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Unravelling the biochemical basis of blood group ABO and Lewis antigenic specificity

Walter T.J. Morgan and Winifred M. Watkins[1,*]

Department of Haematology, Imperial College School of Medicine, Hammersmith Hospital, London W21 ONN, United Kingdom

The ABO blood-group polymorphism is still the most clinically important system in blood transfusion practice. The groups were discovered in 1900 and the genes at the *ABO* locus were cloned nearly a century later in 1990. To enable this goal to be reached intensive studies were carried out in the intervening years on the serology, genetics, inheritance and biochemistry of the antigens belonging to this system. This article describes biochemical genetic investigations on ABO and the related Lewis antigens starting from the time in the 1940s when serological and classical genetical studies had established the immunological basis and mode of inheritance of the antigens but practically nothing was known about their chemical structure. Essential steps were the definition of H as the product of a genetic system *Hh* independent of *ABO*, and the establishment of the precursor–product relationship of H to A and B antigens. Indirect methods gave first indications that the specificity of antigens resided in carbohydrate and revealed the immunodominant sugars in the antigenic structures. Subsequently chemical fragmentation procedures enabled the complete determinant structures to be established. Degradation experiments with glycosidases revealed how loss of one specificity by the removal of a single sugar unit exposed a new specificity and suggested that biosynthesis proceeded by a reversal of this process whereby the oligosaccharide structures were built up by the sequential addition of sugar units. Hence, the primary blood-group gene products were predicted to be glycosyltransferase enzymes that added the last sugar to complete the determinant structures. Identification of these enzymes gave new genetic markers and eventually purification of the blood-group *A*-gene encoded *N*-acetylgalactosaminyltransferase gave a probe for cloning the *ABO* locus. Blood-group ABO genotyping by DNA methods has now become a practical possibility.

Keywords: **ABO blood groups, Lewis blood groups, A, B, H, Le^a and Le^b antigens, blood group-active ovarian cyst glycoproteins, *ABO* and *Lewis* genetic loci: *A, B, H* and *Le*-gene specified glycosyltransferases**

Introduction

In the autumn of 1938 one of us (W.T.J.M) took up an appointment in the London laboratories of the Lister Institute of Preventive Medicine with the intention of carrying on with immunochemical research on bacterial antigens, the topic on which he had been engaged for the previous ten years. However, the clouds of war were already beginning to gather over Europe and with the onset of hostilities in 1939 it became evident that the growth of large scale bacterial cultures would be dangerous in bomb-threatened London. In searching for an alternative topic that might be relevant to the war effort he was influenced by the plans for an Emergency Blood Transfusion Service that were set up in the U.K. early in 1939 in anticipation of war casualties. He considered that the experience gained through the bacterial antigen work could be used to understand more clearly the antigen-antibody reactions leading to incompatible blood transfusions and he therefore set about learning blood group serology and investigating sources of blood group active materials. The second author (W.M.W) joined the group at the Lister Institute in 1942 when work on the purification of substances carrying blood activity was just beginning. The unfolding story of the chemical nature of the ABO and Lewis blood group antigens, the biosynthetic pathways leading to their formation and the glycosyltransferase products of the blood group genes continued at the Lister Institute until its closure in 1975 and was further pursued at the Medical Research Council's Clinical Research Centre on the outskirts of London until 1989.

*To whom correspondence should be addressed. W.M. Watkins
Tel.: + 020-8383-2171; Fax: + 020-8742-9335;
E-mail: w.watkins@ic.ac.uk

[1]We wish to dedicate this article to the memory of two outstanding contributors to the ABO story, Elvin A. Kabat (1914–2000) and Raymond U. Lemieux (1920–2000) who both sadly died this year.

