactose (Rege et al., 1963), it could be argued that, since two different biochemical syntheses are involved, two enzymes of different specificities and, consequently, two loci, are concerned, as suggested, on other grounds, by Bodmer and Parsons (1962).

However, if two separate loci are involved, it would be expected that recombination between the I^A and I^B genes would produce both I^{A_1B} and I^{A_2B} configurations and that, providing there were no selective differences, these would exist in the ratio of the I^{A_1} and I^{A_2} gene frequencies. This expectation is not realized since the above cases all involve only I^{A_2B}. Thus, these observations suggest that two enzymes controlled by separate loci are not involved in the synthesis of A-and B-substances and that, unless a simple amino acid substitution can alter the specificity of an enzyme so that the addition of galactose to preformed H substance is changed in such a way as to involve N-acetylgalactosamine, alternative forms of the one enzyme will not provide a satisfactory explanation. Also, though many gene-enzyme systems have been studied in micro-organisms and gene

mutations have been correlated with amino acid substitutions in the appropriate enzymes, no case of a mutant enzyme being functional, but with altered specificity, has been reported. Therefore, an explanation proposing that a simple amino acid alteration in an enzyme can convert an α-galactosyl transferase to an α-N-acetylgalactosaminoyl transferase would appear to be very unlikely.

A one gene-one enzyme-one antigen hypothesis, with only one active enzyme being specified at a locus, could account for two antigens seeming to be determined by allelic genes - the one being the product of the enzyme specified by the active allele, the other being the activity of the precursor substance in the presence of an amorphic allele, e.g. if, in the ABO system, there were only two groups A and O. However, with blood groups, alternative forms of an antigen, and also a complete absence of antigenic activity, appear to be a common feature, as is shown in the list below.

System	Antigens
ABO	A, B, H
Rh	C, c, -
Rh	E, e, -
MNSs	S, s, -
Lewis	a, b, -
Duffy	a, b, -
P	P, Tj^a, -
Kell	K, k, -
Kidd	a, b, -
Lutheran	a, b, -

Whereas the Le(a-b-) and Lu(a-b-) phenotypes are most probably due to gene interaction, amorphic genes have been invoked to explain the other 'minus-minus' phenotypes. (See Race and Sanger, 1962, for discussion.)

Since an hypothesis involving either one locus or two loci does not seem capable of explaining all of the data about the genetical control of the ABO blood group antigens, assuming there is a gene-enzyme-antigen relationship, it appears that such a scheme for the conversion of genetic information into blood group antigens is not satisfactory.

Perhaps the polypeptide product of the A gene has the capacity to attract and bond particular carbohydrate units to itself and this unit, when attached to the H substance macromolecule, confers A specificity. Amino acid substitution within the polypeptide might then be sufficient to alter the attraction for N-acetylgalactosamine to that for galactose, as has been suggested (Wagner and Mitchell, 1964).

However, irrespective of the biochemical pathways which translate genetic information into blood group substances, it seems that recombination within the ABO blood group locus in Man may occur, very rarely, to give a gene which specifies two antigens accepted under Bernstein's theory as being

controlled by allelic genes.

This discussion has been published elsewhere (Boettcher, 1966).

Since at least four pedigrees which cannot be explained under Bernstein's theory for the inheritance of the genes controlling the ABO blood groups have been found, attempts have been made to ascertain further pedigrees which disagree with the theory.

The four pedigrees at variance with the theory have each involved an A_2B individual who appears to be, genotypically, $\underline{I}^{A_2B}\underline{I}^{O}$ (Kossovitch, 1929; Haselhorst and Lauer, 1930, 1931; Seyfried et al., 1964; Yamaguchi et al., 1965). It seemed, therefore, that, in looking for further pedigrees, these might be best ascertained through an A_2B propositus. Before presenting the results though, it seems advisable to discuss why, if there are pedigrees involving A_2B individuals that are inconsistent with Bernstein's theory, more than four have not been detected or, if they have been detected, why they have not been published. Unless reasonable arguments can be proposed to answer these two questions, it would seem that obtaining pedigrees of A_2B individuals is pointless.

Since the four exceptional pedigrees all involve A_2B individuals it seems reasonable to suggest that cases of unusual inheritance of ABO blood groups do not involve as great a proportion of A_1B individuals as of A_2B individuals; for the probability of ascertaining four exceptional A_2B pedigrees and no unusual A_1B pedigrees, with the hypothesis that the two types of pedigrees should be ascertained in the proportion of A_2B:A_1B individuals in the population, is approximately 0.01 (assuming that the A_2B:A_1B ratio is about 1:3).

