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A STUDY OF THE SECRETOR SYSTEM, WITH SPECIAL REFERENCE TO ANTIGENIC SPECIFICITIES OF THE NEWBORN AS DETERMINED BY PLANT AGGLUTININS

by

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INTRODUCTION

During the second and third decades of investigation in the field of blood group serology, before the era of the Rh system and its vexed terminology, a large number of German and Japanese workers (as well as a few Americans) were engrossed in discovering sites of A/B antigen. Tissues, organs, fluids, glands, and secretions were extracted, titered, absorbed, and otherwise subjected to antigenic identification. Once its areas of occurrence were plotted, and the phenomenon of antigenic secretion securely tied to a genetic locus of dominance-recessive expression, the secretor system aroused little further interest except as a non-adaptive curiosity.

Recently, however, it has again emerged in the fore of research interests, due to the stimulation of several related developments:

1. A changed concept of the devious nature of the ABO(H) system and its intricate interrelations with the Lewis and secretor loci.

2. The discovery and verification of ABO incompatibility; its manifestation in erythroblastosis fetalis on one hand, and in suspected increase in infertility, on the other.

3. Interest in genetic polymorphisms as evidence of stability through compensatory selective pressures, or transient states moving toward the reduction in frequency of one gene or the other. No polymorphisms are considered patently non-adaptive at the present time.

4. The discovery of associations between ABO blood group

frequencies and disease, followed shortly by correlations of the non-secretor phenotype with certain susceptibilities to infection and mucosal erosion. Since the clearest associations between blood group frequencies and disease are those of the upper gastrointestinal tract, where maximum concentrations of soluble antigens are to be found, the implication of the secretor system was inevitable.

5. The observation of varying racial frequencies in the Se gene, providing an additional polymorphic index for assessments of intermixture, migration, and possibly processes of environmental selection.

There is no occasion for determining secretor or Lewis status in the course of ordinary hospital care and the treatment of disease, therefore the search for correlations in the hospital record-room (so effective a means of data-gathering in cases of malignancy, ulcer, and anemia, and ABO) cannot be utilized in secretor studies. This area of investigation must painfully accumulate its data without the large-scale help of blood banks and civil defense programs. For this reason, data on secretor are accumulating more slowly than those relating to many other traits.

It is significant that this thesis, which draws its body of data almost entirely from medical journals, genetic, clinical, and hematologic symposia, and immuno-serological research, is not out of place in the province of physical anthropology. This situation clearly reflects the new occupation of that field with the internal mechanisms of natural selection, which contribute importantly toward the solution of their micro-

evolutionary problems. The mathematicians, geneticists, and serologists have become the most respected contributors to the science and are functioning as the teachers of the coming generation. It is a cause of alternate concern and healthy progress that physical anthropologists, to function effectively, must now relate their activities to the sphere of the hospital, the blood bank, and the Drosophila laboratory as well as the "field" and the museum.

It remains only to be noted that this dissertation, largely completed in the summer of 1961, has included only a very few more recent references of exceptional interest and significance.

Part I

A REVIEW OF THE LITERATURE

CHAPTER I

BLOOD GROUP SPECIFIC SUBSTANCES IN ORGANS AND BODY FLUIDS

Subsequent to the discovery in 1900-1901 of the ABO blood group system by Karl Landsteiner, no attempt was made to locate corresponding antigenic substances other than on erythrocytes until a decade had passed. At this time, van Dungern and Hirschfeld (1910) carried on immunization experiments with dog-kidney suspensions, with promising but inconclusive results.

At approximately the same time Moss (1910) and Gräfe and Graham (1911) located antigens in human serum by inhibition techniques. Schiff (1924) later reinforced the earlier findings by specific precipitation tests. In 1926, seminal fluid was demonstrated to inhibit the agglutination of erythrocytes from individuals of the same blood group by Yamakami; the saliva of one individual was also successfully employed in antibody absorption. Landsteiner and Levine, the same year, demonstrated that spermatozoa had the capacity of absorbing specifically blood group agglutinins from immune serum, which finding was later confirmed by other workers.

Kritschewski and Schwartzmann (1927), as well as Witebsky (1927), observed that organ (spleen, liver, kidney) cells evidence the same specific antigenic properties as erythrocytes in inhibiting agglutination. Witebsky and Okabe (1927), working with alcoholic extracts, demonstrated

the presence of A factor in liver and kidney by means of complement-fixation reactions. Kan-Iti Yosida (1928) recovered A and B antigens from water-soluble extracts of various organs and secretions. Thomsen (1930) also reported group specific substances in the cells of tumors, both benign and malignant.

In 1929, Hirszfeld, Halber, and Laskowski ran a comprehensive series of investigations on alcoholic extracts of organs and secretions, employing the complement fixation reaction. Schiff (1931), and Friedenreich and Hartmann (1938) also contributed to a systematic determination of the antigenic components of various organs. Most generously endowed, they observed, were stomach, submaxillary gland, and pancreas, while moderate amounts were found in kidney, parotid gland, liver, prostate, lungs, spleen, and myocardium; smaller amounts were located in the large intestine, and only traces in the testes. Small quantities found in the brain were thought due to contamination by blood, in view of occasional negative results of testing.

As the result of studies by Lehrs (1930), Putkonen (1930), Yosida (1928), Schiff (1931), and others, soluble group specific substances were found in tears, sweat, urine, digestive juices, bile, milk, and pleural, pericardial, peritoneal, and amnionic fluid. Large quantities were also discovered in the fluid of pseudomucinous ovarian cysts.

Throughout these years of investigation, it had been repeatedly observed that secretions might demonstrate positive blood group character

in some individuals, but react negatively in others. These findings were considered artifactual or accidental until Schiff and Sasacki (1932a) conclusively established that the presence or absence of water-soluble blood group substances in the secretions of glandular or mucous tissue is controlled by a pair of allelic genes, then designated S and s.[1] S was observed to be dominant in its effect to s. The two categories were distinguished as Ausscheider and Nichtausscheider.

Schiff's (1931) interpretation of the secretor phenomenon assumed the erythrocytes as the primary locus of blood group specificity. He hypothesized that the water-soluble form of antigens represented an excreted product resulting from the natural degradation of the cellular alcohol-soluble form. In order to explain the lack of antigens in non-secretors, he proposed a "blood secretion threshold" (Blutsekretschranke); according to his theory, a high antigen concentration (retention) must be anticipated in the plasma or organs of such individuals. However, earlier investigations had conversely revealed antigen concentrations too low to maintain such an explanation, nor was any difference exhibited between secretors and non-secretors as regards the content of alcohol-soluble group antigens in organs.

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[1]In order to prevent confusion of secretor genes with the Ss genes of the MNSs system, the former will be referred to as "Se" and "se" rather than the S and s of Schiff's terminology.

Schiff's theory also necessarily assumed that the water-soluble form, before excretion, must be derived from the alcohol form by some process of transformation. No such transformation under normal physiological or experimental conditions has been observed.

Friedenreich, in 1937, opposed the Schiff hypothesis with his claim that the secretor-nonsecretor distinction depends on the presence or absence of group antigen in the secretory glands and that the Se gene might be considered a complementary gene necessary for the production of antigen in the glandular tissue. A quantitative agreement between the content of water-soluble group antigen in glandular tissues and their respective secretions was conclusively established by him with Hartmann in 1938. They postulated two systems, relating respectively to the water-soluble and alcohol-soluble (or lipoidal) forms; the distinction between individuals with regard to secretor-nonsecretor character lies in the prosence of water-soluble group antigens. In erythrocytes and almost all non-glandular organs of all individuals, whether secretors or non-secretors, the lipoidal form is present in roughly equivalent amounts. Water-soluble substances cannot be readily obtained from erythrocytes (Lewis serum antigens apparently represent an exception) or non-glandular organs.

Three years later, Hartmann's book Group Antigens in the Human Organs reviewed the Schiff-Friedenreich controversy, and set forth explicitly all known quantitative and comparative data on both water- and

alcohol-soluble antigens in organs and secretions. It reviewed current techniques as well as extending her own findings.

She concluded:

1. The content of water-soluble antigen is different in secretors and nonsecretors, the former exhibiting a comparatively high concentration of antigens in various glandular organs and secretions; the latter type, negligible amounts or no antigens whatsoever.

2. The secretions contain water-soluble antigens exclusively, erythrocytes only alcohol-soluble antigens (Lewis was later demonstrated to except this rule).

3. The concentration of water-soluble group antigens in secretors varies markedly from organ to organ, while alcohol-soluble antigen remains relatively constant in amount.

From this, it appeared that the ABO polysaccharides might be referred to two distinct genetic systems, the first of which relates to alcohol-soluble antigens, and is uninfluenced by Schiff's S gene; the second system is manifested in the water-soluble antigens, isolated in exceptional quantities in most secretions of the digestive system. Nonsecretors, far from being devoid of water-soluble antigens, exhibited on an average one-third of the concentration to be found in the same organ of secretors. The S (Se) gene was thus considered the complementary factor, the presence of which is essential for the quantitatively "complete" development of the water-soluble antigens in the organism.

The work of Hartmann and her Copenhagen colleagues thus introduced a new concept of secretor, in so far as the Se gene now appeared to influence the antigenic character of the entire organism.

Up until the decade of the 1950's, most research employed the standard immunological procedure of inhibition of agglutination to determine the presence or absence of antigens in various tissue extracts or secretions. Occasional studies employed the precipitin test (which necessitates specific concentrations of the interacting antigen-antibody substances), as well as complement fixation. Another modification detected antigens by the ability of cells first to absorb an agglutinin, and then to yield it up by elution. This relatively tedious method, like complement fixation, never came into wide usage.

In 1956, Coombs and his colleagues introduced the useful technique of mixed cell agglutination for detecting antigens upon the surface of single tissue cells (epithelium; later platelets, leucocytes, spermatozoa, and a variety of organ cells dissociated from their tissues). In principle, the antigens upon the cellular surface capture and retain their specific antibodies; erythrocytes added subsequently cling to those cells which are holding their appropriate agglutinins. Thus a suspension of isolated epidermal cells of group A, when incubated with anti-A, retain that antibody throughout washing. Mixed agglutinates are formed with the addition of A (but not B or O) red cells. Högman (1959a, 1959b) has recently employed this technique to detect antigens in various embryonic cells grown in tissue cultures.

The publication in 1950 by Coons and Kaplan, of a technique of antigen determination in tissues by the use of fluorescent antibodies, brought with it the possibility of topographical studies of the distribution of various blood group substances throughout the tissues and organs of the body. Pre-treatment of the sections with alcohol or water permits the distinction of alcohol- from water-soluble forms.

In 1957, Glynn, Holborow, and Johnson reported on the findings of blood group substances in a variety of human tissues, including gastric and duodenal mucosa. They found secretors to be distinguishable from nonsecretors by A/B and Le^a endowment in epithelial linings, especially marked in the stomach and duodenum. The mucosa of secretors displays antigenic specificity corresponding to the A/B erythrocyte type; non-secretors fail to evidence the A/B antigens. However, traces of Le^a substances were detectable in some secretors (the Lewis erythrocyte status of such subjects was unfortunately not clarified). H substance in varying amounts appears ubiquitous in occurrence; it is qualitatively independent of type and secretor status in the superficial mucosa,[2] if not always quantitatively.

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[2]The authors find the presence of H substance in nonsecretors understandable "if H is a precursor of Le^a substance." However, that H is <u>not</u> a precursor of Le^a but rather is a transformed product of Le^a is assumed by the accepted interpretation of the "Bombay" type individuals, whose secretions and cells are rich in Le^a while lacking H. Le^a is thus assumed to represent the substrate from which the X + Se genes unite their influence to produce H. That H can also be produced from the basic substrate has been pointed out in accounting for H substance in leleSe genotypes. No worker, however, except these authors has suggested H as the Le^a precursor.

The same tissue may thus contain H and sometimes traces of Le^a irrespective of blood type and secretor status, but the presence of Le^a in significantly large amounts is exclusive to secretors. A and B substance, although limited in secretors to the superficial mucosa, is invariably present in the neck cells irrespective of secretor status. Both antigens are also found in deep parietal cells in the alcohol-soluble form without secretor-nonsecretor distinctions. Failure to detect a corresponding substance in group O individuals, in these sites may, the authors believe, be due to the absence of a specific anti-O serum.[3]

Szulman, in 1960, reported a further comprehensive series of fluorescent antibody investigations in the distribution of A and B antigens in the tissues of human organs. Unfortunately he did not undertake to investigate H substance. The author noted a high degree of correlation of his results with those arrived at by earlier extraction and inhibition methods of Putkonen, Hartmann, and Friedenreich and Hartmann.

The presence and distribution of antigens in mucus closely reflects the secretor status; its staining is unaffected by treatment with ethanol. The distinction between mucous and serous elements is here emphasized.

The universal staining of the intimal lining of the vascular bed unexpectedly illustrates the widespread nature of these antigens. The

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[3]In the light of recent findings, it is more likely due to the lack of any such specific antigen, since O is functionally an amorph.

virtual absence of blood group antigens from aqueous extracts of the parotid (a serous gland) and its presence in submaxillary and sublingual tissues in quantities proportional to their mucous cell populations, had led Tasiro (1938) to the conclusion that the antigen was mucus-borne. Szulman's research confirms this early observation.

The small, ubiquitous alcohol-soluble fraction in deep cells and glands most likely corresponds to the vascular intimal antigen. Its scarcity (except in the case of erythrocytes) and intimate binding to the tissues underlines the difficulty of studying it chemically by extraction. The difficulties involved in the isolation of alcohol-soluble group substances from erythrocytes are well known;[4] histochemical studies may provide another and more fruitful approach.

The author concludes that the presence of antigens found in mucus, sperm, and milk are determined by the Se gene (along with X); antigens in endothelium and stratified epithelium, as well as that in perspiration and pancreatic secretion are apparently independent of the Se gene. In the case of mucus-borne antigens, the control is incomplete, since A and B substances are regularly found in the deeper layers of the gastrointestinal mucosa of nonsecretors. Szulman finds it simplest to suppose that the absence of secretion is due to the suppression of production of this form of antigen by the sese genotype. While the suppression is more or less complete in salivary glands and other mucus-secreting

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[4]W. T. J. Morgan, in Polysaccharides in Biology (1956), p. 150.

apparatus, it stops short in the depth of gastrointestinal mucosa. The traces of antigenic substances observed in most nonsecretor salivas serves further to illustrate the ordinary lack of complete suppression.

An alternative possibility suggested by Hartmann (1941) is that the difference between secretors and nonsecretors lies in an enzymatic difference, manifesting itself in an unequal ability to break down or destroy certain polysaccharides. Nonsecretors, in possessing such an enzyme, could convert water-soluble group antigens into products without serological reactivity. Only the deepest glandular deposits would escape such conversion. Schiff and Buron (1935) believed they had located such an enzyme in the stomach cells, but their work has not been confirmed. In such a case, the presence of H and Le^a substance in nonsecretors would necessarily represent products of enzymatic degradation rather than the unconverted products of the early developmental levels of Ceppellini (1959) and Watkins (1959).

CHAPTER II

GENETICS OF SECRETOR SYSTEM

A knowledge of the ABO allelomorphs and their mode of transmission has been with us since Bernstein (1924). These genes are known to have two forthright expressions: the determination of the alcohol-soluble polysaccharides bound to the protein erythrocyte stroma, and the activation of the corresponding specificity of ABH water-soluble mucopolysaccharide secretions. This dual function is not considered a true example of pliomorphism since apparently the primary action of the gene is, in both cases, the attachment of the same short chemical side-chains to the main molecule which determines serological specificity.

The Se gene is unique in serological systems by virtue of the fact that it adds no information of its own, but rather serves to activate (with the X gene) those mechanisms responsible for the determination of ABH specific structures in mucus-producing cells.

The hereditary nature of the Sese system was first demonstrated by Schiff and Sasaki (1932b), who found that in a series of 50 families with 149 children, the ability to secrete water-soluble A and B substances was transmitted as a single Mendelian dominant, Se (then called "S!"). Its recessive allele, se ("s") resulted in the nonsecretor phenotype in the homozygote, sese. The secreted antigens were found to correspond specifically with those found on the red cell surface.

With the discovery that certain absorbed cattle sera would agglutinate O cells preferentially, it was observed by Schiff (1934) that the majority of persons belonging to group O were secreting a substance in their saliva which inhibited the agglutinating action of such sera. When it was further noted that some A and B individuals secreted not only A and B soluble substance but this "O" antigen as well, the interpretation first put forth (Thomsen, 1930) was that the substance represented the product of the O gene in heterozygous form (AO, BO). Irrefutable evidence that the so-called O substance is not in fact the specific product of Bernstein's O gene was derived from the observation (Sasaki, 1932) that individuals of group A_1B, who do not possess the O gene, nevertheless, if they carry the secretor gene, secrete the so-called O substance in addition to A and B. This observation led Morgan and Watkins in 1948 to suggest that this substance is the basic mucoid on which the A and B genes impress their specific chemical and serological structures. They suggested that henceforth it be referred to as "H" ("heterogenetic") substance.

By 1955, Watkins and Morgan, in reviewing the O (H) problem, could set forth several current theories concerning these antigens and their genetic determinants:

1. The ABO and Se genes control the production of A, B, O, and H antigens, both alcohol- and water-soluble forms. The O gene gives rise to a product on the O cell which is exclusive to the erythrocyte membrane and is not present in any secretion. This antigen alone will inhibit

"true anti-O" occurring in rare human donors, such as that discovered by Boorman, Dodd, and Gilbey (1948). Such anti-O's cannot be inhibited by the soluble antigen of secretions (H). Since both "O" and "H" substances are found on the group O erythrocyte surface, extracts of such cells (accomplished with organic solvents) will inhibit both anti-O and anti-H, the latter being the ordinary agglutinins from most plant, animal, and human sources.

2. The O gene gives rise to a number of partial specificities, and various anti-O sera are directed against one or the other of these structures rather than the whole O complex. Some writers also held that H may be similarly expressed in forms of varying specificity, and may arise independent of the action of the O gene.

3. O antigen can be produced not only by action of the gene O, but in varying amounts by a series of allelomorphic A and B genes (Hirszfeld, 1947); H substance could also presumably be produced by A, B, or O genes.

4. A and B genes impose their characteristic structures upon a basic mucoid, H, which produces variations in the degree of their effectiveness of transformation. Thus a series of substances corresponding to the A and B (and subgroup) specificities result. Since O is an amorph rather than a simple "recessive," there is no comparable transformation upon the cell surface. Sera which Boorman, Dodd, and Gilbey as well as others held to be detecting the product of the O gene are rather detecting one or several of a number of unrelated antigens on the cell

surface, or one of the several specificities of H, including the unconverted substrate.

5. The H character is determined by an allelic system Hh, genetically independent of the ABO locus. It controls the manufacture and deposition of H in secretions as well as its appearance upon erythrocytes. The Se gene controls only A/B substances in secretions, and is probably closely but not absolutely linked to the Hh locus (Watkins and Morgan, 1955).

The above list is not exhaustive, but indicates the main viewpoints of respectable serological opinion. At present writing, six years later, a molecular borderline between O and H specificities has not yet been clearly enough drawn to permit the structuring of genetic models; that O is indeed an amorph seems increasingly more probable, and the existence of a true O substance less so. The present writer holds with those who believe that a concept of molecular heterogeneity of various H and anti-H specificities is sufficient to account for serological data. The precise nature of these specificities will be discussed below.

The original concept of the secretor phenotype (like that of PTC "taster," Sutton et al., 1962) as determined by a single pair of allelomorphs exhibiting simple dominance and recessivity is apparently due both refinement and qualification, according to several workers. During the last few years, Levine et al. (1955), McNeil and Kreutzer (1955), Clarke et al. (1960), Morganti (1960b), and Matsunaga (1959a) have suggested various modifications of the genetics of the secretor systems.

These may be summarized as follows by way of introduction; some revisions will be reviewed more intensively below:

1. Addition of Xx system of Levine:

In 1955, Levine et al. published the pedigree of a family demonstrating the Bombay phenotype, the propositus having no A, B, or H antigens expressed on her erythrocytes or in secretions, yet transmitting to her offspring both the B and the Se genes. The developmental pattern was interpreted as a metabolic block, resulting from the homozygote xx, acting epistatically at the developmental level at which the Se gene (with the X gene) transforms the basic mucoid precursor into H. The lack of H in turn prevents the further formation of B substance. This explanation was anticipated by Ceppellini as early as 1952, in discussing the original Bombay phenotype.

2. McNeil and Kreutzer (1955):

(a) The same year that Morgan and Watkins proposed an Hh secretor system linked to the Sese locus, McNeil and Kreutzer independently set forth a similar revised system, with Hh governing H in secretions, ABO the erythrocytic antigens.