The ABO blood group system

Landsteiner recorded in a footnote in 1900 [1] his observation that the red cells of some of his colleagues in the pathological anatomy institute in Vienna were agglutinated by the sera of the others. He had been studying serological differences between different species but this was the first demonstration of individuality of blood in members of the same species. In a more detailed paper published in 1901 [2] Landsteiner described the classification of human bloods into the three groups, now called A, B and O, and his pupils Decastello and Sturli [3] discovered the fourth, and rarest group, AB, in 1902. A characteristic of the ABO blood-group that makes knowledge of the serological reactions so important for safe blood transfusion is the existence of antibodies directed to the A and B antigens that are always present when the corresponding antigen is missing. Extensive family studies in the years following Landsteiner's discovery demonstrated that the antigens are inherited characters [4]. In 1924 Bernstein [5] predicted that the mechanism of inheritance involved three alleles, *A*, *B*, and *O* at a single locus, and in 1930 Thomsen *et al*. [6] extended this to include alleles corresponding to the A_1 and A_2 subgroups of A. We now know that there are many more different alleles at the *ABO* locus but the cloning of the *ABO* genes [7] has shown that the general principle of multiple alleles at a single locus, as enumerated by Bernstein [5], is the correct basis of inheritance of this blood group. The ABO gene locus has been mapped by linkage analysis to human chromosome 9q34 [8].

The presence of soluble substances in human secretions with the same serological specificities as the A and B antigens on the red cell was first noted by Yamakami [9] in 1926 but it was not until 1930 that Lehrs [10] and Putkonen [11] realised that the character was dimorphic and that individuals could be classified as blood-group secretors and non-secretors. The ability to secrete A and B antigens in saliva was shown by Schiff and Sasaki [12] to be inherited as a Mendelian dominant character, controlled by a gene, now called *Se*, and its allele *se*. Thus secretors carry the genes *SeSe* or *Sese* and non-secretors are homozygous *sese*.

Sources of blood group A and B substances

Early studies aimed at characterisation of A and B antigens from red cells met with limited success. Schiff and Adelsberger [13] and Landsteiner and van der Scheer [14] extracted red cells with ethanol and other organic solvents but the isolated materials had little activity and were never satisfactorily purified. In truth no methods were available at that time which would have enabled membrane components present in minute amounts to be identified. However, the idea arose that the blood-group substances existed in two forms and that on red cells they were lipoidal or lipoprotein molecules, insoluble in water or saline, whereas in tissue fluids and secretions they were present in a different water-soluble form. Carbohydrate was not at that time recorded in analyses of red cell membranes. In 1930 Schiff [15] found that commercial peptone of animal origin contained a substance which possessed human blood group A specificity and Landsteiner and Harte [16] established that the purified material from this source contained amino acids and carbohydrate firmly bound to each other; it was however, left open as to whether the carbohydrate or peptide moieties were responsible for specificity. It was at this stage in the early 1940s that the Lister group started their investigations in this field. Preliminary studies were carried out on A-active hog gastric mucin [17] but the aim from the beginning was to correlate the chemical nature of the antigens with the human blood-group genes and hence it was considered important to find a good source of human material and to use individual samples of known blood-group status rather than pooled materials. A search of the literature revealed that Yosida [18] had recorded the presence of blood-group activity in ovarian cyst fluids. Physiologists in the 19th century had been interested in the fluids in these cysts and Hammarsten in 1882 (cited in [19]) classified those that were rich in carbohydrate and protein as "pseudomucinous", and those which contained only protein as "serous". Since it was known that sometimes these fluids could reach a volume of many litres, a series of ovarian cyst fluids were examined to test their potential as a source of material for biochemical studies on the antigens. The results showed [20] that the presence of blood-group activity in the cysts corresponded to the ABO red cell and secretor status of the patient and that cyst fluids from so-called pseudomucinous, but not serous cysts, were excellent and convenient sources of blood-group substances. These fluids thus became the starting materials for the characterisation and isolation of the blood-group determinants that was to continue at the Lister Institute for the next 25 years. Elvin Kabat and his colleagues in New York also started to use these materials in the 1960s for the isolation of blood-group active oligosaccharides.

Production of antibodies directed towards artificial A and B antigens

The preparations from commercial peptone and hog mucin examined in the 1930s had strong serological activity but were not immunogenic. A spin-off arising from the bacterial antigen work was the demonstration that polysaccharides, such as cherry gum and gum accacia, could be rendered immunogenic by complexing them with the protein component of the O-somatic antigen of the Shiga bacillus [21]. Application of this technique to A-active preparations from peptone and hog gastric mucin revealed that the resultant complexes induced the formation of powerful high titre anti-A immune serum in the rabbit [22]. Subsequently A-active preparations from human ovarian cysts were found to be equally immunogenic when complexed with the Shiga protein [23] and analysis of the antisera [24] showed that, by suitable absorption, specific anti-A_1 reagents could be prepared from the potent anti-A sera

that initially agglutinated both A_1 and A_2 red blood cells. Rabbit anti-B sera were similarly produced by combining a purified preparation from a group B ovarian cyst with the Shiga protein and, as supplies of high-titre human anti-A and anti-B sera were not readily available at that time, a small contribution of the Lister biochemists to the war effort was the supply of powerful rabbit anti-A and anti-B sera to the Royal Navy for blood-grouping sailors on board ship when blood was urgently required for transfusion of casualties suffered in action [25].