The great majority of data on ABO pedigrees has involved pedigrees taken at random from the population and, therefore, the proportion of these involving an A_2B propositus would be about 1 in 150, so that many thousands of such pedigrees would have to be screened before an appreciable number of A_2B propositi would be included. Of the 1,367 pedigrees involving AB individuals included in the 10,628 pedigrees summarized and reported by Wiener (1945), only about 170 involving A_2B mothers would be expected, and these would be the pedigrees capable of providing evidence for the existence of the $\underline{I}^{A_2B}$ gene, since exceptional pedigrees involving an A_2B father could perhaps be explained by non-paternity. Among the 1,367 pedigrees, referred to above,

were the two exceptional pedigrees ascertained by Haselhorst and Lauer (1930) and Kossovitch (1929). Among 45 pedigrees involving A_2B individuals included in another table summarizing pedigrees which provide data on the inheritance of the sub-groups of blood group A, there is one which is an apparent exception to Bernstein's theory, but this can be explained by non-paternity (Wiener, 1945).

It appears, therefore, that if an A_2B individual, genotypically $\underline{I}^{A_2B}\underline{I}^{o}$, can occur, in spite of the large number of pedigrees checked for the inheritance of the ABO blood groups, relatively few pedigrees which could provide evidence for the existence of an $\underline{I}^{A_2B}$ gene have been examined and that, among these, two cases suggesting the existence of such a gene have been reported. It should be noted, too, that, due to the blood groups of marital partners, one would not expect to be able to detect all pedigrees involving an $\underline{I}^{A_2B}\underline{I}^{o}$ individual.

In more recent years, with the very wide acceptance of Bernstein's theory, relatively few pedigree studies have been carried out simply to check on the inheritance of the ABO blood groups. However, a number of mother-infant blood group studies, involving large numbers of individuals, have been carried out and one might expect that an A_2B mother

($\underline{I}^{A_2B}\underline{I}^{O}$) with an O child would have been found among them, if this occurs not too infrequently. Assuming such a combination does occur, there may be two reasons why it has not been described. First, it is possible that the technician carrying out the blood group typing might decide that the infant's blood group "must be" A_2 and that the A_2 antigen is so poorly developed that it is undetectable. Wiener (1945) states "In new-born infants, because of the incomplete development of the agglutinogens, an A_2 blood may be mistaken for group O". Secondly, since mother-infant studies are carried out mainly on data from maternity hospitals one wonders whether such a determination would be published, since it would be an exception to an accepted theory and could be taken as a case of "mixed-up" babies.

For the above reasons, it does not seem unreasonable to accept the existence of an $\underline{I}^{A_2B}$ gene, contrary to Bernstein's theory, and that, due to the random method of pedigree ascertainment used in the early pedigree studies, relatively few pedigrees able to provide critical information have been analysed and, further, that if recent studies had detected individuals with such a gene, they might not have been reported. If such a gene exists, examination of the pedigrees of large numbers of A_2B individuals might reveal further examples.

A_2B propositi of pedigrees ascertained to investigate the genotypes of A_2B individuals were women who had reported to the Red Cross Blood Transfusion Service, Adelaide, for ante-natal blood testing within a four month period in 1964, and three A_2B individuals known to me.

Of twenty women who were contacted through the Blood Transfusion Service fifteen replied favourably to circulars requesting co-operation in testing the blood groups of relatives. The other five did not reply. The families of the three A_2B individuals known to me co-operated in having their blood groups determined.

Finger-prick specimens of blood were taken into physiological saline and the blood groups of the donors were determined from these using the testing fluids.

Of the eighteen pedigrees, two were discarded. One, an aboriginal pedigree, had a sibship of ten, in five of whom non-paternity could be shown by blood groups (in this case venous specimens had been obtained from all members and tested) and the other, where the propositus had no living genetical relation.

Thus, sixteen pedigrees have been examined for the genotypes of the A_2B propositi. In each pedigree sufficient members were available to permit the genotype of the A_2B propositus to be inferred, and in each case it was $\underline{I}^{A_2}\underline{I}^{B}$. So that, as yet, no case of an individual of genotype $\underline{I}^{A_2B}\underline{I}^{o}$ has been detected in this study. However, this study is still proceeding.

CHAPTER VI.

THE NATURE OF THE SECRETOR GENE

A number of suggestions for the possible role of the secretor gene has been proposed. Schiff (1931) regarded the alcohol-soluble antigens of the red cells as the primary factor and the water-soluble form as a degradation product of them and proposed that the organism excreted them in this way. This concept appeared to agree with his observation that it was possible to alter the pure alcohol-solubility of the A-substance in sheep blood into water-solubility by saponification (quoted in Hartmann, 1941). Schiff accounted for non-secretors on the basis of a threshold concentration, by assuming that the concentration of water-soluble antigens in non-secretors is lower than that in secretors. However, on this basis, greater amounts of alcohol-soluble antigen in the serum would be expected in non-secretors than in secretors, which has been shown to be contrary to observations (Friedenreich, 1937).