(b) As a result of his studies on abnormal ratios of A/B:H secreted antigens, McNeil explains antigenic discrepancies as indicating genetic combinations such as would result from allelomorphs (now renamed $Se^{H}se^{H}$, and $Se^{A}se^{A}$ or $Se^{B}se^{B}$, etc.) at two loci, heterologously paired (e.g., $Se^{H}Se^{H}/se^{A}se^{A}$ or $Se^{H}Se^{H}/Se^{A}se^{A}$).

3. Clarke, McConnell, and Sheppard (1960) noted that the frequency distribution of amounts of antigenic secretion results in an almost Gaussian curve. McNeil's "aberrant" category is interpreted as composed of extremes of their unimodal distribution. They agree that the inherited component of the variance in antigenic secretion appears to be polygenic, but not necessarily as described by McNeil.

4. Morganti, in 1959, reported the discovery of sufficient numbers of exceptions to expectations ("paradoxical" secretions) in antigens in saliva and milk, as to render invalid the classical phenotypic dichotomy of secretor-nonsecretor. This dichotomy they denote a "conventional abstraction," founded upon arbitrary criteria.

The same year Lawler, also studying saliva and milk, interpreted her findings as in no way invalidating the hypothesis of an inherited system determined by the alleles Sese, although irregularities of a different sort were noted.

5. Matsunaga (1959a) interpreted variations in the amount of H secreted as a result of the incomplete dominance of the Se gene over its se allele, resulting in diminished secretory function in the heterozygote.

The writings of McNeil et al. (1957a, b, 1960) on "aberrant" salivary secretion will be discussed in some detail, since the work has aroused considerable interest. For the past several years, these authors have been quantifying relative amounts of antigens secreted in both normal individuals and those who have suffered spontaneous abortion. It has

been the contention of these writers that a high degree of correlation exists between the incidence of "aberrant" secretion (that is, abnormal ratios of amount of A/B substance secreted : amount of H substance secreted) in parents, and the incidence of abortion (miscarriage).

In 1957, they enumerated 34 examples of blood group "conflicts" among an aborter group of 47 selected couples. Either aberrant secretion or heterospecific blood group (usually mother group O, father group A or B) may be defined as conflict. Frequently both conditions occur together. In a series of 23 control couples, only two were categorized as incompatible or in conflict, by virtue of heterospecific blood group (and these were unusual examples: a B mother and A father, and an O mother and A_2 father, the latter a combination which has never been reported to produce an erythroblastotic child). A complete breakdown of A, B, and H secretion titers is given for the 70 couples studied, as well as the immune titers of α and β antibodies.

Their total of 20 aberrant secretors out of the 47 aborters presents an impressive figure, especially in view of the fact that none of the 23 control couples exhibit salivary aberrancy. Since either parent apparently may manifest the aberrancy, causal factors are imputed to be genetic and not immunological, for each parent plays a different immunoserological role. There was no case reported in which both parents secreted aberrantly, in view of which McNeil suggests (1957b: 478) "that aberrant secretion per se causes the conflict" leading to abortion. No theory is

offered as to the precise mechanism of fetal involvement, nor is any attempt made to determine the blood group or secretor status of the fetus, although several techniques are available (e.g., direct ABO typing of erythrocytes bled from the heart, or mixed cell agglutination of epithelial cells by the Coombs method; meconium testing for secretor after the fifth month).

In order to account for unequal secretion levels of antigen, the authors propose a new genetic system, composed of two sets of alleles, at two loci, one of which determines the secretion of A/B antigens, the other set, H antigen. In a table, observed secretory incompatibilities are related to proposed genotypes. The authors suggest that secretors homozygous for the Se^H gene will, for instance, secrete more H substance than heterozygous ($Se^H se^H$) persons, and will demonstrate an aberrancy if the other loci (say Se^A) carries the $Se^A se^A$ or $se^A se^A$ genotype. Imbalance of genetic endowment thus results in antigenic imbalance. Phenotypic manifestations of these proposed heritable determinants were not checked by pedigree studies.

Since only one substance (H, as detected by their Lotus extract) could be detected in Group O secretors, it has been impossible to determine their status as aberrant or normal secretors. The authors predict the discovery of a second factor in O secretions by which aberrancy can be defined. It is not impossible that an investigation of two or three specificities of H (which appear to vary independently) could provide

TABLE 1

Proposed Genotypes and Secretory Incompatibilities

Number	Genotype	Secretion of H	Secretion of A
1	$Se^{H}Se^{H}/Se^{A}Se^{A}$	Secretor	Secretor
2	$Se^{H}Se^{H}/Se^{A}se^{A}$	Secretor	Partial
3	$Se^{H}Se^{H}/se^{A}se^{A}$*	Secretor	Nonsecretor
4	$Se^{H}se^{H}/Se^{A}Se^{A}$	Partial	Secretor
5	$Se^{H}se^{H}/Se^{A}se^{A}$	Partial	Partial
6	$Se^{H}se^{H}/se^{A}se^{A}$*	Partial	Nonsecretor
7	$se^{H}se^{H}/Se^{A}Se^{A}$*	Nonsecretor	Secretor
8	$se^{H}se^{H}/Se^{A}se^{A}$*	Nonsecretor	Partial
9	$se^{H}se^{H}/se^{A}se^{A}$	Nonsecretor	Nonsecretor

*These genotypes are aberrant secretors. Source: McNeil et al., 1957b: 477.

their awaited factors; the meaning of such observed variations is another question.

Of the many further criticisms which might be made, five appear most relevant here:

1. Reagent

Their single anti-H reagent, extract of the seeds of Lotus Tetragonolobus, exhibits reaction-patterns which differ from most other anti-H reagents (see below). Demonstrably, a number of minor serological specificities exist, and all inhibition testing should ideally be

carried out with at least one reagent from each of three defineable species of anti-H. The use of Lotus anti-H (as well as eel serum, of the same anti-H specificity) has been criticized for its lack of affinity for H substance by many workers, quoted in Section IV.

2. Comparability

In 1943, Wiener and Belkin wrote: "Naturally, it is never possible to compare the titers of the group-substances of salivas of different blood groups, just as it is not possible to compare directly the agglutinins of A and B sera." (p. 468)

Seventeen years later, in 1960, Clarke, McConnell and Sheppard cautioned: "No comparison can be made between the mean scores of H, A, or B, since these are arbitrary, depending upon the antibody used." (p. 296)

These strictures were obviously not attended by McNeil, whose theory rests directly upon such comparisons.

3. Use of Coomb's anti-human globulin test to determine α and β immune antibodies

McNeil (1957b: 472) states, "In instances of ABO conflict, almost all of the persons had high titers of such [immune] antibodies. Coombs titers were included in the studies on this group in order to confirm an immune type of response. Immune antibodies were not found in the control group simply because ABO-conflicts rarely occurred."

This passage is unique in serological literature in its claim that

immune α and β antibodies can be reliably detected by use of the Coombs anti-human globulin reaction. The value of this test for indicating the presence of the antibodies of the Rh, Kell, Duffy, S, Diego, and other systems is well known. However, as concerns the antibodies of the ABO system, the following quotations are typical:

Reepmaker and van Loghem (1954: 153): "No satisfactory explanation can yet be given for the fact that the anti-globulin test is nearly always negative, even in cases with typical [erythroblastotic] hemolytic anemia."

Kochwa et al. (1961: 874): " . . . there has been no method thus far to study such [ABO] antibodies in unselected pregnancies in advance of delivery."

McDuffie and Kabat (1956: 69): "Results indicate that no proper distinction can be made between such [ABO] antibodies on the basis of their being 'natural' or immune [by Coombs test]."

Further, the presence of α immune antibody, the most common offender in ABO erythroblastosis, has been frequently reported in normal persons. Reepmaker et al. (1954:159) noted "the great prevalence of α hemolysins in normal subjects due to vaccines used in innoculation."

Zuelzer and Kaplan (1954a: 178) note: "In both homospecific pregnancy and heterospecific pregnancy, the maternal serum occasionally exhibits immune properties against A. . . . in all cases the nature of the antigenic stimulus was obscure."

In view of such findings, it is hard to accept the absolute correlation

of blood group conflicts with immune antibodies, especially as detected by the anti-globulin technique.

4. Irregularity of secretion-pattern from hour to hour and day to day

Most investigators of secretor phenomena have assumed a relative constancy of secretion rate at different times and under varying conditions. In regard to some individuals such confidence is apparently not misplaced. The writer, however, tested the secretion levels of eight individuals repeatedly over a period of five months in the course of another project, with the following results:

(a) Six of the eight secretors demonstrated H as well as A/B variations in secretion to an average of 1-2 tubes, with a maximum variation of three tubes' inhibition titer in either or both antigens. There was a greater tendency to vary at different hours during the day than on different days, although a full year cycle could not be completed. Altogether, 38 testings were made on each individual, at various hours of the day, and under varying conditions. Two antigens were noted to vary independently in the same subject.

(b) Two individuals, both women of approximately thirty years of age and both of group A_1, demonstrated the most intense fluctuations. The H antigen output of subject AW varied as much as 4 tubes between an early morning peak and a mid-afternoon plateau upon four consecutive days; the H antigen of subject VR varied from day to day by from 3 to 4 tubes. In both cases the A antigen remained relatively stable, more so

in the first case than in the second (1-2 tube variations only, within limits of technical error).

(c) Variations in H antigen level were found to be 1-2 and occasionally 3 tubes less by use of Ulex extract than by the use of Lotus. Such differences could be ascribed to one or both of two causes, first, the tendency of Lotus to maintain a higher inhibition threshold, and secondly, the possibility of variation of one species of H in the presence of a more stable species. In either event, it is evident that these subjects ranged from aberrant to normal secretion-patterns in the course of a day, if secretion is defined by use of Lotus anti-H.[1]

5. Genetic determinants

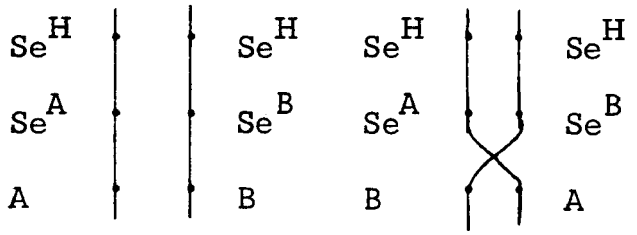
According to the genetic model of Ceppellini (1959) as well as that of Watkins (1959), the existence of a gene Se^A poses an ontogenetic impossibility, since the gene determining secretion per se and those determining A/B specificity function on different developmental levels. The Se gene accordingly activates the X gene to synthesize mucoid H, which is only later transformed to A substance under the influence of the A (I^A) gene. The existence of the Bombay (O^h) phenotype, which carries the "xx" homozygote responsible for the suppression of the formation of H substance from the substrate, although the Se gene is present as proved by its transmission to offspring, clearly demonstrates that the

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[1]In correspondence, McNeil concurs tersely that "H seems to fluctuate in some people." He makes no further reference to the implications of such an admission for his conclusions.

secretor gene has no characteristic phenotypic effect of its own. In the event of the correctness of McNeil's genetic hypothesis, the O^h phenomenon could not be explained.

The presumed linkage of the $Se^{A/B}$, Se^H and ABO loci presents another difficulty. Assuming a group AB secretor individual with the genotype $Se^HSe^H/Se^ASe^B/AB$ according to McNeil, unless these genes were fixed by absolute linkage, meiotic irregularities such as crossing-over, translocations, inversions, etc., would shortly separate them as follows, effecting an individual, say secreting B substance, but with A substance on erythrocytes, and incapable of producing either the α or β antibody.



At left, genetic linkage according to McNeil is shown broken by cross-over.

That one worker (Morganti, 1960a, b, below) has reportedly found such instances in profusion, does not make them the more convincing.

In 1960, McNeil and his colleagues undertook analysis of a further series of secretors of group sA_1, A_2, A_1B, and A_2B phenotypes. The secretion titers of 120 subjects representing the four groups above were compared as to capacity for inhibiting so-called anti-A_1 and anti-A_2 fractions of anti-A (B) antiserum. Most A_2 secretions (30 out of 36) reacted much as did the 63 A_1 secretions. Six "irregular" A_2's and all 9 A_2B's, however, reportedly failed to secrete significant amounts of the A_1

fraction, that is, they appeared to be secreting a substance of specifically A_2 quality.

Although the technique used in inhibiting the agglutinins is not described, apparently residual anti-A, after exposure to A_2B saliva, was observed to agglutinate A_1 but not A_2 red cells. In explaining the apparent lack of A_1 antigen and resultant failure of inhibition, McNeil invokes a "suppressor" gene, analogous to xx or yy, which functions when B is the partner of the A_2 gene in the A_2B heterozygote.

A simpler explanation has been current in serological literature since Lattes and Cavazutti in 1924 partially absorbed anti-A with soluble antigen so that it was reactive with A_1 but not the antigenically weaker A_2 cells. This work was repeated and extended by numerous workers during the decades of the 30's and 40's, and will be discussed below.

The only well-verified qualitative A_1-A_2 distinctions may be made by the use of anti-A_1 antibody which may appear occasionally in the serum of an A_2 or A_2B, or "absorbed" antiserum (above), or by the use of Dolichos biflorus extract. There is no reference in serological literature to a specific anti-A_2; reagents differentiating A_2 cells do so by virtue of weaker endowments of A antigen, or by the stronger H component.

In 1958 and 1959, there appeared a comparative study of ABH

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[2]A survey of early work with α and α_1 agglutinins is given by A. S. Wiener in his book, Blood Groups and Transfusions, 3rd ed. (1946), Chapter XII, pp. 198-218.

substances secreted in saliva and milk, along with their genetic interpretations, by G. Morganti and his colleagues, of Milan.

Saliva and milk samples from 301 subjects were examined quantitatively for H and A/B antigens. Antisera employed were eel anti-H and human immune anti-A and anti-B. The authors reported:

1. A "fairly good" correlation between the amounts of identical antigens present in the two secretions, in single individuals, although a few subjects seemed to secrete in only milk or saliva.

2. A "significant" correlation usually demonstrable between the amount of H and the amount of A/B secreted, with several exceptions here also.

3. No sharp distinctions between secretors and nonsecretors, in so far as inhibition titers form a Gaussian curve.

The authors conclude that the distinction between secretor and nonsecretor is artificial and probably without genetic basis, as determined by their "more refined techniques." They report, rather, a continuous distribution of individuals secreting more or less blood group substances. Although they admit correlations between the specificities and quantities of substances in the two secretions investigated, they hold that classification of secretor depends upon the individual secretion and antigen examined.

From 1958 to 1960, Morganti, in a series of publications, announced his discovery of "paradoxical secretions" ("secretore paradosso"), that

is, evidence of antigenic substances in secretion which fail to correspond to the ABO classification of the subject.

Taking courage and direction from the publications of McNeil (1957b), as well as Buettner-Janusch et al. (1958), whom he quotes, Morganti presents what he believes to be further evidence of the ambiguity and error in the secretor categories.

Before considering the evidence, it may repay one to recall a quotation from Hartmann (1941), who, after studying thousands of secretions, wrote:

> . . . In no case was the group character — as expressed by the blood groups of the subject — found at variance with the specificity of the water soluble blood group antigens. . . . Nor has a disagreement between blood group and group character of the secretions ever been reported in the literature. So, if it exists in man, it must surely be of very rare occurrence.

Morganti reports 47 group O subjects who inhibit the β agglutinin, 150 O subjects of whom 114 show at least a one-tube inhibition of α agglutinin in both saliva and milk, 140 O subjects of whom 106 exhibit "both paradoxical A and/or B," and 131 of the same group of whom 77 exhibit "paradoxical A." In a comprehensive chart (1960a: 28), he demonstrates somewhat parallel findings of β inhibition in group A, and α inhibition in group B secretions. All subjects, selected in the interests of research on human milk, were newly-delivered obstetrical patients studied during their hospital confinement. In this sense, they may hardly be said to represent "normal" subjects, especially his series of group O

mothers, a large proportion of whom might have been defined by heterospecific pregnancies. Their serological configuration may be in some way related to their recent delivery, a suggestion which apparently did not occur to the author. There is no report of the blood group of any offspring, nor any attempt to determine whether or not "paradoxical secretion: can be related to hereditary factors, or factors of incompatibility.

A second criticism may be leveled against this investigation in its exclusive use of eel serum, which, like Lotus (see below), fails to register consistent levels of H secretion in contrast to other reagents. He further employed the serum of group O individuals for some of his α and β inhibition studies, apparently unaware of the considerable body of literature on the "dual specificity" of the anti-AB ($\alpha\beta$) agglutinin. Thus the presence of A substance could lower β as well as α titer, creating evidence for a "paradoxical secretion."

In 1960, Lawler also reported on her study of samples of milk and saliva from 117 parturient women. By the use of Ulex europaeus anti-H, she found she was able to discriminate reliably between secretors and nonsecretors using milk as well as saliva samples. She found, however, human A substance poorly expressed in milk from secretors of A_1, A_2, A_1B, and A_2B blood groups; B antigen was even less reliably secreted. However, there was no evidence of "paradoxical secretion" reported, and no contradictions of correlations between H substance occurring in milk and in saliva.[3]

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[3]It should be pointed out that the agglutinins of Lotus tetragonolobus

Clarke, McConnell, and Sheppard in 1960 reported the use of sib pair data in the study of secretor in the course of research on the association of quantity of salivary antigen with duodenal ulcer. No direct association was detected, but several inferences concerning the genetic control of the secretor phenomenon were drawn.

In all, 331 individuals, involving 151 sib series, were tested. The anti-H used was extract of Ulex europaeus seeds, in standardized solution. Standardized anti-A and anti-B were also employed.

Confusion due to the tendency of many nonsecretors to secrete minimal amounts of antigen was eliminated by adjusting antisera used in inhibition to the highest dilution which gave a good agglutination of the appropriate red cells after being mixed with an equal volume of non-secretor saliva.

Distribution of antigen titers of secretors was found to be unimodal and Gaussian upon using a suitable transformation, which indicated to the authors that much of the heritable component must be polygenically controlled.

In attempting to eliminate variables, and sort out extraneous sources of correlation, the effects of technique (day-to-day reagent fluctuation,

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(but not Ulex) extract may be inhibited by lactose, which is abundantly present in human milk; thus no accurate evaluation may be made by the use of Lotus anti-H (Watkins and Morgan, 1957). Milk sugars are also inhibitory to anti-Lewis agglutinins.

deterioration of antigen and antibody contingent upon storage conditions, etc.), regression of antigen output with age of subject, and various environmental factors were analyzed and eliminated. It was concluded that the factors determining quantity of antigen secreted must have a strong genetic component.

In discussing the work of Morganti, who, it may be recalled, liquidated the secretor system on the basis of his Gaussian curve for antigen-titer distribution, the authors point out that the Liverpool investigations showed an invariable gap of two and sometimes as many as six dilutions between secretor and nonsecretor inhibition titers, suggesting an antimode such as that originally reported by Hartmann (1941: 53). In 14 sese X sese matings, no secretors appeared among the 52 offspring, nor did any pedigree reveal exceptions to Mendelian expectations. There seems no reasonable doubt but that, although modifying genes may be influencing the amount of antigen secreted, two phenotypic groups are clearly drawn as the result of a single pair of allelomorphs.

Modifying genes which presumably might function in a nonsecretor environment, it appears to the present writer, could be expressed in the minimal amounts of antigen frequently detectable, and/or in the ubiquitous presence of tissue antigens in certain loci (see below).

Clarke reports a number of individuals who could be classified as McNeil's aberrant secretors; such cases represent the arbitrarily-chosen extremes of their continuous unimodal distribution of H:A/B ratios.

Clarke suggests that this character be more specifically defined as those whose A:H or B:H ratio falls outside a given number of standard deviations from the mean ratio of a control series.

Quantifications of H antigen in this study confirmed that group O people secrete more H substance than do group A people, and B individuals still less, with AB salivas being the least endowed. The authors suggest that there may be a difference between homozygotes AA and BB and heterozygotes AO and BO with respect to amount of H secreted.

Since Thomsen in 1932 claimed the ability to distinguish the A and B heterozygotes in erythrocytes by reagents now known as anti-H, there have been few serious attempts to pursue this distinction. Moreau's (1946) negative findings, using red cell samples of authenticated genotype with cattle sera, as well as investigations by Witebsky and Klenshoj (1941) have served to discourage workers from this line of research.

The best-known claim for detection of the O heterozygote was that of Boorman, Dodd, and Gilbey (1948), using their so-called anti-O serum, "Mrs. G." Because two known heterozygous AO parents (phenotypically of group A who produced group O offspring) tested similarly, it was assumed that among their four group A children, two genotypes could be differentiated by the intensity of erythrocyte reaction with the antiserum, one genotype reacting much as did the parents, the other less strongly. The actual genotype was not known. At the time of its discovery, the

antiserum was widely accepted, but confidence in its powers has lagged with the failure of other sera to exhibit a similar capacity for differentiation. The supply of "Mrs. G" was soon exhausted, and no comparable claim has been established since.