The H and Lewis blood group systems

A question that had exercised many blood-group workers in the early part of the 20th century was whether there is a product of the *O* gene analogous to the A and B antigens. In the original Landsteiner definition of the ABO system [2] O was seen simply as the absence of A and B but in the 1930s a number of reagents of human and animal origin had been described which reacted preferentially with group O cells; these were then referred to as anti-O reagents. However, thorough serological testing of some of these reagents with cells of known genotype revealed that, although they did react preferentially with O cells, they also reacted, to a much lesser extent, with red cells from homozygous *AA* and *BB* individuals as well as with cells from heterozygous *AB* individuals [26,27]. Hence if Bernstein's theory of inheritance [5] was correct the donors of these cells could not be carrying the product of the *O* gene since an individual inherits only one of the three *ABO* alleles from each parent. We concluded that the antigen preferentially expressed on O cells must be the product of a gene independent of *ABO*, which we named *H* [27]. The distribution of the H antigen is similar to that of A and B in as much as it is present in saliva and other tissue fluids of secretors as well as on red cells. Following certain ideas of Hirszfeld and Amzel [28] and Witebsky and Klendshoj [26] we further proposed that H is the precursor of A and B antigens and occurs to greater extent on O cells than on cells of other ABO groups because it is unchanged on these cells [27]. The H antigen appeared to be ubiquitously present on human red cells until the discovery of the Bombay O_h phenotype in 1952 [29]. This blood type is characterised by a lack of A, B and H antigens on the individual's red cells and the presence of antibodies to A, B and H in their sera, and we therefore suggested that these individuals were homozygous for a null allele of *H*, namely *h*, and lacked A and B antigens because of the absence of H precursor substance [30].

In the late 1940s the discovery of Le^a [31] and Le^b [32] antigens introduced two more determinants that were shown to occur both on red cells and in soluble form in secretions. Grubb [33] made an observation, which seemed remarkable at the time, that individuals who have Le^a antigen on their red cells are non-secretors of ABH substances in saliva and it was soon shown that those who express Le^b antigen on their red cells are ABH secretors [33,34]. Ovarian cyst fluids were also found to be rich in Le^a and Le^b activity [35]. Le^b was originally thought to be the product of an allele of the gene giving rise to Le^a but Ceppellini, observing that Le^b activity was associated with ABH secretors, suggested that this antigen was an interaction product of the secretor gene *Se* and the Lewis gene *Le* [36], a farsighted prediction which has proved to be correct. Although the relationships between the ABH and Lewis antigens were both obscure and puzzling at that juncture it was clear as early as 1949 that in secretions there were five inter-related blood-group specificities, the products of four independent genetic systems, *ABO*, *Hh*, *Sese* and *Lele*, that awaited chemical identification.

Purification and composition of the ovarian cyst substances

The period 1945–1955 saw intensive efforts, with colleagues Aminoff, Annison, Gibbons and others to purify the active materials from cysts of known blood-group specificity. Our original aim was to obtain pure A, B, H and Lewis substances, free from any other specificity present in the cyst fluid. The procedure that was elaborated involved freeze-drying the cyst fluid and thoroughly extracting the residue with liquid 90%, or better 95%, phenol at room temperature. Three or four extractions were sufficient to completely remove protein and lipid substances from the dried cyst and this left a phenol–insoluble residue that was composed of a carbohydrate-amino acid complex carrying the blood-group activity. Further purification was then achieved by fractionation of aqueous solutions of the residue with organic solvents or by treatment with ammonium sulphate [37]. With help from our biophysical colleague, Ralph Kekwick, individual preparations of A [38] B [39] H [40], and Le^a [41] substances were judged to be essentially free from contaminating protein by Svedberg ultracentrifugal and Tiselius electrophoretic analyses, but to be moderately polydisperse, made up of a range of different sized entities with molecular weights extending from about 200×10^3 to several millions. The macromolecules were then classified as mucopolysaccharides [42] although they would now be called highly glycosylated mucin-type glycoproteins, with *O*-linked oligosaccharide chains. It soon became apparent that, however rigorous the purification procedure, it would not be possible to obtain "pure" blood group preparations with just one specificity; group A preparations, for example, frequently displayed H activity and, if derived from an individual who carried an *Le* and *Se* gene, could also have Le^a and Le^b activity. This finding indicated that the antigenic determinants resided in only a part of the macromolecule and precipitation experiments with specific antibodies later clearly demonstrated that multiple determinants were carried on the same macromolecules [43,44].