Friedenreich (1937) suggested that the lack of antigens in the secretions of non-secretors is due to their absence from the corresponding secretory glands and that the presence

of the secretor gene is necessary for production of water-soluble antigens in these glands. This scheme was compared by him with the occurrence of the F-antigen in various mammals since, in different species, this can occur in only the red cells, or in glands, or in both.

Friedenreich and Hartmann (1938) confirmed the predicted lack of antigens in the secretory glands of non-secretors and, in addition, showed that the glands of secretors possessed amounts of antigen proportional to the corresponding secretions. To contradict further the concept of the secretor phenomenon being one of excretion, they calculated the amount of antigen secreted daily by the stomach in the gastric juice, and estimated that this was about 500 times the amount in the blood passing through the stomach.

Hartmann (1941) considered the possibility that non-secretors possess an enzymic ability to degrade the water-soluble breakdown products of alcohol-soluble antigens to units which do not possess serological activity, whereas secretors do not. However, she was able to show that the rates at which blood group antigens in boiled, sterile saliva were degraded upon incubation with salivary glands under sterile conditions for several days, were the same whether the glands came from secretors or non-secretors, and concluded

that such an explanation (invoking secretors being unable to degrade water-soluble antigens, whereas non-secretors can) was untenable.

Friedenreich's suggestion, that the secretor gene is necessary for production of water-soluble antigens in secretory glands (Friedenreich, 1937), is now accepted.

The genetical pathways leading to the ABO antigens have been summarized in Figure I.1 (p. 11). A precursor mucopolysaccharide possessing Type XIV pneumococcus activity has H specificity added to it due to the action of the H gene. Further specificity can be added to H substance due to the action of the genes I^A or I^B to give the A and B antigens.

Since A, B, H and Le^a substances cross-react with anti-Type XIV pneumococcus serum, as does a cyst preparation from an individual who is a non-secretor of A, B, H and a non-secretor of Le^a and Le^b, "Inactive substance, F1." (Watkins and Morgan, 1959), it is proposed that the genetically independent Lewis gene, Le (Race and Sanger, 1954), acts independently of the H and ABO genes to convert precursor substance to Le^a substance, which can be present or absent in secretions having, or lacking, the A, B or H antigens. However, although the Lewis and ABO genes act

independently, it seems they can add specificity to the same molecules. Precipitating anti-A removes the A and most of the B activity from saliva and cyst fluids from AB individuals, but only the A activity from an artificial mixture of A and B substances (Morgan and Watkins, 1956). Similar results have been obtained with A and H using secretions from A_2 individuals (Watkins and Morgan, 1956/57), and with Le^a and A (Watkins and Morgan, 1959).

The secretor gene, Se, which is responsible for the appearance of the A, B and H antigens in aqueous body secretions, has been shown not to have simply an excretory function. Its presence is thought to enable specific tissues to produce water-soluble A, B or H antigens. Therefore, it is most unlikely that the secretor gene exerts its influence after the A, B and H antigens have been formed.

If the sese genotype corresponded to a block after H substance had been formed, non-secretors of group A and B would not be able to secrete A or B antigenic substances but could secrete H substance. This does not occur.

Szulman's observations (Szulman, 1962), similarly, are not consistent with the Se gene operating after the formation of H substance. Using a fluorescein isothiocyanate-labelled anti-H serum from a "Bombay" individual immunized with

purified human H substance, Szulman (1962) showed that O secretors have mucus-bound H substance in mucous glands with a distribution similar to that of A and B substances in secretors of the respective blood groups, whereas non-secretors do not. From his observations, he concluded that the secretor gene acts at the point of formation of H substance, or its precursor.

The Se gene apparently does not influence the presence or structure of precursor substance, since Le^a substance can be present, with a similar distribution of molecular sizes, in the secretions of both secretors and non-secretors (Brown, Glynn and Holborow, 1959; Kaklamanis, Holborow and Glynn, 1964). Thus, the Se gene appears to function somewhere between the formation of precursor substance and H substance. It does not seem to be responsible for any serological specificity since, in the red cells, similar A, B and H specificity can be conferred to that in the secretions without the Se gene being involved and, also, no additional specificity of the A, B or H substances within the secretions has been described.