If the O gene is, in fact, an amorph, one would expect that phenotypic distinctions between genotypes would have, like Clarke's, a quantitative rather than a qualitative basis. The only plausible alternative would be the event of one species of H being preferentially deposited or transformed rather than another species, in which case the claim of Boorman, Dodd, and Gilbey might still be valid.

A few other claims of heterozygote determination have been offered. Bendich, Kabat, and Bezer in 1947 recovered antigens of dual specificity from the stomachs of heterozygous (but not homozygous) hogs; similar dualities have been reported for human AB antigens (see below), but not AA vs. AO, nor BB vs. BO. Koulumies (1950), working with extract of Cytisus sessilifolius, believed that it reacted with erythrocytes as did Boorman's "Mrs. G"; again there has been no confirmation.

Considering the well-known "dosage effect" in the Rh, P, MN, and other blood group systems, it appears possible that refinement of quantitative techniques will solve the problem of homozygosity vs. heterozygosity. Meanwhile, the varieties of H specificity demand further attention in relation to this question.

Clarke, McConnell, and Sheppard concluded:

1. That sib pair data suggest that the absolute amount of A, B, and H secreted by an individual may be in part inherited, as well as the A/B:H ratio.

2. Much of the inherited component of the variance in the antigen secreted appears to be polygenic.

3. In spite of polygenic involvement, the basic secretor-nonsecretor dichotomy as determined by a pair of allelomorphs remains a valid interpretation.

4. Heterozygote AO and BO individuals may secrete more H substance than their respective homozygotes, AA and BB.

Clarke's 1960 work on ABO genotype differentiation according to antigen output had been prefaced by Matsunaga's report of the preceding year, wherein the latter compared the secretory abilities of heterozygote individuals of Sese genotype with those of the dominant homozygote SeSe.

The distribution of inhibition titers of Sese children born of Se X sese matings was compared with that of Se children born of Se X Se matings where no sese offspring were produced. Appropriate calculations resulted in an average difference of antigen output between Sese and SeSe individuals of approximately 2.8 in terms of the inhibition titer of the saliva. Matsunaga concluded that the gene Se is incompletely dominant over its allele se. He suggested that further differences might be detected in other fluids, organ secretions, and tissues, and predicted diagnostic success with the development of more adequate techniques.

Considering Clarke's work along with Matsunaga's, one is here faced with two variables, and problems such as: how does an AO/SeSe genotype salivary secretion compare with, say, an AA/Sese secretion, confront us. The question of expression of genotype in quantity of antigen secretion is as yet far from resolved.

Since the secretor system is genetically and biosynthetically inextricably interrelated with both the ABO and Lewis systems, these three loci must be understood as a unit. Therefore at this point the Lewis system will be considered.

Three comprehensive interpretations of these interrelationships have been offered in recent years: that of Morgan and Watkins (1959), that of Ceppellini (1959), which agrees closely with the first, and that of Andresen (1961), which differs in several particulars.

In 1946, Mourant detected a new antibody (now anti-Le^a) in the serum of a Mrs. Lewis. This antibody agglutinated approximately 24 per cent of north European bloods, irrespective of ABO group.

Two years later, Andresen suggested that Le^a was a recessive Mendelian character, since both parents of a Le^a positive individual could react negatively. Tests on 464 English families yielded sufficient agreement with such an hypothesis to support his theory of recessive inheritance.

Andresen also, at that time, described another antiserum, anti-Le^b, which appeared to give reactions antithetical to those of anti-Le^a, providing that the cells belonged to groups O or A_2. Anti-Le^b gave weaker

or negative reactions with A_1 cells, and about 12 per cent of the samples did not react with either antiserum. Andresen believed the two reactions to result from two allelomorphic genes, and suggested that the A_1 gene could have an epistatic action on the expression of the Le^b gene.

Grubb, in 1948, demonstrated the close relationship between the Lewis system and Schiff's secretor system, reporting that in a group of 62 persons, all of whose red cells were agglutinated by anti-Le^a serum and were thus presumably of the genotype Le^a/Le^a, were ABH non-secretors, while individuals whose erythrocytes reacted with anti-Le^b only (Le a-b+) were ABH secretors. Those reacting with neither anti-serum were usually, but not always, ABH secretors.

At about the same time, Grubb, as well as Brendemoen, observed that the saliva of all persons of Le^a positive red cells also contained Le^a substance in large amounts. The saliva of most Le^a negative individuals also contained Le^a substance, but in smaller amounts. Grubb also reported that the saliva from all ABH secretors, including those of the phenotype Le(a-b-), contained a substance, Le^b, which inhibited the agglutinating action of anti-Le_b serum.

Several workers established independently that the sera, as well as the saliva, of Le^a positive persons contained the antigen Le^a, while sera of Le^a negative persons did not.

Andresen and Jordal, in 1949, reported an antibody X (anti-Le^X) which agglutinated all blood cells except those of the Le(a-b-) phenotype,

which were presumably of the genotype xx. The majority of British and American workers assumed anti-Le^X to be a mixture of anti-Le^a and anti-Le^b, a combination of exceptionally common occurrence. Andresen identified the Le^b gene with Schiff's S (Se) gene, and Le^a with the recessive s (se).

The following year, with W. T. J. Morgan, Grubb offered a more thorough exploration of the problem, using 212 persons, confirming the close relationship of the Lewis and secretor systems. Of Grubb's blood samples, 22.2 per cent were Le(a+) and all failed to secrete ABH substances. Le^a and Le^b antigens were found in the saliva of their appropriate genotypes, displaying the same general physical and chemical characters as the A, B, and H substances. Two individuals whose red cells typed Le(a-b-) and who were also nonsecretors of ABH were discovered; also one individual of the same Lewis status who secreted H but not Le^a or Le^b. It was suggested that if the Le^a and s genes are linked, the linkage is so close that they constitute one genetic system of Le^aLe^bSs.

By 1950, the reality of this "closely packed constellation" of genes seemed open to question to Morgan, who proposed two sets of allelomorphic genes, S and s, and Le^a, Le^b, and Le^c, which occupy contiguous loci, the Lewis genes being considered multiple allelomorphs.

In 1951, Grubb described the discrepancy between erythrocyte and secretion reactivity to anti-Lewis antisera. One thousand persons were

tested for saliva and cell status against a battery of anti-Lewis sera. Secretion of H substances was detected by inhibition of chicken anti-Shiga immune serum. His findings were reported as follows:

	Saliva	Erythrocytes
Le^a present	90.2%	20%
Le^b present	79.8%	74%
Both absent	1.5%	6%

As a further break-down on antigens in secretion, the following chart was offered:

ABH	Le^a	Le^b	Frequency
+	+	+	75.5%
+	+	-	0
+	-	+	8.3%
+	-	-	0.2%
-	+	+	0
-	+	-	18.7%
-	-	+	0
-	-	-	1.3%

Source: Grubb (1951: 9)

The observation that Lewis substances frequently appear in saliva when they are undetectable on erythrocytes, Grubb attributed partly to the technical difficulties of weak antiserum. The significant suppression of the character Le^b on red cells by the A_1 gene was not observed in saliva.

In 998 cases in 1000 the presence of ABH in secretions coincided with the presence of Le^b substance; the two exceptions secreted ABH but no Le^b. When he then found that secretors of ABH were in fact able to inhibit his anti-Le^b serum, Grubb concluded that the genes Le^b and Se are identical, the Le^b substance being a product of the Se gene. The three functions of the Se (Le^b) gene could thus be summarized:

1. Determination of the secretion of ABH substances
2. Cause the formation of Le^b secreted substance
3. Produce, with the Le^a gene, the Le^a antigen upon erythrocytes.

However, since the close association of ABH with Le^b substances in secretion was not absolute, he suggested that two genes exist which have a secretor effect on the ABH system: one (above) identical with Le^b, the other rare.

It appeared to Grubb that if Le^a and Le^b are indeed allelomorphic, as Andresen held, then Le^b/Le^b individuals, with a frequency of approximately 20 per cent, should not exhibit Le^a substance. However, Le^a substance is present in more than 10 per cent of these cases tested; further, most persons of Le(a-b+) erythrocyte reactivity are secretors of Le^a substance. Therefore he concluded that Le^a and Le^b are not allelomorphic. He also doubted that the Lewis system could be described in terms of three or more allelic genes (Morgan's Le^a, Le^b, Le^c, or Andresen's Le^a, Le^b, Le^x), but suggested rather two pairs, occupying loci not necessarily linked.

In 1952, Jordal and Lyndrup demonstrated that all red cells of newborns type Le(a-b-); however, 90 per cent show Le(X+), the adult distribution, thus differentiating these antisera in at least one instance.

Ceppellini, in 1955, reported his agreement with Grubb's interpretation of the Lewis locus as having two effects: one on saliva status and one on erythrocyte status, with Le^a expressing itself only in ABH nonsecretors on red cells. Using data from 84 families, he supported Grubb's hypothesis with one modification: that the Le^b substance represents a product of interaction between the Se and Le^a genes, rather than a product of the Se (Le^b) gene per se.

Ceppellini summarized his conclusions as follows:

1. It is possible to make a satisfactory serological distinction between two phenotypes: secretion of Le^a in saliva and nonsecretion of Le^a.

2. The statistical study of segregation in families fits the hypothesis of a single pair of alleles (referred to as Le, controlling production of Le^a, and its recessive allele, le).

3. A contingency table[4] between ABO secretor status and the Lewis-saliva phenotype sustained the hypothesis of independence, and excluded the possibility of linkage.

4. If Le^a substance is found in saliva, red cells are usually, but not always, Le(a+) among ABH nonsecretors. These exceptions, noted

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[4]Contingency table devised by Dr. C.A. Smith, demonstrated by Ceppellini, Vth International Congress of the Society of Blood Transfusion, Proceeding, 1955, p. 209.

also by Grubb, were thought to be due to weak antisera or an incomplete penetrance of the character with respect to erythrocytes.

5. ABH secretor status inhibits the presence of Le^a antigen on erythrocytes; in saliva a quantitative diminution of Le^a is seen, so that ABH secretors show an anti-Le^a inhibiting titer of four times less than nonsecretors.

It has become increasingly evident during the last decade of study that Lewis antigens can be understood only as soluble antigens belonging primarily to the secretions and serum and only secondarily as adsorbed erythrocyte antigens. Experience with the J factor in cattle and the R factor in sheep, as well as the ease with which Le^a substance is observed to wash off red cells and the change of Lewis phenotype in infancy, all suggested that the Lewis antigens on red cells could be derived from soluble serum antigens.

In 1955, Sneath and Sneath reported that when a Le(a-b+) patient received a transfusion of Le(a+b-) blood, his erythrocytes subsequently gave a Le(a+b+) typing, having acquired the Le^a antigen. <u>In vitro</u> experiments reaffirmed their interpretation; red cells having either Le^a or Le^b negative phenotypes easily acquire the antigens when suspended in plasma specific for either type.

Mäkelä and Mäkelä in the following year confirmed their findings for the Le^b antigen. They were, however, unable to effect the transformation with saliva containing the Le^b substance. In the process of transforming

A_1 Le(b-) cells to Le(b+) in Le(b+) plasma, they demonstrated that the epistasis between Le^b and A_1 is due to a lack of Le^b substance in the plasma of A_1 persons rather than to any reluctance of the cells to accept the Le^b antigen. They also reported that the plasma of newborns was unable to transform red cell reactions, although cord cells could be rendered Lewis positive by exposure to adult plasma.

Recently (1960) Levine and Celano demonstrated that it is also not possible to transform Le(a-) erythrocytes to Le(a+) by Le^a substance from saliva. This conversion, however, could be achieved by using the tanned red cell technique of Boyden (1951) on Le(a-) red cells, and then subjecting the treated cells to the antigen. These converted cells were then used to immunize rabbits, who produced typical anti-Le^a antibodies.

The last years have seen further clarifications of the Lewis and related systems, largely according to the interpretations of Ceppellini. These theories follow, in the main, the lines laid down by Grubb.

1. Le^b: Ceppellini was first impressed by the fact that Le(b+) red cells were invariably accompanied by the presence of both the Le^a (Le, L^r) and Se genes, and their respective secretions. It was proposed that Le and Se+X act upon and compete for the same basic substrate. Their joint action upon it produces Le^b, a secondary configuration arising from the insertion of both Le^a and H specificities upon the same molecule.

The confusing observation that Le^b apparently can be found in the saliva of Le(a-b-) individuals was resolved by the discovery in 1950 by

Brendemoen who reported an anti-Le^b of somewhat different specificity which was later designated as anti-Le^b2 by Andresen, and anti-Le^bL by Ceppellini. This antiserum cannot be inhibited by Le^b substance in the saliva of ABH secretors of red cell phenotype Le(a-b-); however, it is inhibited by the saliva of some group Le(a+b-) persons. In contrast to this reaction, the other Lewis species of antibody (Andresen's anti-Le^b, Le^bH of Ceppellini) can be inhibited by the former salivas. These two antisera give an identical pattern of reactivity against red cells, differing only in ability to be inhibited by soluble substance. Anti-Le^bH presumably cross-reacts with soluble H; anti-Le^bL does not. Neither reacts with erythrocyte H, an observation which supports the presumption that soluble H and erythrocyte H are not identical.

2. Three-allele vs. two-allele concepts: The Danish group of investigators, along with others, conceived originally of Lewis as determined by alleles Le^a and Le^b, the former recessive and the latter dominant in their effects. These genes, however, cannot express themselves save in the presence of an independent gene X (Le^XLe^X, not to be confused with Levine's Xx "expressor-suppressor" genes which function with Se in the production of H). X produces an antigen recognized by anti-X. Many workers, including Ceppellini and Grubb, believe their anti-X to be anti-Le^a plus anti-Le^b, or, since all Le(a+) and Le(b+) blood samples react with it, that it probably corresponds to the cross-reacting portion of the antibody configuration which often persists in

absorbed anti-Le^a sera. Most American and British workers have accepted the two allele hypothesis: Lele (designated L'1' by Watkins and Morgan), with Le as the dominant Lewis secretor gene, producing Le^a antigen from the substrate.

3. Relation of the Se gene to ABO and Le: Recently, Jørgen Andersen (1958) reported the discovery of an irregular cold anti-A antibody in an A_2Le(a-b+) patient ("Magard"). This antibody appeared to detect a factor resulting from the interaction of the three gene systems Lewis, ABO, and Sese. In the absence of the Le gene, the Se gene appears to modify the A erythrocytic substance so as to react positively with the Magard antibody. In other words, all Magard positive individuals carry the Se and A genes, and give an erythrocyte typing of Le(a-b-); there were no exceptions to this configuration in a large number of individuals tested against the antiserum. The Se gene is thus seen not only to cooperate with the X gene in the production of H in mucus-producing cells, and to cooperate with Le in the production of Le^b specificity, but to modify the A specificity in group A, Le(a-b-) persons.

4. A model for the synthesis of soluble antigens: Watkins and Morgan (1959a), as well as Ceppellini (1959), have independently constructed models showing genetic and physiological pathways and interactions. The Ceppellini chart, slightly adapted, serves to plot developmental levels at which various genes operate in the formation of ABH and Lewis substances, according to his theory.

Pathways are largely self-explanatory. The genotypes lele, sese, OO, and xx are observed to set up blocks against the expression of Le, H, and A/B substances, respectively.

Both Le and X (+Se) can act upon, and perhaps compete for, the basic substrate, which is observed to yield H in the presence of the X and Se genes, and Le^a in the presence of Le.

The concerted action of both Le and X + Se results in Le^b specificity, which is interpreted as a secondary structure produced by the coincidence of Le^a and H configurations on the same molecule.

The Bombay (O_h) configuration will be seen to result from the suppression of A/B formation at the Se-X level by the homozygous xx suppressor effect, which inhibits the manufacture of H. Le^a has been secreted by all known Bombay individuals since they have carried the Le gene; the lele xx person who secretes only the untransformed substrate is anticipated by Ceppellini, but has not been observed as yet.

Evidence for the existence of a basic substrate has been based on studies by Kabat et al., in 1948, and by Watkins in 1956, who both found that A, B, H, and Le^a substances, after short, mild hydrolysis, yield fucose, after which further residues react strongly to horse anti-pneumococcus antibody of Type XIV. It is believed that the removal of the fucose serves to uncover common reactive structures. It is further concluded that specificity per se resides in the spatial integration of carbohydrates, which arrangements are effected by the influence of the respective genes on the orienting protein enzymes.

The fact that mucopolysaccharides (chemically very similar, albeit of different serological specificities) are present in the mucoid secretions of all individuals, respective of ABH secretor status, indicates that blood group genes must effect such a determination of specificity late in their synthesis. That these individual genes do not always act independently, but influence each other in the final formation of the mucopolysaccharide molecule, has been demonstrated by Watkins and Morgan (1959).

In spite of the fact that blood group antigens are reputedly inherited as monofactorial discontinuous Mendelian traits, the macromolecules, due to genetic interaction, frequently exhibit complex "polyvalent" structures.

In 1956, Morgan and Watkins reported that the salivary A and B antigens of the AB heterozygote appear to differ in their molecular structure, although not in serological specificity, from an artificial mixture of the secretions of A and B individuals. Precipitating agents for either A or B specificity are capable of removing not only their appropriate antigen from AB secretor saliva, but much of the other antigen as well, revealing a molecular species of dual specificity. When the resultant precipitate is redissolved, it is possible to recover both antigens. The same precipitating agents, exposed to artificial mixtures of secretions of A and B persons, remove antigens only of their single specificities. In the instance of the AB heterozygote, the allelic genes

appear to collaborate in producing the bivalent macromolecule of soluble antigen, which differs demonstrably from the product arising from either gene in the absence of the other.

During the following year, the same authors, working with cyst fluids and salivas of A_2 individuals, found that such persons produce molecules of dual A and H specificity, although free H molecules are recovered as well. The product was interpreted as demonstrating the lesser efficiency of the A_2 gene as compared to A_1 in its synthesis of A specific substance from its H substrate. Thus in A_2 individuals, more H character is retained in both the soluble secretions as well as on the erythrocytes.

It has further been demonstrated that the Lewis Le^a character, which is inherited independently of the ABO system, can also share macromolecular specificity with A, B, and H characters, exhibiting interallelic as well as intra-allelic interaction between blood group loci. Watkins in 1959 reported that preliminary experiments have supported the assumption that mucopolysaccharide molecules of multiple specificities can occur, such as, for example, could carry A, B, H, and Le^a specificities.

Brown, Glynn, and Holborow (1959) stated that they can distinguish the Le^a salivary substance of Le (a+b-) ABH nonsecretors from that of Le (a-b+) ABH secretors by means of precipitation with rabbit anti-Le^a. They suggest that secretor saliva with Le^a activity demonstrates a

macromolecule with many ABH receptors and fewer Le^a receptors, while in ABH nonsecretors, the molecule has more numerous Le^a surface groups and is thus more easily precipitated by the rabbit antibody.

It is generally accepted that the appearance of ABH and Le^a substances in the secretions depends on the operation of three independent gene systems: Lele (Ceppellini, 1959) or L'l' (Watkins, 1959), Sese (Ceppellini, Grubb) or $S^{t}s^{t}$ (Watkins), and ABO.

The genes L^{t}, S^{t}, A, and B are envisaged by Watkins as transforming genes, controlling certain stages in the conversion from the basic substrate into specific soluble antigens. Their alleles, l^{t}, s^{t}, and O are amorphic, and play no role in the conversions.

According to Watkins, the gene S^{t} in single or double dose controls the conversion of either precursor or Le^a substance to H substance, an incomplete transformation which may leave residual Le^a specificity on molecules which have also acquired H structures.