The purified macromolecules were composed of 85–90% carbohydrate and a peptide moiety made up of some 15 amino acids [42]. Landsteiner and Harte [16] had earlier considered that the neutral A-active polysaccharide from pig gastric mucin

was composed of equimolar quantities of *N*-acetylglucosamine and galactose. Subsequently L-fucose was identified as an additional component of A-substance obtained from pepsin [45]. The human ovarian cyst preparations were each found to contain these three sugars and David Aminoff, then a PhD student in our laboratory, employed the newly introduced technique of paper chromatography to demonstrate the presence of a second amino sugar in the preparations which was identified as *N*-acetylgalactosamine [46]. Initially it had been hoped that careful qualitative and quantitative analyses of the purified cyst preparations would yield clues to the basis of their different blood-group specificities but despite the clear cut serological differences they proved to be very similar in both qualitative and quantitative composition, including a preparation lacking all five antigenic specificities isolated from a cyst originating from a patient who was a non-secretor of ABH, Le^a and Le^b [47]. This latter preparation, which we called a "precursor blood-group substance", differed only in that the content of fucose was low compared with the active substances (Table 1) and suggested to us that the specific structures represented only small parts of the molecules and were appended to this "inactive precursor" substance. The analysis of a similar "inactive" cyst preparation later isolated by Vicari and Kabat [48] again showed the low fucose content. Assays for sialic acid were not well developed in the early 1950s and, although it was shown to be a component of some of the cyst preparations, its removal by mild acid hydrolysis did not change the serological specificity of the preparations and therefore we early concluded that this sugar did not constitute an essential part of the specific determinants.

Indirect methods of structural analysis

When the investigations on the cyst materials started methods were not available whereby oligosaccharides containing more than three or four different sugars joined together by different glycosidic linkages could be separated from each other and this initially ruled out the use of chemical degradation methods that would be the obvious first step today for structural identification. We therefore embarked on some indirect methods of structural analysis in attempts to find out more about the specificity differences. Early observations of Landsteiner and van der Scheer [49] had established for artificial antigens that a simple substance with a structure closely related to, or identical with, the immunologically determinant (haptenic) group of the antigen can combine with the antibody and thereby competitively inhibit the reactions between antigen and antibody. In the 1950s this principle had not been used to find the determinants in naturally occurring antigens, although it forms the basis of many methods employed today. By 1952 we had accumulated a large selection of anti-H reagents of human and animal origin and decided to apply the Landsteiner method to see whether any of the component sugars of the ovarian cyst preparations could inhibit the agglutination of O cells by these reagents. Somewhat to our surprise one single reagent, that from the eel, *Anguilla anguilla*, was quite strongly inhibited by L-fucose and to a greater extent by α-methyl L-fucoside and not by the other monosaccharides. Our first tentative conclusions [50] that L-fucose in α-linkage is more important than the other sugars for H specificity was reinforced when we were given some plant agglutinins (later called lectins) which had recently been shown to be specific for certain blood-group antigens [51,52]. Extracts of *Lotus tetragonolobus* seeds preferentially agglutinated O cells and this agglutination was inhibited by H-active glycoprotein and also by L-fucose and its α-methyl glycoside [53] (Table 2). Moreover, other plant reagents specifically agglutinating A cells were inhibited by *N*-acetylgalactosamine, indicating that this sugar is an important part of the A determinant [53]. These first intimations of the chemical nature of the blood group determinants caused us great satisfaction and excitement. The extremely narrow and exclusively carbohydrate specificity of the plant lectins, and the eel reagent, had not been realised until it was revealed by these simple, but very informative, haemagglutination inhibition tests. In the first experiments the use of monosaccharides differing from L-fucose only by substitutions of OH groups at one or two positions enabled the conclusion to be drawn in 1952 that the configurations of OH-3 and OH-4 in the fucose ring were essential for combination with the eel anti-H reagent [50]. These conclusions are very similar to those reached some 30 years later, using more advanced methods, concerning the importance of OH-3 and OH-4 of the fucose ring in the binding of lectin 1 of *Ulex europeaeus* to the H determinant [54]. It is frequently said that all the early deductions concerning the nature of the blood-group determinants came from experiments on the soluble secreted substances but in fact the very earliest inferences from the lectin inhibition experiments highlighted the carbohydrate nature of the blood-group antigens on the surface of red cells since the sugars were competing in the reaction between red cells and the plant agglutinins. However, the failure of many human anti-H and anti-A antibodies to be inhibited by the simple sugars in haemagglutination tests indicated that the complete determinant structures recognised by the antibodies must be larger than the monosaccharides which combined with the lectins.