Since the Se gene appears to function somewhere between the formation of precursor substance and H substance, and since, without the action of this gene, the same conversion

can occur in the production of the red cell antigens and, furthermore, in the absence of any evidence of additional specificity being added to the precursor substance due to the action of this gene, it appears that the Se gene does not have any direct effect on the chemical structure of the blood group substance macromolecule. In addition, the Se gene does not appear to have any effect on the physical structure of the blood group substance macromolecule, so that the concept of the gene Se being a transforming gene determining a step in the biosynthesis of the blood group mucopolysaccharides (Watkins, 1959) appears to be incorrect. Since the influence of the Se gene on the blood group substance macromolecule is considered to be indirect, it is possible that it functions as a "switch" gene, responsible for "switching-on" the H gene in those cells producing water-soluble antigen, but having no effect in those cells producing alcohol-soluble antigen.

Individuals possessing Le, Se and H genes have the Le^b antigen in their saliva as well as the respective Le^a, H and A or B antigens. Individuals possessing lele or Le, Se and hh genes do not produce the Le^b antigen (Levine et al., 1955). Thus, contrary to what has been suggested (Ceppellini, 1955; Watkins, 1959; Watkins and Morgan, 1959), the Le^b antigen cannot be the product of the interaction of the Le and Se

genes only, as has been pointed out by Race and Sanger (1962). However, it is possible that the Le^b antigen is formed when the H gene adds H specificity to Le^a substance, since the agglutination of red cells having the Le^b antigen by human anti-Le^b serum can be inhibited by two oligosaccharides which each have α-L-fucosyl units joined to each of two adjacent sugars (Watkins and Morgan, 1957), and it is known that H specificity is primarily due to O-α-L-fucosyl end-residues (Morgan, 1959a).

If this concept is correct, removal of fucosyl end-residues from Le(a-b+) red cells should convert them to Le(a+b-).

If Le^b activity is due to H specificity being added to Le^a substance, this offers an explanation for the two types of sera termed anti-Le^b from their reactions with red blood cells:

1. Anti-Le^{bH}, which can be inhibited by saliva of all ABH secretors, whether from LeLe, Lele or lele individuals.
2. Anti-Le^{bL}, which can be inhibited by saliva from ABH secretors who are LeLe or Lele, but not those who are lele.

Antisera of the first type would have their specificity directed mainly against the H antigenic determinants, whereas antisera of the second type would have their specificity directed mainly against Le^a antigenic determinants. This would fit well with the suggestion that anti-Le^{bH} sera are those that react strongly only with O and A_2 cells, whilst anti-Le^{bL} sera are those whose specificity is but little affected by the presence of A_1 (Sneath and Sneath, 1959), since A_2 cells have their H activity modified far less than B or A_1 cells, and treatment of A_2 red cells with anti-A does not interfere with subsequent sensitization of these cells with human anti-H (Richardson Jones and Kaneb, 1961).

Bird (1958) has suggested that anti-H may be thought of as anti-$H+H_1$ by analogy with anti-$A+A_1$, and that anti-Le^b may be anti-H_1. Such a scheme would fit that proposed here, and also the one proposed earlier for the A_1 and A_2 antigens (pp. 97-99).

Anti-A_1 is thought of as being directed partly against the A antigen and partly against the link between adjacent A antigenic units. It will agglutinate both A_1 and A_2 cells (the latter more weakly), and an anti-A_1 reagent can be exhausted by absorption with sufficient A_2 cells.

Within these concepts, anti-H_1 is thought of as being directed partly against the H (or Le^a) antigen and partly against the link between the Le^a antigenic unit and the adjacent H unit.

Race and Sanger (1962) report two powerful anti-H sera from Le(a+b-) donors, which react more strongly with Le(b+) than with Le(b-) cells, and which can be absorbed completely by O Le(a-b-) and Le(a+b-) cells. It is proposed that these two sera contain anti-$H+H_1$ and, similarly to A_2 cells with anti-A_1, absorption with sufficient H antigen can remove all anti-H_1 activity.

It has been proposed that, in the Rh system, compound antigens arise from adjacent portions of certain genes when they are in the cis configuration (Boorman and Lincoln, 1962). Thus, parts of the D and C, C and E and c and e genes in the cis configuration perhaps give rise to the compound antigens G, M and f. For adjacent portions of genes to produce compound antigens, the gene order must be also the antigen order, as has been pointed out (Boettcher, 1964) i.e. when the C antigen is added to preformed D substance a "new" antigenic determinant, G, is formed as well as D and C (Allen and Tippett, 1958).

Two types of antibodies have been termed anti-G:

1. Found in all anti-CD sera which are produced by dce/dce people; reacts with cells having the G and/or D and/or C antigens.
2. Found in those anti-C sera which react with r^G cells; this antibody does not react with D-positive C-negative cells.

The analogies of this scheme with the proposed scheme for Le^b are clear. Thus, the concept that the Se gene acts prior to the H genes, but not directly on the precursor molecule, and acts as a "switch" gene for the structural H gene in the respective secretory cells appears to be quite satisfying. Perhaps the Y gene (Weiner et al., 1957) has a similar relationship with the I^A structural gene in cells concerned with the production of the alcohol-soluble antigens.