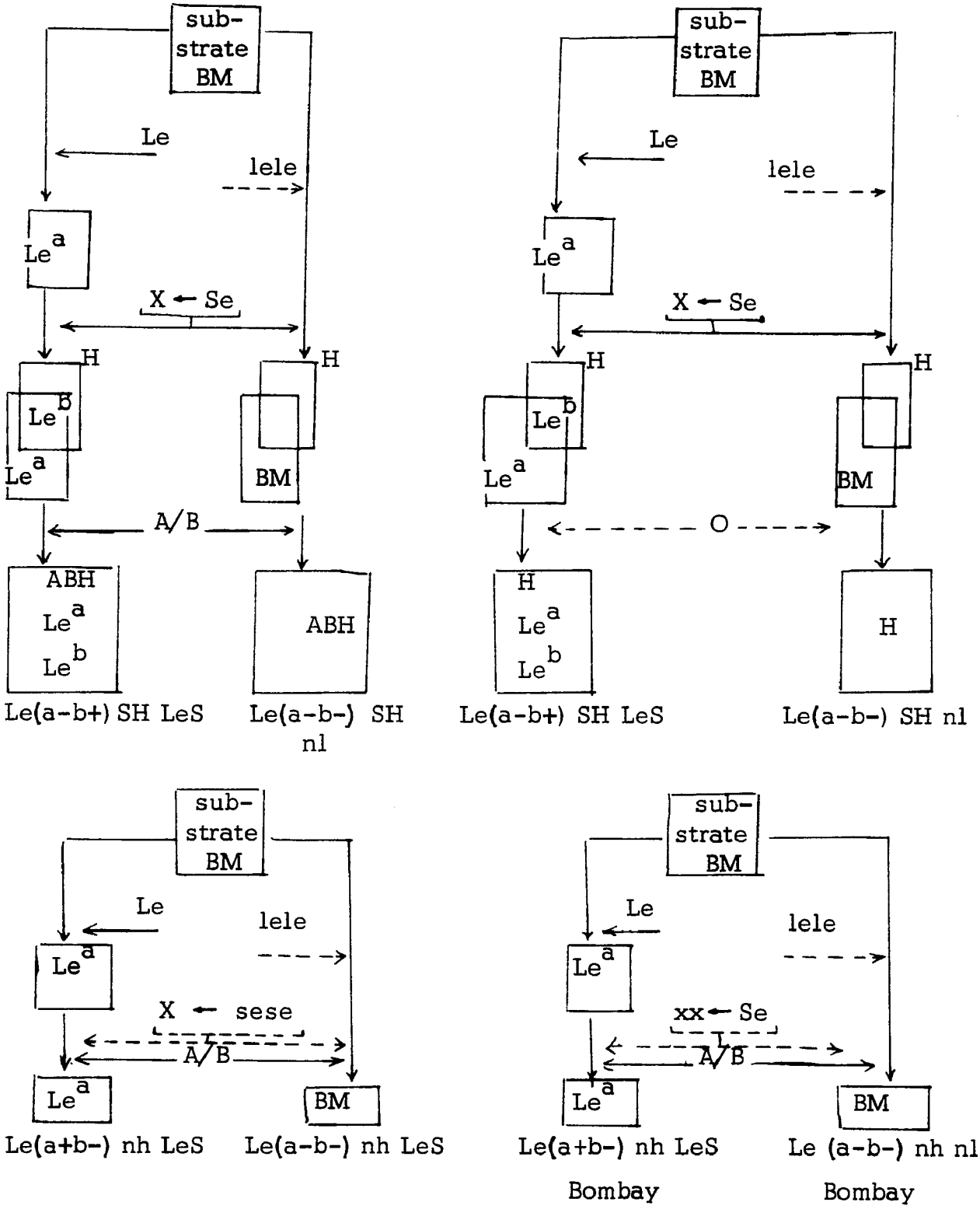
Because this scheme does not account for the appearance of H antigen on the erythrocytes of group O, nonsecretor individuals, it is necessary for Watkins to postulate an independent gene H, possessed by the great majority of people. The gene H is not suppressed by the $s^{t}s^{t}$ homozygote in erythropoetic tissues. Interaction between S^{t} and H in transforming mucopolysaccharide secretions provides a counterpart to Levine's (1955) interaction between Se and X, although less clearly delineated. Watkins hh, like Ceppellini's xx homozygote, results in the "Bombay"

phenotype, individuals who lack ABH antigens on their red cells and in secretions. All those cases so far investigated have secreted Le^a substance, but both investigators postulate the existence of a more rare phenotype carrying the l^1l^1 homozygote, and secreting only the basic unconverted substrate.

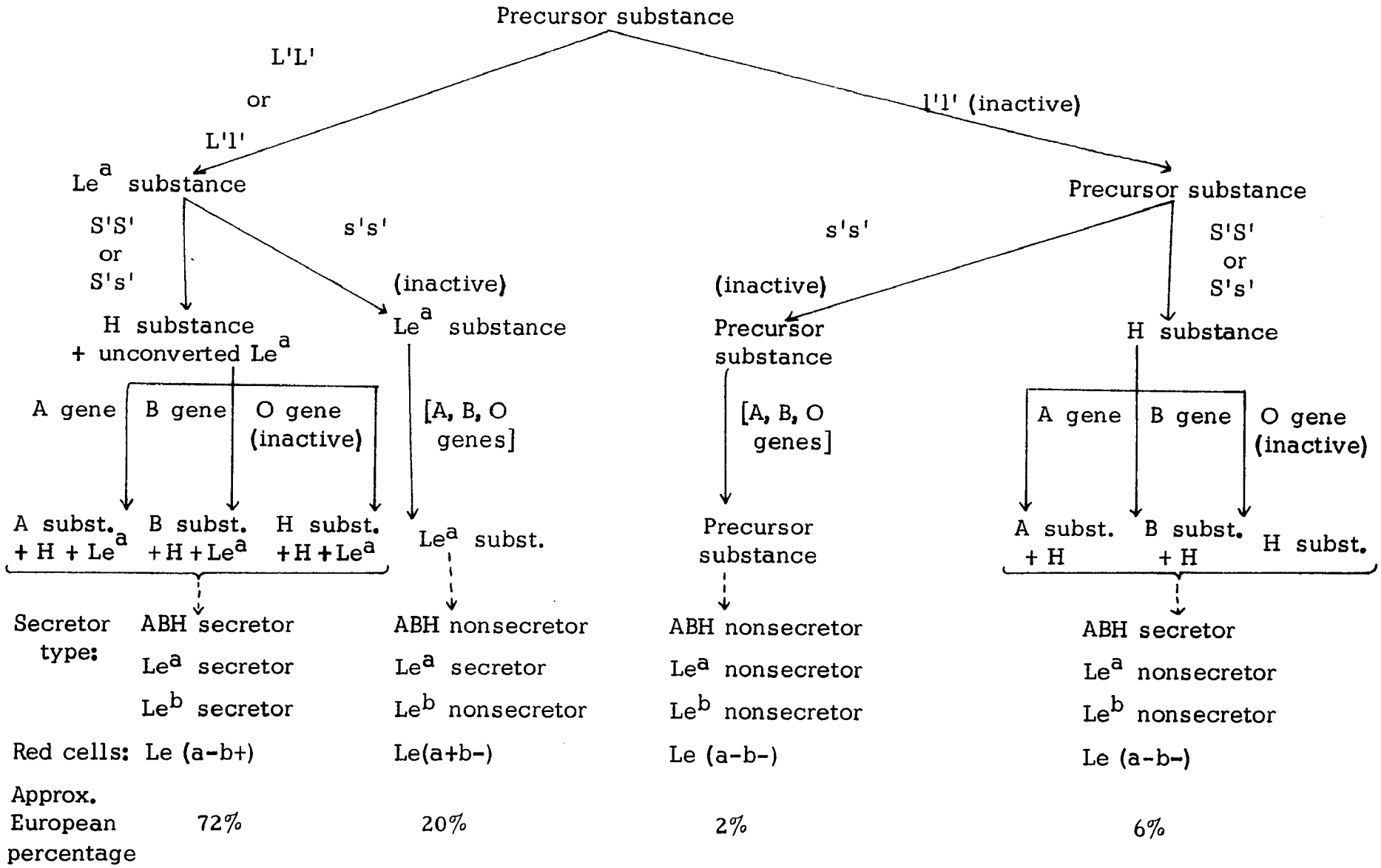
The sequences of transformation of both Watkins and Ceppellini are based upon a reverse order of the process of A and B degradation by enzymatic action (A/B to H to substrate, reacting with horse anti-pneumococcus antiserum of Type XIV). The Se gene, activating the X locus, necessarily functions at a pre-H level; if the sese homozygote constituted a post-H block, A/B nonsecretors would still secrete antigens of H specificity (Ceppellini, 1959: 248). If, however, the xx block functions at a pre-H level, it should be possible to identify the substrate at which the X gene acts. Since secretions of Bombay individuals are all very rich in Le^a substance, Le^a can be assumed as the substance upon which the Se - X collaborative unit works. In the lele genotype, apparently the Se - X unit transforms, instead, the basic substrate. Thus both Le and Se + X act upon and perhaps compete for the basic mucoid. Their joint action produces the Le^b specificity.

Andresen, in 1961, presented a developmental synthesis combining much of his original genetic interpretation with that of Ceppellini, and including Weiner's Y (1957) gene and Levine's (1955) X gene, as well as his own Le^X substance, now produced by the Le gene. His scheme

Scheme of the metabolic patterns which lead to the synthesis of salivary mucopolysaccharides, from R. Ceppellini, "Genetics of Human Blood Factors," Biochemistry of Human Genetics, 1959.



Scheme of Patterns, from Watkins and Morgan, "Possible Genetical Pathways for the Biosynthesis of Blood Group Mucopolysaccharides," Vox Sanguinis 4: 97, 1959.



purportedly accounts for polysaccharide structures in tissues as well as on erythrocytes and in secretions, which various processes he envisages as differing considerably, each from the others. It is outside the scope of this paper to detail his excessively complicated model, except to remark the main points of similarity and difference in relation to other theories, as concerns the Se system.

1. Water-soluble Le^a, Le^b, Le^b2, A, B, H^2, and the Magard factor are derived by complicated processes from one precursor substance, converted under the influence of the genes governing the various systems.

2. The model (presented in only fragmentary form) resembles those of Watkins and Ceppellini in the general derivation of ABH substances, except for a few modifications:

(a) Se and X genes do not function here as a synergistic unit; the Xx system of Levine maintains its position as the implement of transformation of H from the basic mucoid, while the Sese system is moved to a later developmental level, functioning after the formation of A and B antigens under the influence of those respective genes.

(b) The gene Le acts on the basic mucoid to form the Le^X receptor. Le^X is transformed by a series of complicated conversions in "cells" and "glands" to Le^a, and, under the added influence of the Se gene, achieves the various specificities of Le^b. There is no clear definition of the Le^a - ABH secretor relationship, and no explanation of the presence of Le^a in the saliva of ABH secretors.

There are several inaccuracies which are unaccountable, e.g., Andresen's statement (1961: 407): "Provided that ample Le^a substance is formed (partly in sese individuals and partly in babies in whom the secretor ability is not yet fully developed) . . . " Wiener and Belkin (1943) and many others, including the present writer, have remarked that ABH secretor ability is fully developed in the newborn, although antigenic endowment on erythrocytes may be variable.

Ceppellini is misquoted (Andresen, 1961: 405) as holding that "H may be formed in 3 ways: X is able to transform precursor substance, and S' will be able to transform both Le^a and precursor substance to H receptor." However, Ceppellini had previously clearly stated (1959: 248), "No specific products of either Se or se are known; in the writer's opinion, the dominant Se gene does not add any new information of its own to the blood group substances, but only activates also in the muciparous cells the genes responsible for making up of the ABH structures."

It is also of questionable validity, in the opinion of the present writer, to build a theoretic structure of general applicability around the Magard factor, an aberrancy produced in a terminal cancer patient, however interesting and informative may be its deviant specificity.

Since this work serves to becloud the clarity and consistency of the Ceppellini-Watkins models, adding little new and misconstruing existent data, it will not be referred to further.

CHAPTER III

SECRETOR SYSTEM AND NATURAL SELECTION

That there is an unmistakable, statistically significant correlation between the incidence of certain blood groups and pathologies has now been widely accepted, although not without an initial period of skepticism and controversy from many sources. The general biological, as well as genetical and anthropological interest in such associations is considerable, particularly in their bearing on problems of polymorphism ("balanced" and "transient"), selection, and genetic drift. There is good evidence that at least some human polymorphisms, such as ABO, secretor, and taster, are older than the species. From the immense and growing body of clinical and geographical data many further correlations may be expected, and more accurate assessments made of current hypotheses.

The strongest cases to date are those relating to the associations between duodenal ulcer and group O, and carcinoma of the stomach and group A. Data on secretor, technically one of the simplest characters to detect, has been surprisingly neglected, with only the several exceptions to be discussed below.

It has been pointed out by Roberts (1957) that those pathologies which have been correlated with blood group frequency are also those located in the upper gastro-intestinal tract, where maximum concentrations of ABH secretions are to be found. This observation has served to

activate much of the investigation undertaken by the Liverpool group.

Relations between secretor system and disease per se may involve three possible explanations:

1. Pliotropic effects of the Se or se genes, or linkage with genes influencing susceptibility.

2. Antibacterial action of mucopolysaccharide secretions, or protective action of such secretions on surface mucosa in situations of erosion, cellular destruction, ulceration, etc., or metabolic or hematopoietic advantage.

3. Immunologic reactions of complex nature taking place within the tissues or on tissue surfaces sometimes by cross-reaction, and perhaps involving reactions with soluble foods, pollens, bacterial substances (sheaths, toxins, hemolysins, etc.) or viral substances, or as-yet unspecified cellular complexes.

In 1956, Clarke and his colleagues presented data on 514 unrelated duodenal ulcer patients, revealing a significantly higher proportion of nonsecretors among these patients (35.0 per cent) than among 491 controls (24.2 per cent). The distinction is unrelated to sex, although association had been proved stronger between the disease and blood group O than other blood types. Sibship studies were undertaken in order to test for a correlation between the incidence of ulcer in secretors and nonsecretors. These investigations revealed a higher incidence of nonsecretors in duodenal ulcer patients than in their unaffected sibs.

Wallace et al., in 1958, obtained comparable data in Scotland, noting the high incidence of nonsecretors in all groups requiring surgery. Doll, two years later, reported almost identical results, his preliminary figures on stomal ulcer revealing an even higher proportion of nonsecretors than his figures on duodenal ulcer.

In 1959, Clarke and his Liverpool group reported that the secretor character as well as the gene O has a negligible effect on susceptibility to gastric ulceration, probably none at all. It seemed likely, in view of the marked differences noted as regards sex, social distribution, age of onset, and various precipitating factors, that the two types of ulceration have different genetic backgrounds, and predispositions may be inherited independently.

Several hypotheses have been put forward in attempts to explain the relationship between duodenal ulcer, nonsecretor status, and group O. These are roughly classifiable according to their mechanical or immunological bases, as suggested by Clarke.

1. Theories of mechanical protection:

Aird et al., in 1955, suggested that the high incidence of blood group O as well as nonsecretors in duodenal ulcer patients might relate to the lack of protection against ulcerogenic agents affecting the duodenal mucosa otherwise afforded by the A/B antigens in secretion. However, there is probably not much difference between individuals in the total output of secretion, since most ABH nonsecretors are secretors of Le^a

substance. It is hard to conceive of any manner in which substances so similar in chemical structure as A, B, H, and the Lewis substances could effect such diverse results except immunologically, or why, if A/B antigens prove efficacious on duodenal mucosa, they are not equally protective in the stomach.

Using the presence of fucose, a constituent of all blood group substances, as an index to the total amount of antigens present, Clarke found that most secretors and nonsecretors do, in fact, produce comparable amounts of soluble substance in saliva. Further, he found that the level of fucose concentration is similar in the saliva of duodenal ulcer patients and in the saliva of controls. There was also no significant difference between the mean salivary titer of A, B, and H substance in the secretions of duodenal ulcer patients compared to their unaffected sibs. Such findings argue against any theory of quantitative protection by blood group substances in ABH secretors.

An investigation now in progress (1961) by the Liverpool group on correlations between duodenal ulcer and the small percentage of persons who secrete neither ABH nor Lewis substances so far indicates a particular susceptibility in this phenotype.

Several workers have attempted to correlate susceptibility to ulceration due to the erosive effects of impaired gastric and duodenal digestive secretions with blood group frequency.

Køster (1955) suggested that group O individuals might have a higher

maximum acidity output (i.e., more parietal cells) than group A persons. Brown, however, found that in 276 patients with duodenal or gastric ulcers there was no correlation between maximum acid output, blood group, and ulceration of any particular site.

Sievers (1959), in investigating patients with impaired gastric secretion, found a correlation with blood group A rather than O. In O patients, 86.1 per cent produced free acid as compared to 73 per cent of group A patients.

Data from Sievers, arranged by Clarke (1961: 97):

	Blood Group			
	B	A	O	Totals
Acid secretors	29	111	167	307
"Achlorhydrics"	13	41	27	81
Total	42	152	194	388

The blood group distribution of the sample closely resembled those of a control group. Secretor status was not determined in the three studies cited above, giving a telling example of the neglect which is often accorded this factor.

2. Theories of immunological protection:

A variety of accumulating evidence, not yet clearly correlated, points to the probability of an immunological etiology for ulcer.

Since Witebsky and Neter in 1935 noted that secretor activity, which is strongly evident in the region of the stomach and duodenum,

decreases in the lower regions of the intestine, it has been reiterated many times. Such attenuation is probably the result of digestive activity. In any event, it is one consideration in locating sites of most active immunological reaction. It should be noted that correlations between blood group and disease are most clear-cut in these regions.

Clarke, in 1961, pointed out that several ulcerogenic drugs, such as steroids, aspirin, and phenylbutazone, are known to block antigen-antibody reactions.

In 1957, Cain published the attractive theory that the brush border of nonsecretor duodenal mucosa might be immunologically weakened or damaged by the ABH agglutinins ingested as food (peas, beans, lentils, etc.), while in secretors such activity would be inhibited. Apparently, however, lectins are inactivated by cooking and are seldom eaten raw, at least in this culture which boasts the world's monopoly on ulcers. The immunological effect of many foods customarily eaten raw is unknown.

Coombs et al. (1956), as well as Szulman (1960), Glynn and Holborow (1959), Högman (1959), and Selsnick have demonstrated ABH antigens in many types of epithelium, including duodenal mucosa. They have reported ABH antigenic endowment to vary considerably here in accordance with secretor status.

Szulman reported as follows, in his study employing **fluorescein**-conjugated antibodies:

Secretors: Intense staining of the mucus-producing goblet cells

of the villi is evident; staining of cells in the crypts of Lieberkühn and especially Brunner's glands is also noted.

Nonsecretors: The goblet cells of the villi are invariably free of antigen. In the crypts of Lieberkühn the goblet cells secrete variable amounts of antigen, with the most active cells in the lower half of the crypts. Antigen-laden mucus is also found at times in the Brunner's glands.

Also using fluorescein-labeled antibodies as well as Coomb's mixed cell agglutination method, Selsnick states that she finds ABH antigen sites on the striated border of epithelial cells in secretors, but only very rarely in nonsecretors.

Glynn and Holborow (1959: 151), investigating duodenal tissues by fluorescein-staining, noted that "the effects of the secretor status appeared to be confined to the specificity of blood group antigens in the superficial epithelium of both stomach and duodenum," while neck cells absorbed stain in both secretors and nonsecretors. H antigen was found to be more variable in its distribution, and less closely correlated with secretor phenotype, as might be expected.

In discussing the etiology of ulcer, Helmbold (1959) proposes the reverse situation from that which obtains in carcinogenesis (see below). Local abnormalities in cellular components stimulate the organism to produce auto-antibodies which erode and destroy surface tissues. Where such destructive antibodies are inhibited (as best evidenced in group A

secretors) the tissue regenerates; in nonsecretors and group O persons, presumably the antibodies continue their erosion unimpeded, and an ulcer forms.

Although this model probably presents gross oversimplification, in a general way it coincides with observed fact; there has not been, however, any trace of such "antibodies" which accomplish their destruction immunologically.

The problem of ulcer and its relation to serological phenomena can be solved only with a greater knowledge of tissue antigens, their nature, specificities, and reaction-patterns, and especially what differences obtain between those two entities known as "water-soluble" and "alcohol-soluble" antigens, respectively.

Cameron, in 1958, reported a strong association of both benign and malignant tumors of salivary gland tissue with blood group A, from a study of 341 case records.

Osborne and De George (1962), in view of Cameron's findings, have currently extended this investigation in order to check his correlation in an American population. They also hoped to determine whether differences could be ascertained between tumors of the parotid (an albuminous gland, and a poor secretor of mucopolysaccharides) and the submaxillary gland (a predominantly mucinous structure with rich antigenic output).

Analysis of 525 cases revealed that the excess of salivary tumors correlated with blood group A are those characterized by mucinous

secretions (mucoepidermoid tumors, adenolymphomas, and mixed tumors), while those of non-mucinous neoplasms (parotid adenomas) do not show significant blood group association. They conclude that the above associations relate to genetic constitutional differences in susceptibility which appear to involve mucus-producing elements. They note a possible correlation of blood group B as well as A with certain rare types of salivary malignancies.

They further remark that salivary gland enlargement observed in cases of malnutrition, as well as the prevalence of malignant malnutrition in southeast Asia accompanied by a high frequency of submaxillary gland tumors may be manifestations of a hitherto unsuspected relationship: that of mucus-secreting elements of glandular epithelium under nutritional stress, with ABH antigenic specificity.

Attempts to correlate secretor status and malignancy have otherwise not revealed significant findings.

A Liverpool series of 219 cases of carcinoma of the cervix revealed a normal secretor, as well as ABO, distribution.