Table 1. Analytical figures (typical values) for preparations of human ovarian cyst blood group substances.

Substance	*Nitrogen (%)*	*Fucose (%)*	*Acetyl (%)*	*Hexosamine (%)*	*Reducing sugars (%)*
A	5.4	19	9.0	29	54
B	5.6	16	7.0	24	52
AB	5.6	17	–	26	54
H	5.3	18	8.6	26	54
'inactive'	5.4	1.6	–	28	49

Modified from Morgan [42].

Table 2. The inhibition by H-substance and simple sugars of the agglutination of group O cells by anti-H agglutinins in Eel serum and extracts of *Lotus tetragonolobus* seeds.

	Minimum amount of substance giving complete inhibition	
Inhibiting substance	*Eel serum ($\mu g/0.1$ ml)*	*Lotus tetragonolobus ($\mu g/0.1$ ml)*
Human H-substance	0.1	8
L-fucose	8	8
Methyl α-L-fucopyranoside	2	4
Methyl β-L-fucopyranoside	32	15
D-fucose	>1000	1000
Fucosyl$\alpha(1 \rightarrow 2)$-fucose	4	8
Fucosyl$\alpha(1 \rightarrow 3)$-fucose	15	–
Fucosyl$\alpha(1 \rightarrow 4)$-fucose	64	–
D-Arabinose	250	500
2-Deoxy-L-talose	125	250
D-Arabinose	250	500
L-galactose	>1000	32
D-galactose	>1000	>1000
D-digitoxose	>1000	500
D-ribose	>1000	1000
N-acetylglucosamine	>1000	500
N-acetylgalactosamine	>1000	>1000

Compiled from [50,53].

No lectins that were as reliable and specific as the anti-A and anti-H reagents were available in the early 1950s for the detection of B determinants but on the basis of weak inhibition by melibiose, raffinose and stachyose of precipitation of soluble B-substance by human anti-B serum Kabat and Leskowitz [55] in 1955 suggested the role of α-1,6 galactosyl groupings in B specificity. The part played by α-linked galactose later proved to be correct although not the inference of an α1,6-linkage. Similar precipitation inhibition experiments with *N*-acetylgalactosaminides confirmed the role of this acetyl-amino sugar in A-specificity [55].

A second approach that strengthened the deductions concerning the immunodominant sugars in the A, B and H structures was inhibition with monosaccharides of the destruction of blood-group activity by microbial enzymes [56]. It had been known since the 1930s that certain bacterial extracts destroyed the A, B or H specificity of secreted substances (reviewed in [57,58]) but their mode of action was unknown. Treatment of the ovarian cyst blood-group active glycoproteins with the crude microbial extracts usually resulted in extensive release of all the component monosaccharides but by attempting to inhibit enzymic loss of blood-group activity with simple sugars we found that destruction of A-specificity by certain bacterial or protozoan extracts, was inhibited by *N*-acetylgalactosamine, destruction of B specificity by D-galactose, and destruction of H specificity by L-fucose [56]. We therefore deduced that the degradative enzymes were, respectively, an α-*N*-acetyl-D-galactosaminidase, an α-D-galactosidase and an α-L-fucosidase that split off terminal sugars (and hence are what would now be termed exo-glycosidases) and that they were being inhibited by an excess of the products of their own action.