Figure VI.1. summarizes the conclusions concerning possible genetical pathways leading to H, A, B and Lewis antigens in the saliva presented here.

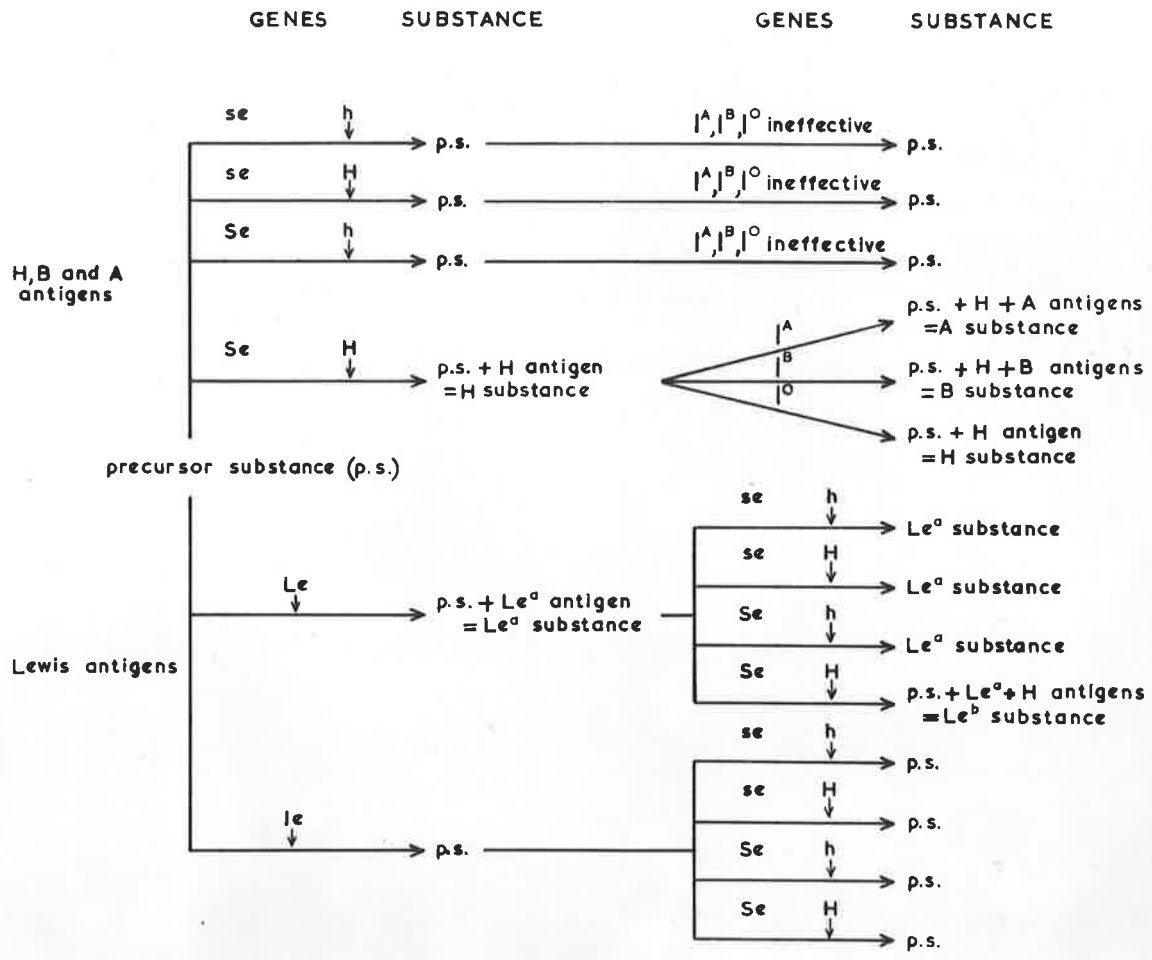


FIGURE VI.1. POSSIBLE GENETICAL PATHWAYS LEADING TO H, A B AND LEWIS ANTIGENS IN THE SALIVA. A way of summarizing conclusions from the text.

All individuals have a pathway for H, A and B antigens and one for Lewis antigens. The two are not mutually exclusive.

The secretor genes are considered to act prior to the H genes, but not directly on the precursor molecule.

Le^b antigen is considered to arise from the addition of H antigenicity to Le^a substance.