However, in working with the anti-H agglutinin in an extract of a Laburnum species (L. watereri prokop), Speiser, Baumgarten, and Kaserer (1954) reported elevation in the levels of H substance secreted in the salivas of cancerous patients. An average titer of 1:5900 for salivary inhibition of the plant extract was reported for 16 afflicted group O persons; 19 healthy controls demonstrated an inhibition titer of

1:3000. Twelve group A patients averaged twice as high an inhibition titer as a control group. The site of malignancy was not specified. Even considering the smallness of the series, these figures indicate the necessity of investigating further the relationship between the secretor system and malignancy.

Two further studies should be mentioned in this connection. In 1959, Barber and Dunsford reported on a terminal carcinomatous patient whose serum contained enough blood group A substance to neutralize commercial anti-A before it could agglutinate her unwashed red cells. She also demonstrated a marked excess of H substance in her serum. The woman, who belonged to group A_2, was a secretor, afflicted with carcinoma of the cardia of the stomach. She died five days after admission.

Glynn, Holborow, and Johnson (1957) described a case of stomach cancer in which the malignant cells, stained specifically for A substance, revealed an unusual accumulation of that substance, more than had been demonstrated in any normal tissue.

These findings strongly suggest that the affected cells are either accumulating or manufacturing group specific substance; whether the cancer is stimulated by the excess of antigen, or whether the mucopolysaccharide overload collects as a result of the condition, is not, as yet, clear, but underlines Osborne's suspicion of a relationship between certain blood groups and susceptibility in mucoid tissues.

Helmbold (1959), in attempting to explain the association of group A with neoplasms in a variety of sites, offers a theory which is included here since it involves the immunological activity of soluble antigens. In brief, he accepts the immunological theory of carcinogenesis, as enunciated by Green (1959, 1961). Alterations in carcinomatous cells involve a disturbance of the "cellular antigenic complex," including blood group character, from its normal biochemical state. The organism reacts to the abnormal complex by elaborating antibodies capable of inhibiting growth or destroying such "cytotoxic" cells. According to the theory it appears that these antibodies carry a specificity directed against the abnormal A/B cellular component as well as protein components. The subject, having normal A/B antigens also present (in larger and more available stores in secretors) will inhibit the abnormal antibody complex, presumably by cross-reaction, thus inactivating his own protective mechanism. The development of the neoplasm thereafter continues unimpeded.

In order to account for the preponderance of group A individuals among carcinomatous patients, it must be assumed in the light of Helmbold's theory, either that the abnormal anti-A elaborated by the organism is less efficacious than anti-B in the destruction of malignancy, or that the neoplasm having an A-like component renders the production of abnormal anti-A more difficult in the environment of a group A host. A larger incidence of nonsecretors is assumed by Helmbold, who feels

that such phenotypes offer no interference to the protective antibody in the destruction of afflicted tissue. In individuals of group O and presumably of group B, he hypothecates a lack of inhibition of protective antibody, both B and H antigens reputedly failing in this function. As was discussed above, evidence points to a possible correlation of carcinoma with secretor phenotype, rather than nonsecretor, in view of which this theory is difficult to reconcile with what little evidence obtains.

In 1960, Jørgen Anderesen and Lauritzen reported their findings on a diabetic series of 992 individuals treated in Copenhagen. They observed a significant excess of group O patients among the males and females below age 30. After age 30, the ABO distribution did not show significant correlation. The excess of group O in all age groups was significant for men but not for women. This was explained by the fact that a higher percentage of females develop diabetes after 30 than males. These associations are in contradiction to those found by McConnell and Fraser Roberts in 1956, who reported evidence of an excess of group A in a British series. Craig and Want in 1955, in a Scottish study found, like Andersen, a group O excess. The possibility of different geographical areas fostering unequal sensitivities or diabetic "types" is possible, but as yet purely speculative.

Of interest to this dissertation is the highly significant correlation of nonsecretor status, Le(a+), in males as well as females, associated

with the disease (P = 0.0003, reported by Andersen and Lauritz). No explanation is offered for the association, nor does it clearly relate to any of the above theories.

As to the possible antibiotic effects of ABH secretions, we have two suggestions: in the work of Glynn, Glynn, and Holborow (1956, 1959), and in that of Dold (1957).

Glynn, Glynn, and Holborow, in 1956, reported investigations on streptococcal infections, rheumatic fever, and incidence of secretor status. Whether or not rheumatic fever is immunologically determined, a host factor, influencing the milieu of the streptococci during the period of throat infection, might well be a contributing factor in the development of the rheumatic fever involvement. Secretor status accordingly might prove one of the determining characteristics of the host.

A study of 253 patients with rheumatoid arthritis along with 460 healthy school children who served as controls, showed approximately 22 per cent nonsecretors in both groups. 450 patients with verified cases of rheumatic fever demonstrated a significantly higher percentage of nonsecretors (28.7 per cent). Since Fraser Roberts and Thomson (1934) and Wilson and Schweitzer (1954) have produced evidence in support of an hereditary factor in rheumatic fever, their findings could substantiate a concept of nonsecretor susceptibility, secretor status being also genetically determined. The authors suggest that during the streptococcal throat infection that precedes an attack of rheumatic fever, some

haptenic polysaccharide material in throat secretions might be adsorbed onto the microorganisms and thus rendered antigenic. According to this hypothesis, it seemed reasonable to expect a differential distribution between secretor status of persons with and without rheumatic fever. The correlation might relate to either secretors or nonsecretors, depending upon whether it was the ABH series of substances, or Lewis substances, to which the hypothetical hepten might be related and which became antigenic and, accordingly, protective.[1] There was also the possibility that the secretion of the hapten would be correlated not exclusively with one group or the other, but more with one than the other. If this were so, a weighting in one direction or the other (a situation which most resembles the observed distribution) might be anticipated.

It was further found that the significant preponderance of nonsecretors in rheumatic fever occurs principally in blood groups A and AB; the authors will not attempt an interpretation of this observation until the accumulation of further data.

Recent work by the same group, published in 1959, provisionally advanced the hypothesis that rheumatic fever can develop only in individuals of genotype Sese or sese. The hypothesis is as yet unsupported by detailed family studies. In such a case, the expected incidence of

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[1]The finding (Brown, Glynn, and Holborow, 1959) that there are two detectable Le^a specificities, one in ABH secretors and one in nonsecretors, may reinforce this hypothesis, insofar as the Sese genes will influence the molecular specificities of Le^a as well as ABH, and the proportion of surface of "polyvalent" macromolecules devoted to each specificity.

nonsecretors in a population with rheumatic fever would be 31.4 per cent, which is not significantly different from the observed figure of 29.1 per cent. On the basis of this hypothesis, the writers demonstrate that the incidence of rheumatic fever in the parents and sibs of the patients with the disease should not, on hereditary grounds, be more than 13 and 16 per cent, respectively, greater than in the general population.

In 1957, as well as in a series of previous publications, H. Dold reported studies on the antibiotic effects of gastric juice, saliva, milk, bile, urine, and other antigen-bearing exudates. He found that they produced inhibiting and modifying effects upon many microorganisms, largely pathogens. Agars mixed with such secretions and used as media resulted in attenuated growths and modified characteristics of bacterial colonies. Fresh tissue fluids and secretions of various laboratory animals as well as humans gave impressive indications of antibiotic action.

Unfortunately, there is no mention in his article of secretor-nonsecretor distinctions, and no consideration as to whether such factors could have influenced his observations.

It is beyond the scope of this dissertation to discuss the excessive ramifications of the problems relating to heterospecific pregnancy and ABO hemolytic disease of the newborn. However, that the secretor factor may play a significant role in the etiology of the disease, as well as endowing a selective advantage in withstanding its ravages, has been suggested in at least four connections:

1. The view that it is the fetal ABH secretions which effect the production of immune A/B antibodies in the mother rather than the leakage of erythrocytes into the maternal circulation. This sensitization may take place during gestation, or during the third stage of labor, or both.

2. The opinion that soluble antigens in the fetus impart a selective advantage in increased protection from invading antibodies by inhibiting them, or by protection of red cells by antigenic coating.

3. The possibility of greater hematopoietic activity and erythrocyte regeneration in secretors.

4. The possibility that a higher proportion of "aberrant" secretors who secrete abnormal ratios of A/B: H substances are found in families with high abortion rates.

The problem of ABO incompatibility has long been a vexed one, since Dienst as early as 1905 suggested that differences between maternal and fetal blood types might result in symptoms of toxemia in the event of accidental sensitization of the mother. Much more recently, with the existence of ABO erythroblastosis securely established, the secretor system has been investigated both as a factor in the etiology of ABO hemolytic disease, and as a protective mechanism.

Until the last few years, understanding of the phenomenon has been handicapped by a lack of precise information on the complex nature of antibodies ("natural" vs. "immune" proving an often contradictory and

blurred dichotomy) as well as by lack of studies relating to antigenic specificity.

Wiener et al. (1960), in a series of investigations on iso-immunization of group O volunteer donors, noted that soluble blood group substances proved antigenically more effective than erythrocytes.

In 1945, G. H. Smith published observations on 46 heterospecific pregnancies, of which 40 showed elevated maternal iso-antibody titers. All 6 who failed to exhibit increase in titer had nonsecretor infants, the other 40, secretors. Their findings were shortly confirmed by Jacobowicz and Bryce. Smith assumed (as did Sheppard as recently as 1959) that the antigenic stimulus is provided by the passage of soluble substances elaborated by the fetus, across the placenta during gestation.

In the early 1950's, the problem was reviewed in detail by Zuelzer and Kaplan, whose studies indicated that the stimulation of maternal-antibody titer takes place not during gestation, but rather during labor. Postpartum immune responses are elicited by fetal soluble antigens admitted into maternal circulation by absorption of amnionic fluid from the retroperitoneal space during the third stage of labor.

That these responses are limited to mothers of secretor infants substantiated Smith's conviction of the primacy of soluble antigens as the instrument of maternal sensitization. However, whereas Smith claimed that such responses occurred regularly in all secretors, Zuelzer and Kaplan pointed out that only in a minority of cases has the birth of

ABO incompatible offspring been followed by measurable antibody response. Observations further indicated to them that the relationship between maternal antibody response and infant secretor status holds not only for saline agglutinins, but for all types of antibody reactions investigated.

This explanation accounted for the rise in maternal antibody titer, but not for fetal involvement, particularly of the firstborn, incurred before any rise in maternal titer had been effected. Such occurrences have frequently been reported, and present one of the most striking distinctions between erythroblastosis due to Rh or Kell incompatibility and that due to ABO conflict.

Zuelzer and Kaplan envisaged the normal penetration of the placenta by both bivalent (saline) and univalent (reacting in macromolecular or colloid medium) antibodies independently, although the latter they felt to be more readily transferred, and more destructive. Since many so-called "normal" individuals carry immune α and β, this theory occasioned no contradiction. The absence of the antagonistic antibody in most cord sera in incompatible pregnancies indicates that such antibodies are inhibited in the fetus after crossing the placenta; thus effective destruction of maternal hemolysins by fetal tissue antigens is suggested as the chief mechanism of erythrocyte protection. The secretor status, which might be suspected of playing a major role in this connection, appears irrelevant to the investigators, since they claim that most individuals, irrespective of secretor status, produce sufficient A/B antigens in

tissues and organs for the purpose of protection. Zuelzer and Kaplan based this claim on their failure to demonstrate antagonistic antibodies in cord sera of nonsecretors as well as secretors, a failure which was not shared by other workers, such as Kochwa, Rosenfield, Tallal, and Wasserman (1961), or Wiener and his colleagues. The degree of effective injury accordingly is a function of the ratio of amount and titer of maternal antibody reaching the fetus to amount of fetal tissue antigens capable of absorbing it. Qualitative considerations are also present, such as the capacity of particular antigens for the inhibition of particular antibodies. In some instances, Zuelzer and Kaplan noted an increasing resistance to inhibition by Witebsky (A/B) substance of maternal hemolysins. They suggested that symptoms of erythroblastosis may be linked to the presence of these maternal antibodies with special immune characteristics, along with a "factor of individual susceptibility." They did not stress any cumulative rise in maternal titer with each successive birth, which would place a family of afflicted secretors at a serious disadvantage. Wiener (1960) however, noted that in firstborn erythroblastotic offspring the manifestations tend to be mild, while subsequent children born to a sensitized mother demonstrate more severe afflections engendered by immune antibodies. The clinical severity, Wiener reported, remained unchanged in 10 families, but seemed to increase in 20 which he observed.

In view of Zuelzer's conviction that "no evidence was found that the secretor property added to the degree of protection," it is interesting to

note that the combined figures on secretor-nonsecretor distribution among erythroblastotics from three studies present approximately a 10 per cent increase in secretor frequency above expectations:

	Se	sese	total
Zuelzer and Kaplan	25	3	28
Crawford, Cutbush, and Mollison	11	0	11
Wiener, Freda, Wexler, and Brancato	51	7	58
Total	87	10	97

From Levene and Rosenfield (1961), p. 134

Although the incidence of nonsecretors is lower than random distribution would warrant, it is Wiener's observation that more nonsecretor infants demonstrate high bilirubin levels, while his secretor infants were not jaundiced. This he explained on the basis that once a mother became sensitized, the large amounts of soluble antigens available to secretors would give them an advantage.

A major breakthrough in the problem of antibody determination was achieved in 1959 when Abelson and Rawson divided alpha and beta antibodies into two different physical forms, separable by cellulose ion exchange column chromatography:

1. a gamma-1 globulin, with a molecular weight of approximately 1,000,000. Under ultracentrifugation, a sedimentation constant of 19S was determined. Most so-called normal group A and B responses produce such 19S antibodies.

2. A gamma-2 globulin, with a molecular weight of approximately 160,000, and a sedimentation constant of 7S. These 7S antibodies are seldom produced by persons of type A or B, but are found as immune responses in type O sera.

The rarity of fetal impairment in pregnancies involving group B mothers with A children, and A mothers with B children had long argued for some distinctive property characterizing the antibodies of group O. Wiener and Wexler (1954) and Unger and Wiener (1954) stressed the importance in the etiology of ABO erythroblastosis of Wiener's "anti-C" cross-reacting $\alpha\beta$ antibody occurring in group O individuals. When 37 cord sera were tested, all with high titers of $\alpha\beta$ agglutinins, against individual A and B antigens, in only 8 were α and β agglutinins found to be separable.

In 1957, Haberman and Aguilar recovered such $\alpha\beta$ antibodies of dual specificity in eluates from erythrocytes of affected infants.

Fudenberg, Kunkel, and Franklin, in 1957, suggested that the placental barrier might be permeable to 7S but not 19S maternal antibodies, the decisive factor being molecular size.

Using the new technique of column chromatographic separation of serum proteins, Kochwa et al., in 1961, moved another step in the direction of a solution to the problem of the etiology of ABO incompatibility.

In contradiction to the claim of Zuelzer and Kaplan that most ABO isoagglutinins are capable of penetrating the placenta, but are rendered

harmless by fetal antigens, Kochwa et al. held that most ABO antibodies are incapable of reaching infant circulation, and thus play no role in the pathogenesis of erythroblastosis. In a large series of afflicted infants, they recovered only the 7S gamma-2 globulin fraction in cord sera, an antibody which could be duplicated in titer and identity in the respective maternal sera.

Out of 73 group O pregnancies, 53 demonstrated placenta-crossing 7S antibodies. Yet it is known that only 1 in 200 infants carries erythroblastotic symptoms. Further investigations led to the conclusion that overtly affected cases are those in which invading antibodies prove resistant to inhibition by blood group specific substances. In incompatible but unaffected infants, the antibody is inhibitable by soluble antigens and thus attenuated or destroyed.

The writers accept secretor advantage in ordinary protection against incompatible 7S antibodies. They further accept post-partum maternal sensitization by secretor infants as described by Zuelzer and Kaplan. They report an increasing severity of the disease with subsequent secretor births, as noted by Wiener, only occasionally, and do not consider the picture typical.

Two questions are thus left largely unanswered. One relates to the practical circumstances under which an uninhibitable 7S fraction may be manufactured by the maternal physiology. The other relates to the more theoretical aspect of the problem, that is, how, if it is the post-partum

sensitization by soluble antigens which stimulates the major maternal immune response, can these isoagglutinins then fail to be inhibitable by those very major structural groupings in response to which they are constructed? An answer based on "heterogeneity of combining sites" is not fully explanatory.

In conclusion, the secretor factor appears to play the dual role of effecting some maternal sensitization, and then supplementing fetal protection against errant antibodies in at least a majority of situations of incompatibility.

Studies apparently will have to consider the following factors:

1. Stimulation of maternal physiology; mode of sensitization;
2. Ability of mother to manufacture 7S antibodies;
3. Ability of antibodies to cross placental barrier
   a. placental permeability,
   b. molecular size of antibody;
4. Ability of antibodies to resist inhibition by A/B substances;
5. Amount of A/B substances provided by fetus (secretor status);
6. Ability of antibodies to combine with fetal erythrocytes and hematopoietic tissues;
7. Ability of the infant to deal with a hemolytic syndrome.

In 1958, Vos and Kirk made an exceptional (and largely ignored) contribution to an understanding of the mechanisms of protection functioning in ABO incompatibility. It is now recognized that while the secretor

character is fully developed, A and B erythrocytic antigens of the newborn are relatively weaker than those of adults. Nevertheless, there have been few attempts to explain these attenuated reactions in the greater proportion of cases, and particularly the frequent failure of erythrocytes to react to anti-A and anti-B agglutinins partially neutralized with A/B group specific (Witebsky) substance.

The writers found that if adult A_1 or B cells are strongly agglutinated by anti-A or anti-B sera, following which the agglutinates are broken up and washed repeatedly, clumping will reform spontaneously in saline. However, this agglutination can be inhibited if the sensitized washed cells are exposed to a solution of A/B substance from saliva or commercial sources. First the ability to agglutinate in saline disappears; upon further exposure, agglutination in serum or other macromolecular medium is also inhibited. Finally even the anti-globulin reaction becomes negative. If the explanation here were a mere removal of the antibody from cell receptors, after washing the erythrocytes would be indistinguishable from the original cells before sensitization. However, such cells, known as "fully protected," exhibit characteristic reactions unlike those of either normal "unprotected" or sensitized cells. Sensitized cells are known to be osmotically more fragile than unsensitized cells, but after "protection" they are found to be more resistant to hemolysis than are normal cells. The reactions of sensitized adult cells protected with A/B substance resemble in many respects those characteristic of cells of

erythroblastotic infants. Protected cells of group A_1 give barely trace reactions when exposed to potent partially-neutralized immune antiserum. In this respect they resemble A_2 cells; such reactions may contribute to the frequent observation that more cord erythrocytes type as A_2 than expectation would warrant.

A clear distinction between A/B antigen concentrations in sera of secretors and nonsecretors was noted by Hartmann (1941). The presence of soluble antigen was especially marked in the serum of group A persons when their inhibition of anti-A was indicated by employment of A_2 (rather than A_1) erythrocytes. Since soluble antigens are, however, present in the serum of nonsecretors as well as secretors, although in smaller amounts, it is possible that some protection might transpire here as well. It should be recalled that Wiener noted more severe hemolytic manifestations in nonsecretor newborns than in secretors. The functioning of this mechanism, along with the inhibition of antagonistic antibody by the same soluble substance, may help to account for the large majority of incompatible pregnancies in which no symptoms can be detected.

Subsequent findings by Vos and Kirk (1958) are reported to suggest that protection of the nature described above has been shown to take place in at least some incompatible infants of group O mothers.

In 1947, Gurevich, Polishuk, and Hermoni observed that the well-known "enhancing" action of serum media on incomplete (immune) antibodies could not be demonstrated when the serum of most infants under 6 months of age was used. In four out of 52 sera, however, enhancement was noted.

Currently, Constandoulakis and Kay report that fetal AB serum (or group A or B sera) when used as a medium for cells, effects a dramatic diminution of agglutination if the donor of the serum is a secretor; such diminution is far less marked if the donor is a nonsecretor. Sera from secretor adults do not effect this phenomenon. This effect may be noted by the use of either infant or adult cells, but is more marked in the case of the former.

It is possible that Gurevich's four exceptional sera belonged to non-secretors, in which case these two observations, made 15 years apart, may well refer to the same phenomenon.

Further, since the most obvious difference between nonsecretor and secretor sera lies in antigen content, both investigations may relate to Vos and Kirk's "protective" action, with resultant diminution of agglutination in the case of secretors. If so, we have further demonstration of protection against serological reaction in vivo in secretor infants, and the resultant advantage to such phenotypes.

In conclusion, it should be pointed out that the precise route of transfer of antibodies from maternal circulation to the fetus may occur directly through the placenta (as usually assumed) or, as Brambell and co-workers have suggested (1951), by way of the chorionic lumen into the amnionic fluid and thence to the fetal alimentary tract by swallowing. Brambell et al. demonstrated that such a transfer does, in fact, occur in the rabbit, and, further, antibodies have for some time been known to be

ingested and absorbed via the gut in a variety of newborn mammals. It is not impossible that 7S agglutinins may be directly absorbed in this manner by the human fetus, although no evidence for it exists as yet. Since soluble antigens occur in amnionic fluid, as well as in the alimentary canal, they would again function protectively insofar as the antibody was inhibitable by them, and perhaps even more effectively.