APPENDICES

A. ABO BLOOD GROUP AND SECRETOR GENE FREQUENCIES

During the studies on the ABO antigens present in salivas carried out to provide data for this thesis, specimens from 1,085 university students have been tested. The data are presented in Table A.1. The ABO blood groups and secretor status were determined from in general, finger-prick blood specimens and saliva specimens given at the same time, though some individuals were blood-grouped from venous specimens.

Most A and AB individuals have had their A sub-group determined, but some have not. Therefore, in determining the blood group phenotype and blood group gene frequencies on the total data, there has been no subdivision of A or AB, but the data where the sub-groups have been determined are given as well.

When determining the secretor status of individuals two techniques have been used. The first is where the inhibition titres of all antigens in each saliva have been obtained. The second is where the inhibitions of only a particular dilution of the testing fluids have been determined. Since it might be argued that the second technique

TABLE A.1. 1085 UNIVERSITY STUDENTS CLASSIFIED ACCORDING TO ABO BLOOD GROUP AND SECRETOR+ STATUS

AUSTRALIAN-BORN STUDENTS

	MALES									FEMALES								
	O SEC	O NON	A SEC	A NON	B SEC	B NON	AB SEC	AB NON	TOTALS		O SEC	O NON	A SEC	A NON	B SEC	B NON	AB SEC	AB NON	TOTALS
METHOD (i)	156	49	156	66	41	9	17	5	499	(i)	54	13	35	7	6	2	4	0	121
METHOD (ii)	45	13	46	9	8	2	4	1	128	(ii)	34	18	29	10	11	1	1	0	104
TOTALS	201	62	202	75	49	11	21	6	627		88	31	64	17	17	3	5	0	225

EUROPEAN-BORN STUDENTS

	MALES									FEMALES								
	O SEC	O NON	A SEC	A NON	B SEC	B NON	AB SEC	AB NON	TOTALS		O SEC	O NON	A SEC	A NON	B SEC	B NON	AB SEC	AB NON	TOTALS
METHOD (i)	37	4	42	12	20	1	4	2	122	(i)	10	2	7	0	4	0	3	0	26
METHOD (ii)	9	1	8	2	1	0	0	0	21	(ii)	8	2	3	0	0	1	2	0	16
TOTALS	46	5	50	14	21	1	4	2	143		18	4	10	0	4	1	5	0	42

ASIAN-BORN STUDENTS

	MALES									FEMALES								
	O SEC	O NON	A SEC	A NON	B SEC	B NON	AB SEC	AB NON	TOTALS		O SEC	O NON	A SEC	A NON	B SEC	B NON	AB SEC	AB NON	TOTALS
METHOD (i)	12	3	7	2	6	1	2	0	33	(i)	1	0	1	0	2	0	1	1	6
METHOD (ii)	1	1	1	1	3	0	0	0	7	(ii)	0	0	1	0	1	0	0	0	2
TOTALS	13	4	8	3	9	1	2	0	40		1	0	2	0	3	0	1	1	8

(i) TOTAL DATA

BLOOD GROUP FREQUENCIES

	AUSTRALIAN-BORN STUDENTS					EUROPEAN-BORN STUDENTS				
BLOOD GROUP	O	A	B	AB	TOTAL	O	A	B	AB	TOTAL
	382	358	80	32	852	73	74	27	11	185

(ii) THOSE INDIVIDUALS INCLUDED IN (i) WHO WERE EXAMINED WHEN SUB-GROUPS OF A AND AB WERE DETERMINED

	AUSTRALIAN-BORN STUDENTS							EUROPEAN-BORN STUDENTS						
BLOOD GROUP	O	A_1	A_2	B	A_1B	A_2B	TOTAL	O	A_1	A_2	B	A_1B	A_2B	TOTAL
	335	241	80	70	22	9	757	62	61	10	27	8	2	170

+ SEC = SECRETOR NON = NON-SECRETOR

/ METHOD (i) IS WHERE INHIBITION TITRES OF SALIVAS WERE DETERMINED (see text).
METHOD (ii) IS WHERE ONLY ABILITY OF A SALIVA TO INHIBIT CRITICAL CONCENTRATIONS OF TESTING FLUIDS WERE DETERMINED (see text).

is less sensitive than the first and, therefore, it might not detect some secretors who have low concentrations of antigens, the results obtained when using the two techniques have been kept separate.

The blood group frequencies of the 852 Australian-born and 185 European-born individuals are not significantly different when A and AB are not subgrouped ($\chi^2_3 = 6.90$, $0.10 > P > 0.05$) but, when the blood group frequencies of those individuals within these totals who were examined when the A and AB subgroups were being determined are compared, the blood group frequencies of 757 Australian-born and 170 European-born individuals are significantly different ($\chi^2_4 = 12.64$ $0.02 > P > 0.01$). It can be seen that, in the two groups of individuals, the frequencies of the A_1 and A_2 subgroups are significantly different

Students	Blood Groups		
	A_1	A_2	
Australian-born	241	80	321
European-born	61	10	71
	302	90	392

$\chi^2_1 = 3.861$ $\quad 0.05 > P > 0.02$

The gene frequencies obtained from the data (Table A.1) are given below.

(i) When groups O, A, B, and AB only are considered.

852 Australian-born students	185 European-born students	1037 students
$\underline{I}^A = 0.2627$	$\underline{I}^A = 0.2637$	$\underline{I}^A = 0.2628$
$\underline{I}^B = 0.0\emptyset69$	$\underline{I}^B = 0.1072$	$\underline{I}^B = 0.0739$
$\underline{I}^O = 0.6704$	$\underline{I}^O = 0.6291$	$\underline{I}^O = 0.6633$

(ii) When groups O, A_1, A_2, B, A_1B and A_2B are considered.

$\underline{I}^{A1} = 0.1905$	$\underline{I}^{A1} = 0.2337$	$\underline{I}^{A1} = 0.1978$
$\underline{I}^{A2} = 0.0751$	$\underline{I}^{A2} = 0.0469$	$\underline{I}^{A2} = 0.0704$
$\underline{I}^B = 0.0662$	$\underline{I}^B = 0.1196$	$\underline{I}^B = 0.0756$
$\underline{I}^O = 0.6652$	$\underline{I}^O = 0.6039$	$\underline{I}^O = 0.6544$

When the data on secretors are divided into those of Australian-born and European-born, and males and females, the numbers in the classes for European-born students are too small for sensitive statistical evaluation. Therefore, the conclusions derived from consideration of the data provided by the Australian-born students have been applied to the data of European-born students.

Within the sexes there is no significant difference between the frequencies of secretors and non-secretors of the different blood groups given by the two methods of classifying secretors (for males, $\chi^2_7 = 5.07$, $.70 > P > .50$; for females, $\chi^2_6 = 8.81$, $.20 > P > .10$). Accordingly, the data derived from the two methods have been pooled. Between the sexes there is no significant heterogeneity of the total numbers of individuals in the eight classes ($\chi^2_7 = 11.49$ $0.20 > P > 0.10$). Accordingly, the data for males and females have been pooled. Within the four blood-groups there is no significant difference between the numbers of secretors and non-secretors ($\chi^2_3 = 2.92$, $.50 > P > .30$). So the data have been pooled to provide two classes, secretors and non-secretors, within the groups of Australian-born and European-born secretors.

It can be seen that the frequencies of secretors and non-secretors within the groups of Australian-born and European-born students are significantly different.

Students	Secretors	Non-secretors	
Australian-born	647	205	852
European-born	158	27	185
	805	232	1037

$$\chi^2_1 = 7.74 \qquad 0.01 > P > 0.001$$

The secretor gene frequencies obtained from the data (Table A.1) are given below.

852 Australian-born students	185 European-born students	1037 students
$\underline{Se} = 0.5095$	$\underline{Se} = 0.6180$	$\underline{Se} = 0.5240$
$\underline{se} = 0.4905$	$\underline{se} = 0.3820$	$\underline{se} = 0.4760$

Both the ABO blood group gene frequencies, where the genes $\underline{I}^{A1}$ and $\underline{I}^{A2}$ have been considered, and the secretor gene frequencies for Australian- and European-born university students studied here have been shown to be significantly different.

It is not considered appropriate in this thesis to speculate on why these significantly different gene frequencies have been found, or the effect on the "Australian" ABO blood group and secretor gene frequencies of migrants, who now comprise about 10% of the total population, and who have come from regions with different frequencies of these genes.

It is not suggested that the gene frequencies reported here are representative of the frequencies for Australian residents. Rather, it is suggested that they are not since, first, there is a higher proportion of European-born individuals in this sample than in the whole population and, secondly, there are very few southern Europeans included in this sample in comparison with their proportion in the population.

However, the gene frequencies for Australian- and European-born university students have been pooled to give frequencies obtained from a sample of Australian-domiciled university students.