During the last decade, evidence has been accumulating that ABO incompatibility may function as a selective agent not only in its manifestation in hemolytic disease of the newborn, but may contribute to infertility at the level of preconception, or fertilization, or very early embryonic development.

Chung and Morton, in 1961, offered a statistical analysis of data on about 3000 families, collected by various workers. They reported an average reduction in fertility, as expressed by the number of children per family, in ABO incompatible matings, of 6.3% - 3.2%. This means the elimination of 9.4+ - 4.6% of incompatible zygotes, or 2 per cent over-all loss of all zygotes. At this level, such a loss would constitute a major selective force.

Matsunaga (1959b) summarized data on Japanese matings and resultant offspring as on the following page. Assuming that fertilization is the same in both compatible and incompatible matings, Matsunaga calculated the mortality rate of incompatible heterozygous children from all causes (abortion, stillbirth, infertility, erythroblastosis) as 21 per cent. This

Effect	Compatible	Incompatible	P
Abortions/pregnancy	10.34%	15.30%	<.001
Childless couples	9.8%	18.15%	<.001
Av. # living children/ per mother	2.6 ± 0.1	2.2 ± 0.1	<.001
Change of pregnancy resulting in living child	0.799	0.696	---

from Matsunaga (1959b: 410)

indicates an extremely powerful selection pressure due to ABO incompatibility.[2] The net effect of this selection is to decrease the frequency of the genes A and B and increase the frequency of the gene O.

The 5 per cent excess of abortions per pregnancy in incompatible matings indicated above represents late fetal death, and is probably due to isoimmunization. The effect appears more marked with number of offspring per family. Note that the number of childless couples who are compatibly mated totals only half the number of those incompatibly mated. These infertile couples may represent either failure of fertilization (sterility) or early fetal death.[3]

Gershowitz, Behrman, and Neel (1958) specified four factors or

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[2]Figures such as those above reveal that the incidence of fetal deaths and ABO hemolytic disease is considerably higher in Japanese than in United States populations, illustrating the importance of the genetic composition of populations. Thus, the frequency of incompatible matings for ABO and Rh in Japan is 44 per cent and 0.5 per cent, respectively, while in the United States the figures are 36 per cent and 13 per cent.

[3]This discussion is taken almost in entirety from W. H. Stone, class notes, 1962, unpublished.

aspects which demanded consideration in their investigation of selection at a preconception level. These aspects are as follows: (1) the existence of antigenic receptor sites on the sperm surface, (2) the possibility of sperm populations bearing different antigenic specificities in heterozygote donors, (3) the presence of soluble antigens in seminal fluid, and (4) agglutinating or inhibiting substances in uterine secretions.

Landsteiner and Levine (1926), as well as Yamakami (1926) and others, reported that washed spermatozoa contained A and B substances in accord with blood type. The antigens were evidenced by their ability to absorb anti-A/ anti-B specifically.

In 1957, Gullbring claimed not only to have identified sperm antigens by means of the technique of mixed cell agglutination, but to have identified two sperm populations in heterozygotes, each carrying the allelic antigenic expression of the haploid chromosome. These populations were also distinguishable by means of agglutination with commercial antisera.

Behrman and his colleagues repeated this work with similar results shortly after (1960); however, it has not been duplicated elsewhere.

Szulman, in 1960, failed in his attempt to stain spermatozoa with fluorescein-conjugated antibodies.

The presence of soluble antigens in the seminal fluid of secretors is well known; the finding has frequently been called into forensic service. In her comparative study of secretions, Hartmann noted that the seminal vesicle contributes a high level of A/B antigen.

Szulman found evidence in staining that the antigenic endowments of epididymus, seminal vesicle, and prostate vary markedly in secretors from those of nonsecretors. In all three glandular tissues, epithelial cells showing specific staining at the luminal tips are visible in sections from secretor subjects alone. Testicular tissue gave occasional feeble outlining of cell envelopes in the tubules, irrespective of secretor status.

Gershowitz, Behrman, and Neel (1958) reported evidence for the presence of anti-A and anti-B antibodies in uterine secretions. Of 35 group O women, 15 demonstrated antibodies; out of 30 group A and 5 group B women, only two gave such evidence. Secretions of 7 AB females failed to yield detectable agglutinins.

Recalling the 160,000 molecular weight of the 7S γ_2 antibodies found in group O incompatible maternal (and cord) sera by Kochwa et al. (1961), this discovery is not too surprising. One hopes that such antibodies may be tested for bivalent ($\alpha\beta$) specificity, as well as for inhibitability by group specific substances, in spite of the difficulties in technique which such minute amounts will entail.

If such antibodies function in selective impedence or immobilization of sperms, a mechanism for the preconception selection against group O, incompatibly-mated individuals may well be established.

Obviously, such a supposition implies secretor advantage in the ability of mates to inhibit such antibodies with the soluble antigens in seminal fluid and so protect sperm. The question may be again, as in

Kochwa's situations of erythroblastosis, one of the differential inhibitability of 7S antibodies by soluble antigens.

The impressiveness of the findings of the Michigan group (Behrman et al., 1960) is emphasized by their final figures: of 102 infertile couples, 87.3 per cent were found to be ABO incompatibly mated, as compared to 38.6 per cent in 171 fertile couples. There were 57 per cent nonsecretors in the infertile group, or well over twice expectancy. This is indeed selection with a vengeance! Unfortunately, we are not told whether males, females, or both manifested the high frequency of nonsecretion. Considering their different immunological roles, this would seem a serious omission.

An investigation by Cresseri and his colleagues (1958) may be appropriate to a consideration of the role of secretor in heterospecific pregnancy. Rats, made anemic by innoculations of phenylhydrazine, were fed a hog gastric mucin fraction with a high content of A and H blood group substances, together with vitamine B_{12}. The animals exhibited a specific hematological response of red cell replacement in comparison with rats receiving the B_{12} alone. Electrophoretically homogenous preparations of A and H substances also gave positive results.

Two possible explanations were suggested:

1. The action is mediated through a modification of the rat intestinal flora, perhaps related to Springer's Bifidus factor activity of blood group substances, or,

2. The effect is a direct one on rat tissue metabolism.

The inference is one of secretor advantage in situations of hematopoietic or hematologic stress, such as would obtain in the erythrocyte destruction of erythroblastosis foetalis.

The present writer knows of no statistics available on the percentage of infant recovery in relation to ABO blood group or secretor status.

Part II

TECHNIQUES IN THE USE OF PLANT AGGLUTININS

CHAPTER IV

MATERIALS AND METHODS

Since plant agglutinins (lectins) were the primary reagents used in this study, a short history of their adoption into modern serological procedure will preface this section.

Plant agglutinins have been known since 1888, when Stillmark discovered that ricin, a toxic substance extracted from the seeds of Ricinus communis, agglutinated the cells of some animals. Ricin and similar extracts were recognized by Ehrlich to be good antigens, and were extensively used in the early investigation of several important immunological problems, e.g., tolerance studies, antibody inhibition in vitro, local immunization, Danysz's phenomenon, and the reversibility of antigen-antibody reaction.

A complete historical résumé of the earlier work in this field has been given by Bird (1959), Krüpe (1956), and Mäkelä (1957).

Renkonen, in 1948, is credited with ushering in a new era in the investigation of plant agglutinins; almost simultaneously the first American work in this field was published by W. C. Boyd and R. M. Reguera (1949). Renkonen's series of plants investigated included 99 species, all of the family Leguminosae. Six of the seed extracts showed specific affinities for either A, or A_2 and O cells. By 1955 the total number of species studied was estimated at 1,000; at present writing, this number has probably been doubled.

There are approximately 50 species of plants which have been found to date to yield specific anti-H agglutinins from seed extracts. All belong to one or the other of the following genera: Virgilia, Ulex, Genista, Cytisus, Lotus, Laburnum, and Petteria.

Early investigations on plant agglutinins were carried out by use of saline extracts of the seeds, with red cells also suspended in physiological saline. Mäkelä (1957) and Krüpe (1956) both contend that the agglutination thus formed is identical with that of animal agglutination. The present writer would describe it rather, in strong reactions, as the formation of a tattered skin-like membrant, quite characteristic in appearance, often closely adherent to the tube wall, and differing in appearance considerably from the clumping of cells produced by animal agglutinins. These agglutinations are successfully eluted at 56° C to yield the extract.

Lectins have been used both in partially purified form (Boyd 1960) and directly as extracted from soaked and macerated seeds. There seems to be little advantage to the effort of partial purification by means of organic solvents or sodium sulfate precipitation, since specificity is not improved over that generally obtainable with the untreated extract. Stability, however, may perhaps be prolonged.

In 1954, Krüpe found that extract of Sophora japonica, inactive against B cells in a saline medium, was highly active in AB serum or bovine albumin. Agglutinating activity could also be provoked by the use of papain-treated cells in a saline medium. Hitherto such "incomplete," "blocking" or "univalent" antibodies have been identified with animal

immune antisera. There is a serious doubt as to whether the class of plant extracts termed "incomplete hemagglutinins" by Krüpe are in fact comparable in their biophysical properties; that they respond by a dramatic amplification of titer when used in macromolecular media or with enzyme-treated cells is easily demonstrated.

The phenomenon of "incompleteness" is of interest to this dissertation, since the Lotus tetragonolobus of McNeil (1955b) and the present author belongs to this class of reagents, requiring use in a macromolecular medium to obtain reactions strong enough for practical purposes.

In some instances, Krüpe (1956) reported that saline extracts of such agglutinins became amplified if merely aged for two to three weeks at room temperature; in the case of Sophora japonica he interpreted this as a fermentation of sugars from the splitting of the shell mucoids, which removed the blocking of the receptors. The present writer also found, in the case of Lotus, as well as her Euonymus yedoensis anti-B, that titers were enhanced by long periods of storage at 4° and 20° without evidence of contamination.

By use of a rabbit immune anti-plant-extract serum, the direct Coombs' test for incomplete agglutinins may be successfully performed on cells exposed to such saline seed extracts and then washed. Further, like other incomplete agglutinins, they are less easily inhibited by soluble blood group specific substances than complete agglutinins, an observation which assumes considerable importance when extracts are used in studies of the secretor phenomenon.

Such agglutinins were subsequently found in approximately 5 per cent of species tested. Most saline extracts, while not classifiable as "incomplete" agglutinins, nevertheless can be grossly amplified by the addition of macromolecular media: AB sera, bovine albumin, or polyvinylpyrrolidone (PVP or "kollidon").[1]

Krüpe reported that most, but not all extracts respond to a supplementary media. The present writer found that anti-H agglutinins (Cytisus, Laburnum, and Ulex) can be raised four to eight steps in titer by such additions, without losing specificity to the point of obscuring A_1 - A_2 erythrocyte distinctions. However, the increased reactivity with B cells is confusing for some purposes, and in most instances such high titers are not needed. The rise in titer of Phaseolus anti-A extracts with supplementary media is unusual, being over a thousand-fold increase.

Technically, the great majority of plant extracts are panagglutinins if used in purified or amplified form, and specificity is achieved by a dilution to the point where only the stronger specificities are functional. Thus, some extracts lose usefulness at such great amplification, and, when diluted further with the medium in order to lower the titer, still do not regain the original specificity of the saline extract. Ulex proved to be less useful in its amplified form than as a saline extract in the

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[1] European "kollidon" differs from the American product by its molecular weight of 28, 000, in contrast to Antara K-30, of 40, 000. The American product, used in a 10 per cent solution buffered to pH 7. 00, effects a far greater rise in titer than the European counterpart, judging by figures given by Krüpe and Mäkelä.

experience of the present writer, although some workers disagree. Ulex was most satisfactory when made from fresh seed, soaked and macerated in a Waring blendor, agitated and warmed to 37° for 8 hours, then the extract centrifuged and stored for 48 hours in the refrigerator. If used immediately after extraction, the extract gives non-specific reactions. During this time, a white precipitate accumulates, which is twice removed by centrifugation, and the supernate is stored frozen. Unfortunately for the practice of opening a new frozen vial for usage with each testing in the interests of standardization, Ulex reaches its highest and most specific titer after 6-8 days of "ripening" in the refrigerator. It will then hold approximately the same titer for a week or two if uncontaminated. Apparently a large quantity can thus be made up, aged, and frozen as a whole, which procedure is recommended.

Extracts of Laburnum alpinum and Cytisus sessilifolius are similarly prepared.

Papain-treated cells were found by the present writer to be more useful in working with incomplete agglutinins than PVP-amplified media; however, the use of bromelin according to the method of Pirofsky (1959) is preferred to either. This procedure needs no activator, and the bromelin extract can be added directly to the test without pre-treatment of cells.

In 1959, Margery Gray reported a four to five-fold increase in H activity for bromelin-treated cells, measured by their titers as obtained with Ulex extract. While amplification was generally observed wherever

H antigen was present, it did not reportedly obscure specific differentiation of red cell blood group.

In a series of classical experiments, Landsteiner had proved that very simple substances are capable of acting as determining groups of antigenic structure. It has also been known for some time that a substance of low molecular weight whose molecule nevertheless contains the determining group of an antigen may inhibit an immune serum not produced by that antigen per se; the terminal units of a complex hapten may thus serve as effective inhibitors.

Watkins and Morgan, in 1952, first demonstrated that simple sugars are capable of inhibiting blood group agglutinins. At that time the anti-H of the eel Anguilla anguilla was shown to be inhibited by L-fucose and some structurally-related compounds. The obvious explanation is that the sugar whose structure most closely resembles the specific groups of a red cell receptor attaches itself to the active sites of the agglutinin and blocks them, thus revealing their chemical configuration.

Morgan and Watkins (1953), Krüpe (1956), Mäkelä (1957, 1959), and Boyd (1960, 1962) have all contributed information concerning the specific inhibition of a number of plant and animal agglutinins by various sugars, amino acids, and polysaccharide substances. All amino acids examined have been found to be inactive, as well as most polysaccharide substances. Several simple sugars, however, have proved effective in inhibiting agglutinins from both plant and animal sources, and thus defining their structural specificities.

A year after their work on the inhibition of eel anti-H, Morgan and Watkins demonstrated that the anti-H agglutinin of Lotus tetragonolobus was inhibited by L-fucose, unlike the anti-H agglutinins of Cytisus sessilifolius and Laburnum alpinum. These observations indicated that although L-fucose is an important determinant of H specificity, it is not the only one.

Krüpe, in 1956, reported that salicin, a glucoside of D-glucose and saligenin, inhibited the agglutinins of Cytisus and Laburnum, but not those of Lotus or eel anti-H. Mäkelä, the following year, observed that Ulex europaeus activity could be inhibited by either L-fucose or by salicin, thereby creating a third category of anti-H specificity.

Plato and Gershowitz, in a recent article, have demonstrated that the anti-H of Cytisus and that of Ulex, while reacting with similar titers in the inhibition of secretor salivas of group A_1, show a higher inhibition titer of Ulex anti-H in groups A_2 and O. B and AB groups reveal higher titers with Cytisus. They conclude that at least two different types of anti-H specificities are involved, with qualitative as well as quantitative differences, a finding which is reinforced by observations of the present writer as well as those of the investigators cited above. It is regretted that Plato and Gershowitz did not include a Lotus tetragonolobus anti-H in their series, since one might reasonably predict a third specificity here.

Since Ulex is inhibited by both salicin and L-fucose, it must carry combining sites directed against most extensive areas of the H substance

than other anti-H's, and could be expected to demonstrate a wider range of H specificity. This is, in fact, the observation of the present writer.

The following quotations are offered in order to demonstrate the general mistrust which has been accorded the use of both Lotus extract and eel serum as anti-H reagents.

That those workers who find evidence of aberrant, partial, and paradoxical secretions (McNeil, Morganti, Buettner-Janusch) used one or the other of these reagents without exception, while those individuals using Ulex, Laburnum, or Cytisus extracts report more nearly normal findings is not in the nature of a coincidence, as will be demonstrated below.

> Ceppellini estimates that with the usual technique, 10 to 30% of group O secretors, depending upon the particular batch of eel serum used, may fail to show any inhibition and, in this series, they will have been scored as non-secretors. An extract of the seeds of Ulex europaeus is a much more satisfactory source of anti-H than is eel serum, and will be used in the future. In the data presented in this paper, however, all the patients were tested using the technique with eel serum that gave 38% non-secretors in controls of group O.
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> R. B. McConnell, in "Selection and the ABO Blood Group Locus" in Effects of Natural Selection, Annals of the New York Academy of Sciences, Vol. 65, Art. 1, p. 20, 1956.

> . . . it is evident that the agglutinin in the Lotus extract has less affinity for H substance than have the Laburnum alpinum and Cytisus sessilifolius extracts. . . . results . . . suggest that the receptor with which the Laburnum and Cytisus agglutinins are reacting is much more closely related to the isolated and purified H substance than is the receptor detected by the Lotus agglutinin.
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> W. T. J. Morgan and W. M. Watkins, "Inhibition of Haemagglutinins in Plant Seeds by

Human Blood Group Substances and Simple Sugars," Brit. J. Exp. Path., Vol. 34, p. 101, 1953.

Fernerhin erkennt man, dasz die Affinität des Lotus tetragonolobus Agglutins zur H-substanz im Vergleich zu der übrigen Anti-H-Reagenzein am geringsten ist.

M. Krüpe, Blutgruppenspezifische Pflanzliche Eiweiszkörper, 1956, p. 80.

. . . the other anti-O antibodies of plant and animal origin as well as the O specific plant hemagglutinins of Laburnum alpinum and Cytisus sessilifolius extracts have combining sites directed against more extensive areas of the blood group O(H) substances, thereby showing greater specificity [than Lotus extract or eel serum] since these materials have been found to be inhibited only by the hog and human substances, but not by any of the simple sugars.

A. E. Kabat, Blood Group Substances, 1956, p. 240.

CHAPTER V

GROUP A SPECIFICITIES

$A_1 - A_2$ antigenic distinctions are of interest to this dissertation in so far as they represent erythrocytic specificities with no discernible salivary counterpart.

It has been demonstrated by several workers, cited below, that little distinction is possible between A_1 and A_2 subgroups in fetus and newborn, yet A_2 offspring have not been known to acquire the hemolytic syndrome of ABO incompatibility. Obviously some differences must obtain, either quantitative or qualitative, which thus influence selection against the A_1 phenotype. It was in the hope of establishing such a distinction that a part of the investigation was devoted to attempts to subtype group A newborn.

A_1 and A_2 cellular antigens in the adult have been distinguished quantitatively by the lesser amount of A and the greater amount of H antigen on the A_2 erythrocyte surface. This observation has been interpreted as the less complete transformation of H into A substance under the influence of the A_2 gene. A_2 and A_2B individuals may occasionally produce anti-A_1 agglutinins which react specifically; specific anti-A_1 receptors are also found in extract of Dolichos biflorus. Such agglutinins argue for a qualitative distinction between A_1 and A_2 antigens.

Wiener and Wexler, in 1952, observed that if A_1 differed from A_2 in quantity of antigen only, then the relative titers of A_1 to A_2 cells should be the same with all anti-A antisera. However, since anti-A sera exist which act strongly on A_1 cells but very weakly on A_2 , while other antisera react almost equally on the two, the difference in behavior of these antigens must be related to qualitative differences in the structure of the antigen molecule. This view is now almost generally accepted.

Along with the observation that iso agglutinin anti-A combines more avidly with A_1 than with A_2 cells is the corollary of the uniformly higher absorptive titers of A_1 erythrocytes. There is the further consideration that, since A_1 cells hemolyze more rapidly than A_2 cells, they are likely to more easily involve erythroblastotic symptoms, as is indeed borne out.

According to Thomsen (1930), differences exist not only in the absorptive capacities of the cells, but in the secretions of individuals of the A_1 and A_2 subgroups. Dahr, however, reported (1938) that the antigens in salivas of A_1 and A_2 secretors absorb anti-A and anti-A_1 in group B isosera with equal readiness.

Wiener and Kosofsky (1941), in checking Dahr, used a human isoagglutinin from a group B as well as an immune rabbit serum. Results showed that inhibition tests on A_1 and A_2 saliva differed strikingly from comparative reactions of A_1 and A_2 erythrocytes. When A_2 test indicator cells were used, almost identical titers were obtained in secreted antigens using the two antisera. When A_1 test cells were used, the rabbit sera

again yielded almost equal titers, although there was somewhat more variation using iso-antisera. On this basis, Wiener concluded that the differences between A_1 and A_2 substances in secretion are small in comparison with those obtaining between A_1 and A_2 erythrocytes, and that the A substances in saliva and blood are serologically distinct. Human B isoserum gave higher titers with saliva than cells, while rabbit anti-A acted more intensely with cells than saliva, at least in instances of subgroup A_1 .

Witebsky and Engasser, in 1949, studied the agglutinating capacity of erythrocytes of 34 newborns of group A, using adult A_1 and A_2 cells as controls. Their four reagents were:

1. A human immune anti-A, with saline diluent
2. A human immune anti-A, with serum diluent
3. Anti-A partially neutralized with blood group specific substance, with saline diluent
4. The above reagent with serum diluent.

They found, using adult and infant cells respectively:

1. Adult cells of subgroups A_1 and A_2 are not distinguishable from cord cells of the same type by use of the first reagent, with saline diluent.

2. If saline is replaced by human serum as the diluent, A_2 cells in the adult are distinguishable from A_1 by a somewhat weaker reaction. Cord cells, under these conditions, more frequently resemble A_2 cells than A_1 cells. Subgroups are not clearly distinguished.

3. The addition of purified A substance results in a lack of agglutina-

tion of A_2 subgroup in adult, as well as of most infant cells, when saline is the diluent.

4. By use of anti-A serum partially neutralized with A specific substance, and cells suspended in normal adult serum, the authors found that about half their cord blood series reacted like adult A_2 cells or weaker, the other half somewhere between A_1 and A_2 adult reactions.

Constandoulakis and Kay (1962) similarly demonstrated that fetal A cells remove some activity of anti-A (alpha + $alpha_1$) against adult A_1 cells, and all or practically all activity against A_2 cells; in other words, in their absorptive capacity fetal cells also react like adult A_2 cells.

Both articles report wide variations in individual reactivities in cord cells.

According to these authors, it is not possible to classify A cells of the newborn into sharply differentiated subgroups. Commercial absorbed anti-A_1 proved similarly useless in this respect. No follow-up to determine the correct subgroups of infants tested was reported.

Since the work of Witebsky, it has been found that A_1-A_2 distinctions may be accurately determined for adult erythrocytes by the use of extract of Dolichos biflorus seeds, a potent and specific anti-A_1, with an affinity for A_1 cells over 500 times as great as that for A_2 (Bird, 1951, 1952a, b, c). Ulex europaeus extract, an anti-H used in standardized adjusted dilutions, will react specifically with A_2 (not A_1) cells. These two extracts will respond reciprocally and thus each serves as a check on the specificity of the other (Boyd and Shapleigh, 1954a).

Blumenthal, Gillert and Wulkow (1959), in studying bloods of 100 group A fetuses from 9 cm to term, found that all erythrocytes resembled more nearly the A_2 form in absorptive capacity, with two exceptions. Attempts at subtyping were unsuccessful, using both plant agglutinins (Cytisus, Laburnum, and Dolichos), and absorbed anti-A_1. No direct agglutinations with saline extract were observed, and 33 of 39 attempts at differential absorption of agglutinins revealed no diminution of titers.

The subtyping of 69 newborn infants of group A in the University of Minnesota series was attempted. The initial assignment of blood group A presented no difficulties with the use of commercial typing serum. Absorbed anti-A did not successfully distinguish A_1 from A_2 infants, most reactions being very weak or negative.

Attempts to subtype cord cells by the use of strong Dolichos biflorus extract proved equally unsuccessful; in less than a fourth of the samples did trace reactions appear. The extract was capable of agglutinating adult A_1 cells to a titer of $> 1:500$. Reactions of the cells to Ulex anti-H in saline extract were variable, and could not be used to distinguish the A_2 subtype; in spite of several strong positive reactions, too many trace reactions appeared to be all of the A_2 subtype.

The A cells were then submitted to the following reagents for comparison:

1. Ulex, standard saline extract.
2. Ulex, a 1:30 saline extract (dilution of the above standardized reagent) used with bromelin, according to the method of Pirofsky.

3. Laburnum alpinum, prepared and used with bromelin in the same manner as Ulex, above.

4. Cytisus sessilifolius, prepared as above.

5. Lotus tetragonolobus, used in 7.5% PVP, also tried in bovine serum albumin.

6. Lotus, used in saline extract with bromelin, as Ulex, above.

7. Dolichos, used in 1 : 50 saline dilution with bromelin.

TABLE 2

A-Cell Reaction Patterns to Seven Reagents,
University of Minnesota Series

	Adult		Infants							
	A_1	A_2	1	2	3	4	5	6	7	8
Ulex in saline	-	4	+	-	±	+	3	+	4	+
Ulex used with bromelin	±	4	2	2	+	2	4	3	4	2
Laburnum with bromelin	-	4	+	2	+	+	4	3	4	2
Lotus in PVP (aged)	-	3	-	2	-	+	-	-	2	+
Lotus used with bromelin	+	4	±	3	+	2	2	+	4	2
Dolichos in saline	4	-	2	4	2	3	-	-	-	+
Dolichos with bromelin	4	-	4	4	3	4	±	-	±	2
Presumptive subtype			A_1	A_1	A_1	A_1	A_2	A_2	A_2	?

The above table indicates typical reaction-patterns to the reagents employed. As will be noted, most reactions to anti-A_1 (Dolichos) and

anti-H are not completely specific and mutually exclusive, but, in most cases, one reaction or the other is sufficiently strong so as to permit a decision. Over a period of six months, 69 group A infants were thus subtyped, of which 16 were listed as presumptive A_2, 45 as A_1, and 8 inconclusive. Some of the last category may belong to the group known as "A intermedius" (reactive with both reagents), but it is unlikely that all 8 could be included here, the frequency being far lower in the Caucasian population.

Ulex and Laburnum reagents used with bromelin were found superior to other anti-H reagents for subtyping. The use of macromolecular media (PVP, Buminex, bovine serum albumin) was noted to lower specificity greatly and allowed for too great a sensitivity.[1] Cytisus used with bromelin was less specific than Laburnum and Ulex; however, the seed was older, which might be accountable. Other workers have not reported a lack of specificity. Since not all cases could be tested with Cytisus, it is not reported herein.

Lotus, used in any medium, reacts with far greater variability than other anti-H reagents, often forming patterns of considerably discordant specificity. The use of Ulex, 1:30 dilution of standard saline extract

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[1]It must be recalled that similar techniques, employing bromelin and macromolecular media effect the spontaneous agglutination of cells sensitized with certain "incomplete" or "blocking" antibodies. Therefore, in cases of maternal-fetal sensitization, where the infant cells may carry such antibodies undetected, such techniques cannot be employed. Positive, indeterminate results should be checked, therefore, by the anti-human globulin technique.

with bromelin, as an anti-H, and Dolichos, 1 : 50 dilution with bromelin as anti-A_1 were chosen as the most discriminating and reliable reagents. Laburnum may be used equally well as a substitute for Ulex.

Unfortunately, there was no opportunity to follow up the subtypings and determine their accuracy at, say, 12 months. The A_1 - A_2 frequency as determined is within expectation for the population.

CHAPTER VI

GROUP O(H) SPECIFICITIES

Group O erythrocytes of the newborn have been investigated with several anti-H agglutinins in the interests of this dissertation. Having H specificities only partially developed, such cells serve as valuable indicators of various anti-H reactivity patterns. It was hoped that by this means, a comparison of Lotus, Ulex, and Laburnum reagents could be made. It was also hoped that distinctions between erythrocyte and salivary antigens of the newborn might be thus more finely drawn.

Formaggio, in 1951, followed early post-natal development of reactivity in cells and secretions of group O infants. For his reagent, he used an anti-H serum produced in a goat immunized with Shigella shiga bacillus. He considered his reagent to be more selective of the product of the O gene than "merely an anti-H." Certainly he correctly concluded that anti-O (H) may contain a complex mixture of antibodies of varying specificity, the proportions of which often differ depending upon the source.

Formaggio reported that his reagent exhibited a titer of 1:4 to 1:16 in agglutinating newborn group O cells; adult cells reacted to titers as high as 1:256. Reactivity titers in the growing child increase for about three years, according to his table, then gradually level off.

Watkins, in 1952, investigated the problem of detection of O(H)

character of group O cord bloods with several anti-O and anti-H reagents, including goat anti-Shiga serum, eel serum, and several human sera characterized as "anti-O" since they were not inhibited by H substance in saliva.

Nine of the 11 anti-O reagents failed to agglutinate group O cord cells, while two agglutinated the cells to approximately adult levels of reactivity. It was at first suggested that these two might contain different species of antibody molecules, distinctive either in specificity or in some physical property, only one of which reacted with group O cells from infants, while the other reacted with adult cells. It was then found that absorption of the two antisera with either group O cells from adults or cord cells removed the antibodies active against both types of cells.

Immune rabbit sera were shown to agglutinate O cord cells, but natural cattle serum, eel serum, and immune chicken and goat anti-H reacted only very weakly, if at all. Four samples tested with Cytisus sessilifolius reacted strongly. Use of an albumin medium and trypsin-treated cells failed to effect an increase in the agglutination titers of cells not reacting to specific animal and human anti-O(H).

At the age of one month, stronger agglutination was observed with all reagents, and agglutination increased until between 6 and 12 months, when adult levels were achieved.

Obviously, assessment of the amount of O(H) component on cells at birth depends upon the reagent employed. Those sera which effect

agglutination must be detecting a structure which is developed at birth, whereas those which fail to react relate to another antigenic specificity not developed in fetal life. Or, Watkins suggests, perhaps the antigen on the infants' cells is masked by other structures, so that only the antibody (agglutinin) molecules of a certain configuration are able to combine with the specific groupings and effect agglutination. In such a case, one would expect the use of proteolytic enzymes to increase titer. Watkins failed to do so, although the present writer was successful in amplifying some titers with bromelin, as discussed in this section.

Red cells from 58 cord blood samples from the Delivery Room of the University of Minnesota Hospitals were studied with plant reagents. Of these bloods, seven were discarded because of an abnormal condition (usually maternal-fetal sensitization) which might have effect spontaneous agglutination in a macromolecular medium, such as PVP, or with enzymes, due to blocked receptors. Plant anti-H agglutinins used included Lotus tetragonolobus, Cytisus sessilifolius, Laburnum alpinum, Laburnum anagyroides, and Ulex europaeus.

The supply of Cytisus was limited, so that it could be used on only a few phases of the investigation. Laburnum anagyroides appeared to exhibit a specificity identical with that of L. alpinum, but reacted more weakly, so was not employed throughout the study.

1. Lotus tetragonolobus (see Appendix)

Lotus extract was prepared by grinding the hulled seeds in saline

according to the method of McNeil. 7.5 per cent PVP was substituted for the prescribed 10 per cent, since the two concentrations gave equal titers with adult cells, and the higher concentration caused the cells to adhere to the walls of the tube.

All extracts used were found to agglutinate pooled adult group O red cells to titers > 500; however, they were adjusted by the addition of their specific diluent to react to a 4+ in their first three double dilutions with adult O cells, as follows:

1:1	1:2	1:4	1:8	1:16	1:32	1:64
4	4	4	3	2	+	-

or to a titer of 32.

Freshly-prepared Lotus extract in 7.5 per cent PVP, which was adjusted to the above strength with adult O cells read as follows in the first tube of the 58 cord blood specimens:

36 samples	negative
13 samples	± to +
2 samples	++

After approximately two weeks of using the above preparation, (stable according to McNeil) which was stored in the refrigerator, it was noted that the anti-H titer of cord O cells appeared to be increasing. Checks run on the same cells with fresh Lotus preparations confirmed this suspicion, that certain receptors on the newborn cells were now being more strongly detected in some instances. Both fresh and aged

Lotus extract in PVP were therefore employed in testing 42 samples of O cord cells, the older extract having aged 3 to 4 weeks, including some 30-40 hours at room temperature. The extract evidenced no cloudiness or other indication of contamination. After a month's duration, specificity was gradually lost, presumably from bacterial contamination and an unmasking of the T-receptor.

Extracts aged 3 weeks gave the following reactions with 42 samples:

6 samples negative

8 samples ± or +

7 samples ++

21 samples +++ to ++++

As can be noted, variability was so great as to make generalizations impossible, except to report that many titers were increased, some more dramatically than others. Thirteen of the 15 specimens of the first category which had given positive reactions were tested, and found to give +++ to ++++ reactions with the aged agglutinin. This would seem to indicate that the same receptors were being indicated, only more strongly.

A third reagent was prepared by using fresh Lotus extract in saline (a 1:50 dilution of the extraction from seed powder), plus a drop of freshly-prepared bromelin solution for every drop of 5 per cent cell suspension. Titers even higher than those above were recorded, but variability was still the rule.

3 samples negative

9 samples ± to +

13 samples ++

25 samples +++ to ++++

There was an indication that aged Lotus extract with bromelin was still more efficacious than fresh extract, but time did not permit a study of this phenomenon.

2. Ulex europaeus

Ulex extract was prepared in the form of a fresh saline seed extract, pulverized in a Waring blendor, shaken, centrifuged, and passed through a Seitz filter. It was adjusted to the same strength as Lotus (titer of 32 against pooled group O red cells from the same three persons).

The following results were recorded on 58 group O bloods:

1 sample ± to +

14 samples ++

43 samples +++ to ++++

No advantage was observed in the employment of PVP as a medium or by the use of bromelin. The increase in titer was not marked (2-3 tubes) and, in readjusting to a titer of 32, specificity was frequently sacrificed, the amplified anti-H reacting with all cells.

Most interesting in Ulex reactions was the observation that strongly positive reactions with Lotus (amplified by bromelin) did not always

coincide with strongly positive Ulex reactions; in 11 cases a discrepancy of reactivity was recorded. That is, high Lotus titers were always high Ulex titers, but the reverse did not hold true. The 11 cases listed were negative with the original Lotus reagent, but strongly positive with Ulex. Apparently the H receptors detected by Ulex are different from those detected by Lotus.

Further evidence for a distinction between the species of H detected by Ulex on one hand, and Lotus on the other, was accumulated in 1959. At that time the present writer was engaged in a quantitative comparison between H endowment on group B erythrocytes and endowment of H in the salivas of the same individuals. It has been recognized that blood group B erythrocytes from different individuals demonstrate considerable variability in H component. Twenty-five group B samples, all from blood donors at the University of Minnesota Blood Bank, were selected on the basis of their high H quotient on erythrocytes, as tested with Ulex reagent (at least a ++ reaction with a standard reagent of 32 titer against O cells). These same samples were also tested with Lotus extract in PVP, according to the method of McNeil. Only 15 of the samples showed visible macroscopic reaction, and these were in general far weaker than those produced by Ulex. The two strongest Lotus reactions did not correspond to the strongest Ulex reactions.

Saliva H levels showed no quantitative correlation with Ulex reactions. Secretor saliva successfully inhibited both anti-H reagents to comparable titers, i.e., no variability in H specificity was detectable in soluble H antigen in saliva.

TABLE 3

Typical Reactions of Untreated Adult and Cord Erythrocytes

		1: 1	1: 2	1: 4	1: 8	1: 16	1: 32
	Adult A_2	3	3	2	+	-	-
Cytisus	Cord A_2	+	-	-	-	-	-
sessilifolius	Adult O	4	3	2	+	±	-
	Cord O	4	3	2	+	-	-
	Adult A_2	3	2	+	-	-	-
Laburnum	Cord A_2	+	-	-	-	-	-
alpinum	Adult O	3	3	2	+	-	-
	Cord O	2	+	-	-	-	-
	Adult A_2	4	4	3	2	+	-
Ulex	Cord A_2	+ to 2	+	-	-	-	-
europaeus	Adult O	4	4	3	2	+	-
	Cord O	4	4	3	2	+	-
	Adult A_2	4	4	3	2	+	-
Lotus	Cord A_2	-	-	-	-	-	-
tetragonolobus	Adult O	4	4	4	3	2	±
(PVP)	Cord O	- to ±	-	-	-	-	-
	Adult A_1	4	4	3	3	2	-
Dolichos	Cord A_1	- to +	-	-	-	-	-
biflorus	Adult A_2	-	-	-	-	-	-
	Cord A_2	-	-	-	-	-	-

TABLE 4

Typical Reactions of Adult and Cord Erythrocytes Treated with Bromelin

		1: 1	1: 2	1: 4	1: 8	1: 16	1: 32
	Adult A_2	4	3	2	+	-	-
Cytisus	Cord A_2	3	2	+	-	-	-
sessilifolius	Adult O	4	4	3	2	+	-
	Cord O	4	3	2	+	-	-
	Adult A_2	3	2	+	-	-	-
Laburnum	Cord A_2	3	2	+	-	-	-
alpinum	Adult O	4	3	2	+	-	-
	Cord O	3	2	+	-	-	-
	Adult A_2	4	4	4	3	2	+
Ulex	Cord A_2	3	2	+	-	-	-
europaeus	Adult O	4	4	4	4	3	2
	Cord O	4	4	3	2	+	-
	Adult A_2	4	4	3	2	+	-
Lotus	Cord A_2	±to 2	+	(highly variable)			
tetragonolobus	Adult O	4	4	3	2	+	-
	Cord O	+ to 3	+ to 2	(highly variable)			
	Adult A_1	4	4	4	3	2	1
	Cord A_1	2 to 3	+ to 2	+	-	-	-
Dolichos	Adult A_2	-	-	-	-	-	-
biflorus	Cord A_2	-	-	-	-	-	-
	Adult O	-	-	-	-	-	-
	Cord O	-	-	-	-	-	-

CHAPTER VII

SALIVA AND MECONIUM

Salivas:

The salivas of 53 secretor newborn infants were studied in their reactions to plant agglutinins.

To judge from the literature, other workers have not shared the considerable difficulties which were experienced in collecting the saliva samples. The following reference is typical: "A thick cotton swab is rubbed over the floor of the mouth until the cotton is saturated. The wet cotton swab is then forced into a narrow tube in order to squeeze out the saliva." (Wiener, 1943: 278)[1] These instructions were not found possible to follow on the newborn infants at the University of Minnesota Hospital nursery. Instead, a cotton-tipped sterile applicator-stick dipped in sterile saline was swabbed around the gums and under the tongue; with gentle massage enough saliva was gathered to becloud a few cubic centimeters of saline when repeatedly expressed. The solution was boiled for 15 minutes, centrifuged, and decanted. Such supernate kept well in the refrigerator or frozen. No quantitative studies were possible by this method. However, the antigens were strong enough to give clear-cut

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[1]In spite of the fact that crying babies salivate more freely, it was not considered justifiable to pinch babies in order to promote a more abundant secretion.

reactions even in still further dilutions. Salivary antigens, as reported by Wiener and Belkin (1943), are apparently fully developed at birth. The secretor-nonsecretor dichotomy could be clearly established by inhibition of A/B or H antigens.

Significantly, two group A, two group B, and one group O salivas inhibited the anti-H of Ulex and Laburnum, but not the anti-H of Lotus extract in PVP. Had Lotus been the only reagent employed, these A and B infants would have been classified as aberrant or partial secretors, the group O as a nonsecretor.

The saliva of a presumptive A_1 infant failed to inhibit Dolichos extract, although anti-A immune isoserum was inhibited. All other group A secretor salivas of both subgroups removed the lectin successfully. In working with meconium extract, it became increasingly evident that the Dolichos extract and human anti-A were absorbed by different antigenic receptors although considerable cross-reaction may be possible.

All other reagents reacted predictably, and in the manner of adult salivas.

Meconium:

Meconium, the first stool of the newborn, produced within the first 36 hours after birth, is a dark greenish to black viscous or rubbery material, odorless and in large part water-soluble. It collects in the intestine from the fifth month of development, representing an accumulation of desquamated cells, secretions of the alimentary tract, lanugo,

TABLE 5

Reactions of 53 Infant Secretor Salivas

	Inhibited	Not inhibited	Total
Group O			
Dolichos	1	25	
Lotus	25	1	
Laburnum	26		
Ulex	26		26
Group A			
Dolichos	19	1	
Lotus	18	2	
Laburnum	20		
Ulex	20		
Anti-A	20		20
Group B			
Dolichos	0	6	
Lotus	4	2	
Laburnum	6		
Ulex	6		
Europaeus yedoensis	6		
Anti-B	6		6
Group AB			
Dolichos	1		
Lotus	1		
Laburnum	1		
Ulex	1		
Europaeus yedoensis	1		
Anti-A	1		
Anti-B	1		1
			53

Note: Controls of adult secretor and nonsecretor salivas were included in tests using all reagents in order to check efficacy and specificity.

fatty material from the vernix caseosa, and amnionic fluid, ingested and circulated through the fetus. In secretors, it is exceptionally rich in blood group antigens.

Yosida (1928), Putkonen (1930), and Witebsky and Satoh (1933) all reported blood group activity in meconium. Witebsky and Neter (1935) noted that the amount of blood group activity disappeared rapidly after a few days; this blood group destroying power was thought to be enzymatic, produced either by the body or intestinal flora, or both. In 1951, Buchanan and Rapoport studied the inhibiting action of meconium antigens on a human "anti-O" as well as anti-A and anti-B isosera. They observed a lack of Rh and MN activity in the substance.