B. PUBLISHED PAPERS

1. The Rh 'deletion' phenotypes and the information they provide about the Rh genes.

2. The Rh antigens of anthropoid apes in relation to Rh 'deletion' phenotypes.

Boettcher, B. (1964). The Rh 'Deletion' Phenotypes and the Information they Provide about the Rh Genes. *Vox Sanguinis, 9*(6), 641-652

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The Rh Antigens of Anthropoid Apes in Relation to Rh "Deletion" Phenotypes

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IN A RECENT PAPER concerning the Rh "deletion" phenotypes, produced by *D*--/*D*-- and other cells (Boettcher, 1964), a three-gene model based on the current concepts of the genetical control of the ABO red cell antigens (Race and Sanger, 1962) has been proposed for the genetical control of the Rh antigens. A sequential arrangement is envisaged whereby a precursor substance has D antigenic specificity added to it by the action of the enzyme product of the Rh *D* gene. Further specificity is normally added to this substance by the action of the enzyme product of the *C* gene, and so on. To account for the absence of phenotypes corresponding to *–C–/–C–* and *––E/––E* from the "deletion" phenotypes detected so far, it has been proposed that C specificity cannot be added to the antigenic unit unless specificity controlled by the *D* locus has been added previously, i.e., an individual homozygous for an amorphic allele at the *D* locus would have the phenotype ---/---, irrespective of the *C* and *E* alleles possessed. (The *d* gene is not considered an amorph.)

Furthermore, it has been suggested (Boettcher, 1964) that the Rh antigens of different groups of primates might be a reflection of the evolutionary development of the Rh genes. Gene duplication, with modification of the "new" gene, has been proposed (Boettcher, 1964) as the mechanism involved in this development.

Recently Wiener, Moor-Jankowski, and Gordon (1964) have described the Rh blood types of anthropoid apes, with the interesting result that only the c antigen of the Rh series has been detected on red cells obtained from each of nine gibbons. If the gibbon's genotype is truly *–c–/–c–*, this would be a contradiction of the model described (Boettcher, 1964). Accordingly, tests have been carried out to determine whether any form of Rh D antigen can be detected on gibbon red cells.

Blood was obtained from a female and a male whitehanded gibbon, *Hylobates lar* (groups A_1B and A_1, respectively, as determined from both red cell and serum tests) and tested against a series of saline and incomplete anti-D, -C, -c, -E and -e testing sera. Cells could be classified as positive only with anti-c sera. It is of interest that the gibbon red cells would not agglutinate in the presence of papain. In the presence of this enzyme, not only did incom-

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TABLE 1. REACTIONS OF RED CELLS WITH SPECIFICALLY ABSORBED AND ELUTED AHA ANTIBODIES

Cells	Reaction* with anti-			
	nl, pdl, dl	nl, pdl	dl	nl
DCe/DcE	4	4	4	4
dce/dce	4	4	4	4
D––/D––	4	4	4	–
Dc–/Dc–	4	4	4	–
Gibbon	4	4	4	–
---/---	4	–	4	–

*4 = strong agglutination. – = no agglutination.

plete Rh antisera fail to agglutinate the cells but also the usual antispecies reactions failed to occur. However, when ficin was used, gibbon cells behaved normally.

Since the antigen LW ("D-like") bears some relationship to the antigens determined by the Rh genes and because it has been detected on the red cells of various monkeys and anthropoid apes (Levine and Celano, 1962), the gibbon red cells were tested against guinea pig anti-LW. Anti-LW reacts strongly with red cells having the D antigen, including *D––/D––* cells, and either weakly or not at all with cells classed as d. The ---/--- cells of Vos *et al.* (1961) and of Levine *et al.* (1964) do not react with anti-LW (Levine *et al.*, 1962; Levine *et al.*, 1964), nor do the cells of Mrs. G. and Miss B. (Levine *et al.*, 1963) and Mrs. V. W. (Swanson and Matson, 1964), whose Rh antigens appear normal otherwise. The gibbon cells were agglutinated by anti-LW and, therefore, possess the LW antigen.

Further tests on the gibbon cells were carried out using ficin-treated red cells and specifically absorbed and eluted acquired hemolytic anaemia (AHA) antibodies, described previously (Weiner and Vos, 1963). These antibodies are (1) anti-nl (normal) which reacts with red cells having Rh antigens of the D, C, and E series; (2) anti-pdl (partially deleted) which reacts with red cells lacking some of the expected Rh antigens, e.g. *D––/D––* and *Dc–/Dc–* cells; and (3) anti-dl (deleted) which reacts with red cells lacking all Rh antigens, i.e. ---/--- cells. The gibbon cells fell into the pdl (partially deleted) group with *D––/D––* and *Dc–/Dc–* cells, since they reacted with anti-dl and with anti-nl + pdl but not with anti-nl (Table 1). Since Weiner and Vos (1963) have shown that anti-pdl does not recognize the LW antigen, gibbon cells must have another antigen in common with *D––/D––* and *Dc–/Dc–* cells that is not possessed by ---/--- cells.

Thus, these results suggest that gibbon cells have antigens of the Rh D and c series which are qualitatively different from those of humans. This is similar to the situation in chimpanzees, orangutans, and gorillas (Wiener, Moor-Jankowski, and Gordon, 1964). Consequently, the Rh antigens of the gibbon do not contradict the Rh gene model and its suggested evolution, described earlier.

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