Since meconium was saved routinely at the University of Minnesota Hospital nursery, it was possible to study 124 samples from secretors. Less than half of these were also represented by saliva and cord blood from the same infants.

Meconium was collected as soon after birth as possible. It was thoroughly mixed and agitated with applicator sticks in about five times its volume of saline, and centrifuged at high speed. The clear supernate was decanted, and stored frozen until testing.

One drop of the meconium solution was added to two drops of antibody or lectin in inhibition studies. All agglutinins were standardized to a titer of 32 with pooled adult cells of the appropriate type.

The reactions of meconium antigens demonstrate certain marked differences from those on erythrocytes or in saliva.

Most interesting was the response to Dolichos biflorus extract. This specific anti-A_1 reagent has proved reliable in innumerable subtypings of adult group A, for which purpose it is sold commercially. It will be recalled that the Dolichos specific receptor is poorly developed on the erythrocyte of the newborn, and often needs the amplification of enzymes in order to effect a clearly visible agglutination reaction of A_1 cells.

By contrast, salivary antigens which easily inhibit the agglutinin are found in the secretions of both A_1 and A_2 subtypes, as reported by Bird and others. One group O saliva sample, which inhibited the Dolichos lectin, provided the only irregularity, except for a group A saliva which perhaps carried antigen too weak for detection. Although it has proved true that most A_1 and A_2 salivas inhibit Dolichos alike, the present writer has noted two A_2 adults whose salivas will not inhibit the Dolichos agglutinin. (The individuals did not appear in this study.)

In the report of inhibitions with meconium solution, it will be seen that responses were highly erratic. Seventeen out of 49 group O, and 8 out of 18 group B meconium samples inexplicably inhibited Dolichos. Perhaps this is an artifact created by degradation products; since A and B degrade to H, the expectation would be rather less inhibition of the lectin than more. Most unexpected of all, four nonsecretor meconium samples absorbed Dolichos, a finding which suggests that some substance other than ABH antigen, such as Lewis or a degradation product, might be involved.

TABLE 6

Reactions of 124 Meconium Samples from Secretors

	Inhibited	Not inhibited	Total
Group O			
Dolichos	17	32	
Lotus	46	3	
Laburnum	49	0	
Ulex	49	0	
Anti-A	0	49	
Anti-B	0	49	49
Group A			
Dolichos	52	0	
Lotus	45	7	
Laburnum	52	0	
Ulex	52	0	
Anti-A	51	1	52
Group B			
Dolichos	8	10	
Lotus	17	1	
Laburnum	18	0	
Ulex	18	0	
Anti-B	16	2	
Euonymus yedoensis	18	0	18
Group AB			
Dolichos	4	1	
Lotus	5	0	
Laburnum	5	0	
Ulex	5	0	
Euonymus	4	1	
Anti-A	5	0	
Anti-B	3	2	5

Euonymus europaeus seed extract is listed by Krûpe as a source of both anti-H and anti-B specificities. It elicits only a trace reaction with A_1 erythrocypes. The plant is one of the rare species yielding specific agglutinins which is not a legume.

In the course of preparing extracts of Euonymus europaeus as well as another species, Euonymus yedoensis, in order to test inhibition of saliva by a plant agglutinin having dual specificites, the present writer devised a method by which a stable and specific anti-B, with no trace of anti-H could be prepared. The somewhat painstaking preparation will be given in the Appendix. The procedures involves, in brief, a careful removal of the fruit wall, washing the inner seed with chloroform, and extracting with saline. The extract was employed by adding one part of extract to three parts of 10 per cent PVP, or, in the typing of erythrocytes, it is used diluted with saline, with the addition of bromelin. Extract of E. yedoensis proved somewhat stronger and more specific than that of E. europaeus.

In over several hundred testings, it was proved to be a completely reliable, discriminating, and stable reagent, which increases its titer of approximately 32, to 64 and 128 upon aging for two to three weeks in the refrigerator.

Euonymus yedoensis gave interesting reactions in the course of this study on newborn cells and secretions. While group B cord erythrocytes type somewhat more weakly than adult B cells with commercial anti-B,

the anti-B of E. yedoensis gives an equally strong typing in the newborn. It is inhibitable in all B and AB secretor saliva.

Most importantly, as will be noted below, in four meconium samples which showed H but no B antigens by the use of anti-B isoserum, E. yedoensis was completely inhibited. It was not inhibited by meconium of any other blood group. Hitherto, the lack of B antigen had been hypothesized as missing due to degradation processes; however, if so, only one species of antigen was degraded. It has long been recognized that B is immunologically a complex antigen, which can be demonstrated to contain at least three components and perhaps more. Further work with these lectins is surely indicated here, especially with Cotterman's new B_1 antigen.[2]

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[2]Charles Cotterman, personal communication.

CHAPTER VIII

DISCUSSION AND CONCLUSIONS

Three kinds of distinctions are here under consideration:

1. Distinctions between erythrocyte and salivary antigens in general.
2. Distinctions between adult and infant antigens, both erythrocytic and soluble.
3. Distinctions between various specificities as detected by plant lectins, and by lectins as compared to iso-agglutinins.

Distinctions between so-called alcohol- and water-soluble antigens have been recognized by several workers.

Koscielak and Zakrzewski (1960), in a comparative study of ethanol-extracted red cell substance and body-fluid A substance, reported that the erythrocytic substance fails to exhibit the following reactions:

1. No O, H, M, N, P or influenza-virus inhibiting activity.
2. No N-acetylyhexosamine color with Ehrlich's reagent.
3. No destruction by hot 0.05 N sodium carbonate in 15 minutes. However, when erythrocytic extractions are treated with hot hydrochloric acid of pH 2.3 for 8 hours, the resultant substance gives reactions characteristic of mucoid A.

Kochwa, Rosenfield, Tallal, and Wasserman (1961) pointed out that certain immune iso-agglutinins which are resistant to inhibition by soluble blood group specific substances provide a further distinction between

erythrocytic and extra-erythrocytic antigens. 7S antibodies which have crossed the maternal placenta and result in ABO erythroblastosis usually are of this type. The expected protection afforded the fetus by tissue and fluid A/B group substances, which serve to inhibit antibody action in other situations, are here absent. However, erythrocyte antigens react with such antibodies readily.

It has already been noted that soluble antigens are more antigenic than their erythrocytic counterparts.

Two, and even three and four specificities have been claimed for the same molecule of soluble antigen; to prove that erythrocyte antigens differ in this character is not a simple undertaking. Krüpe (1956) in checking cellular antigens found that A_2 erythrocytes completely saturated with anti-H will still absorb human anti-A; he concluded that different receptor-sites were involved, with single specificities. However, if a mixture of molecular species were involved (as is the case in soluble antigens), with some compound and some simple sites, his observations would not differ.

The work of Formaggio (1951), Watkins (1952), Constandoulakis (1962), Witebsky and Engasser (1949), and others treated above, have all indicated that important distinctions exist between adult and infant antigens. Including the findings of this dissertation, several generalizations may be made:

1. The A/B agglutination reactions in saline of fetal or newborn cells tend to be somewhat weaker than those demonstrated by adult cells.

Nevertheless, ABO group distinctions as such are clearly definable from early embryonic development by means of agglutination effected by immune high-titered antisera. This phenomenon is also expressed (Constandoulakis and Kay, 1962) as an increased ability of adult cells to absorb more anti-A and anti-B than fetal or infant cells of the same group.

2. A_1-A_2 subgroup distinctions are not discernible in the fetus or newborn by ordinary techniques, which are effective only several months after birth. Rather, implementation by bromelin is necessary in order to assign A cells to a subgroup.

3. Certain macromolecular media can effect diminution of agglutination of fetal/newborn cells. This medium is most effective when it consists of AB serum from secretor infants. The present investigation has indicated that secretions in the newborn are unusually rich in H substance, although apparent deficiencies in A/B antigens may be encountered.

4. Cord cells show extreme variations in antigenic endowment, which usually involve all antigens. Thus cells tend (occasionally) to be heavily endowed, approximating the adult condition, or, more often, moderately endowed or weakly reactive with all reagents.

In view of theories calling for the transformation of H into A/B antigens during fetal life, it might be expected that a weak A or B endowment would be accompanied by a richer H deposition, as in the case of adult A_2. This did not prove to be the case, although it is possible that untransformed H could not be indicated by the anti-H reagents in use here. Two samples of fetal erythrocytes, one from a four-month,

one from a six- to seven-month fetus, showed no increase in H, using the several plant agglutinins under study, but rather a diminution. Using commercial antisera, one tested clearly as an A; the other as an O, with agglutination in the latter case effected only by a highly concentrated Ulex extract amplified by bromelin, and then agglutination was weak.

5. Adult cells contain a wider spectrum of heterogeneous antigens than fetal /newborn cells. Anti-H reagents directed against cord erythrocytes give inconsistent and variable patterns of reactivity, some of which appear to change during the first few months of life, and some of which do not.

6. Salivary antigens of infants are by contrast stable, and more comparable to adult antigens in reactivity patterns. The secretor-nonsecretor dichotomy is consistently demonstrable in meconium and saliva, as indicated by the presence of H antigen. In most meconium secretor samples, H, as detected by Ulex, is present in impressive quantities, often inhibiting the lectin in dilutions as high as 1: 1000. Antigens on the cell surface are far from proportional in quantity.

Distinctions between various species of H antigen have been dealt with above in considerable detail. It was observed that both Laburnum and Ulex extracts performed with complete reliability the detection of secretor status in saliva and meconium, while Lotus failed to detect five of 53 salivary H and 12 of 124 meconium H antigens, or about 10 per cent each group. It should be again pointed out that a trace inhibition reaction would not appear in the tabulation, since no dilution

of less than 1: 5 was tested. The use of dilute antigens was necessitated by the difficulties of collecting infant saliva and the method chosen for handling meconium.

The results of investigation on H may be summarized by stating that at least three and probably more specificities of H exist which may vary independently, one (that detected by Lotus) demonstrating more variability than the other (Ulex). These variations may best be studied on infant red cells of group O, on the adult group B cell, and in day-to-day fluctuations in salivary antigen output, wherein that species detected by Lotus may be observed to vary more markedly. These H substances, although closely related, are not immunologically identical; while many of them cross-react with some reagents, by the use of other reagents, they are separable. These differences may be largely a reflection of variations in the analytical properties of the reagents. Like group A and B, H is patently unhomogeneous, with subgroups and probably intermediate types not clearly fitting any categorization. This will be especially true of substances such as those in meconium which have undergone degenerative changes to varying degrees.

It should finally be noted that A and B antigens as indicated by commercial anti-A and anti-B isosera were missing in several meconium samples; in three cases Euonymus yedoensis indicated the presence of a B specificity not indicated by the anti-B isoagglutinin. This situation, in which H is invariably present but not A or B, resembles that found in human milk by Lawler. Rather than claim aberrancies or

paradoxes here, the present writer will invoke only antigenic degradation, undetected trace reactions, or a species of antigen other than that detected by the particular reagent employed.

A review has been presented of the considerable evidence for a close relationship between the secretor system and ABO selection, both on preconception and developmental levels, as well as in several elusive correlations with infectious and pathological states. The complex nature of these relationships is still under investigation, and many aspects are still unknown or uninterpreted.

In view of the data presented herein, it appears that fetal and infant erythrocytes, with their attenuated ABH antigenic endowments, are less likely to effect maternal sensitization than the high levels of soluble antigens demonstrated in secretions. The concentrations of H substance in saliva and meconium are remarkably high. Whether or not this H substance can function in the protection of erythrocytes in the manner described by Vos and Kirk has not, as yet, been investigated. If so, it might provide an added explanation for the reportedly more severe affliction of non-secretor erythroblastotics than of secretors.

Differential mortality and differential fertility provide the two circumstances in which natural selection functions to change gene frequencies in populations. We have noted that the secretor system may well be involved in both, although details are largely undefined.

In ABO erythroblastosis, a 10 per cent excess of secretors over

expectation out of about 100 well-authenticated cases has been noted. Yet non-secretors are said by Wiener to be more severely afflicted. Implications are that the Se gene in its phenotypic manifestation serves both to initiate at least some of the erythroblastotic process by maternal immunization, and then to protect the fetus by antibody absorption from too-serious affliction. Non-secretors, on the other hand, may be selected against on the preconception level (witness the overwhelming percentage of non-secretors among infertile couples: 57 per cent). Thus there seems to be selection pressure in both directions, against Se in the frequency of a debilitating erythroblastosis, and against se in the frequency of infertility.

As far as aberrant secretion is concerned: there is no real evidence against McNeil's observation that amounts of at least one species of H, or A/B, antigen may vary unaccountably in certain situations (which being "cause" and which "effect" is another problem). Further, it has not been demonstrated by reproducible techniques that the primary spermatocyte in AO males can or cannot lose A antigen, thus creating two distinct sperm populations, A and O, each being susceptible to different immunoserological reactions in the same (uterine) milieu. If such a process could result in A and O spermatozoa from an AO father, then his aberrant secretion (having less A antigen) in seminal fluid, might indeed promote A-sperm infertility in so far as it would resemble a non-secretor situation, without sufficient A antigen to inhibit maternal

anti-A. But this situation would obtain only with a shortage of seminal fluid A (not H) substance, and only in the instance of aberrant secretion in the father. McNeil and his followers do not discriminate between the roles of the sexes, although group O mothers cannot demonstrate aberrant secretion, producing only one antigen.

Assuming that the hypothesis of seminal fluid A protection of sperm is correct, one could substantiate Gullbring's A and O sperm populations by looking for a more frequent association of A with the Se gene among the offspring of heterospecific matings. Since O-bearing sperm need no protection, they will perform fertilization equally well in any milieu, while A sperm need the protection of a secretor father.

There is another avenue of approach to this problem which has not been investigated. In 1957, Shaw and Stone announced their observation on seasonal fluctuations in human antibody production. It is remarkable that this highly significant finding has been so generally ignored. If it can be demonstrated that the birth dates of children born to ABO incompatible couples tend to cluster in certain months, antibody impedence might be demonstrated as relaxing during those months of diminished output.

It can be calculated that the maximum number of ABO heterospecific pregnancies will occur when the frequency of O(r) is approximately .65. Therefore, one would expect selection to push either the A or O frequency to 1.00, due to the loss of incompatible heterozygous fetuses. In looking at the distribution of the O gene in Mourant's tables, it can roughly be assessed that this .65 frequency is not far from the world's mean frequency

for the gene; certainly it represents a point of extremely common occurrence. This must imply a strong counter-pressure, for which one naturally looks to the Se gene.

Data on Se distribution comprise about sixty reports, some involving as few as 20 to 30 persons to a "population." A large number of these reports are based not on the results of secretor testing per se, but upon interpolations from Lewis blood group frequencies. Several of these workers wisely mistrust their own results.

Having struggled with several Lewis antisera (and not under field conditions), the present writer is frankly too skeptical of the data offered to ask its performance in any very stringent way until more reports have been assembled.

One would like to entertain the notion that the Se gene occurs with highest frequency in those populations where the O gene is also at its maximum frequency; witness Central Australia and aboriginal America. One might hypothesize that as the incidence of ABO incompatibility decreases, selective pressures against Se as a precipitating factor in erythroblastosis accordingly decrease; or, to put it the other way, as Se decreases, so does some selective force for O. But, as was stated above, the data on Se distribution are not yet adequate for manipulation. One can only state that the frequency of the Se gene has only rarely (and dubiously) been reported below .5, and in several populations, approximates or reaches 1. In several instances this accords with O frequency.

The problems still unsolved in this area are diverse, and their solution depends as much upon the improvement and standardization of laboratory techniques in assuring accurate data as upon their mathematical manipulation.

APPENDIX ON METHODS

1. Method for determining secretor factor:

Samples of saliva are collected free of food, sugar, or blood. They are immediately boiled for 20 minutes in a water-bath or beaker in order to destroy digestive enzymes which otherwise weaken antigens in a few hours. Boiling also kills some bacteria and coagulates mucus so that it is more easily removed by centrifugation. After centrifuging at high speed for aa few minutes, the clear supernantant is decanted for use. It can be stored for several days in the refrigerator without deterioration or indefinitely if frozen.

If no quantitative study is being carried out, the following procedure has proved satisfactory in order to assign secretor status:

a) Prepare three tubes, with three drops of physiological saline in each. To the first tube, add a drop of saliva from a pipette, mix well, and carry a drop of the mixture to the second tube. Mix well and carry a drop of the mixture to the third tube. Mix well, and discard a drop.

b) Add to each tube two drops of a Ulex extract, titered to 32.

c) Prepare a control tube with no saliva, just three drops of saline and two drops of Ulex extract. This will indicate whether the mere dilution factor can cause false negative reactions when cells are added.

d) Prepare controls of known positive and negative salivas.

e) Mix all contents well by vigorous shaking of the racks, and let stand for 30 minutes, shaking at intervals, while absorption is carried out. Racks may be also put on a rotator for 15 minutes.

f) After standing or shaking, add a drop of 10% suspension of washed pooled group O cells, which act as indicators of residual anti-H. The cells should be freshly drawn and suspended in fresh physiological saline. Tests may be read after standing at room temperature for 15 minutes, or may be briefly centrifuged at slow speed and read.

If the red cells are not agglutinated, the antigen in secretion has inhibited the Ulex, indicating secretor status. At least the first two tubes should be inhibited for a clearly positive result.

All control tubes should react according to expectation.

2. Preparation of Lotus tetragonolobus extract (McNeil, 1955:1205)

The dry ripe seeds of Lotus tetragonolobus from Munich, Germany were ground up in a pepper mill; 50 seeds for each 25.0 ml. of physiological saline solution. Before adding the saline, the hulls were removed and the yellow-green tinted central portion was ground to a fine powder. The powder and saline were then mixed well and incubated 1 hour at 37° C. Overnight incubation did not seem to harm the result. After incubation, the mixture was shaken one hour on the Kahn shaker. The opalescent supernatant fluid was then decanted into centrifuge tubes and spun at 4000 r.p.m. for 20 minutes. The resulting fluid was only faintly opalescent and this entire product was added to 50.0 ml. of 10 per cent polyvinylpyrrolidone (PVP). This mixture was then allowed to stand overnight at 4 C. during which time a faint precipitate formed. Recentrifugation at 4000 r.p.m. cleared the reagent of this precipitate. The crystal-clear fluid was passed through a Seitz filter without altering its activity. The titer of this reagent diluted to 10 per cent PVP against O cells was found to be approximately 1:64. AB serum was used as a substitute for PVP in the above preparation but was found to be unstable. The PVP preparation has given a stable titer after storage at 4 C. and 25 C. for 3 months. This preparation was taken as stock solution and for use was diluted 1:8 in 10 per cent PVP. Dilution was necessary to avoid a nonspecific agglutination of A_1 cells. Diluted in this manner the reagent will give a 4-plus reaction with O and A_2 cells and a negative reaction with A_1 cells.

Typing of erythrocytes is performed by adding cells on an applicator stick to a 2 per cent concentration, into 0.2 ml. of reagent. The tube is spun in an angle head centrifuge for two minutes at 2000 r.p.m.

3. Preparation of Euonymus yedoensis extract:

Extract of Euonymus yedoensis was prepared by rubbing the seeds between hard towels or gauze until all traces of the oily outer seed-covering was removed, and no further traces of yellow coloration could be noted. The seeds are now a smooth dark brown with no adherent particles of the wall. Any remaining shreds will cause an anti-H reactivity. Seeds are then washed with chloroform and dried.

Approximately 100 seeds were ground in a mortar, and 20 ml. of physiologic saline added. It was noted that the yellowish-green color of the extract results from the large central embryo, while the endosperm is white. The saline was allowed to stand over the ground seed for 24 hours, during which time it was subjected to 3 to 4 hours of incubation in a 37 C. waterbath, as well as several hours of shaking. It was then left overnight in the refrigerator.

At the end of this period, the extract was centrifuged, and the supernatant passed through a polypore filter. This saline extract will give no reaction with human erythrocytes of any group.

One part of the extract was then diluted with two parts of a 10 per cent solution of PVP (K-30) buffered to pH 7.0 with phosphate buffer.

Three drops of this reagent were placed in a test tube, along with a drop of a 5 per cent suspension of fresh washed erythrocytes for testing. The mixture was spun for one minute in a centrifuge at slow speed, and read with gentle shaking. It was noted that after standing 5-10 minutes, readings may become more distinct than when first read; occasional very weak agglutinations with O and A_2 cells disappear, while agglutinations of B and AB cells reach a 3-plus reaction.

Somewhat less satisfactory results were obtained by the use of Euonymus europaeus, which was supplied without the outer fruit wall by Herbst Brothers, Seedsmen, New York. In this case, specificity was less clearly marked.

In secretor or meconium testing, the above extract, (one part saline extraction with two parts of 10 per cent PVP) was used with one drop of saliva or meconium solution being added to three drops of the Euonymus preparation.

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