The loss of Le^a specificity by the microbial enzymes was also inhibitable by L-fucose, thus implicating this sugar in the structure of a second determinant [56]. Soon after the serological and enzymic inhibition tests had revealed the association of L-fucose with H- and Le^a specificity the distinguished chemist, Richard Kuhn, in Heidelberg started an investigation on the structures of a series of fucose-containing oligosaccharides that occur in human milk [59]. A request for these compounds received a favourable response and they gave such clear cut inhibition results in Lewis haemagglutination inhibition tests with a human anti-Le^a reagent [60] that it was possible, from the established structures of the oligosaccharides, to delineate in 1957 the complete trisaccharide determinant responsible for Le^a specificity, Galβ1-3[Fucα1-4]GlcNAc (Table 3 and Figure 3), and to predict a difucosyl structure for Le^b. Hence Le^a was the first mammalian cell surface carbohydrate antigen to be precisely defined in chemical terms.

Proposed biosynthetic pathways

Information that was rewarding both from the point of view of structure and for giving insights into possible pathways of biosynthesis of the blood-group determinants was obtained

Table 3. Inhibition of haemagglutination of Le(a+) red cells by human anti-Le^a serum with fucose containing compounds.

Inhibiting substances	*Minimum amount of substance (μg/0.1 ml) giving complete inhibition*
Human Le^a substance	0.04
L-Fucose	>1000
D-Fucose	>1000
Lacto-*N*-fucopentaose I	
Fucα(1 → 2)Gal(1 → 3)GlcNacβ(1 → 3)Galβ(1 → 4)Glc	>1000
Lacto-*N*-fucopentaose II	
Galβ(1 → 3)[Fucα(1 → 4)]GlcNAcβ(1 → 3)Galβ(1 → 4)Glc	1
Lacto-*N*-difucohexaose II	
Galβ(1 → 3)[Fucα(1 → 4)]GlcNAcβ(1 → 4)[Fucα(1 → 3)Glc	2
Trisacharide isolated from Le^a substance	
Galβ(1 → 3)[Fucα(1 → 4)]GlcNAc	2

Compiled from [60,85]. Abbreviations: Fuc, L-fucose; Gal, D-galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Glc, D-glucose.

from the sequential exposure and destruction of specificities in a single blood-group substance [61,62]. Confirmation of the precursor-product relationship of H to A and B first came from enzyme degradation experiments. Iseki and Masaki [63] made the seminal observation in 1953 that treatment of A substance with an enzyme preparation from bacterium *Clostridium tertium* made the A specificity disappear with the apparent production of O(H) substance. In our laboratory, partially purified enzymes from the protozoan *Trichomonas foetus*, enabled us to demonstrate that the loss of B activity was accompanied by the release of D-galactose, and the loss of A activity was accompanied by the release of *N*-acetylgalactosamine; in each instance this loss resulted in a development of H activity [61,62]. These results therefore revealed that addition of only one monosaccharide was required to bring about the conversion of H to A or B and dispelled any lingering doubts that the H antigen detected on group O cells was the product of an allele of the *A* and *B* genes.

A reagent that gave some useful structural information in early experiments was a horse antibody directed to the Type XIV pneumococcal polysaccharide. Purified blood-group substances of animal [64] and human [38–41] origin cross-reacted to varying extents with this antibody in the undegraded state and reactivity was increased by mild acid treatment. The precipitation of human blood-group substances by this antibody was specifically inhibited by the disaccharide, *N*-acetyllactosamine (Galβ1-4GlcNAc), indicating that it was cross-reacting with this structure in the blood-group substances [65]: a finding substantiated by the subsequent demonstration that the pneumococcal polysaccharide has multiple *N*-acetyllactosamine branches [66]. In enzyme degradation experiments the H-active substances resulting from the removal of *N*-acetylgalactosamine from a serologically ALe^b active-substance and galactose from a BLe^b substance developed Le^a activity and showed increased cross-reactivity with the Type XIV pneumococcal antibody when they were treated with an H-destroying enzyme: thus removal of the fucose involved in H specific structures exposes Le^a and *N*-acetyllactosamine groupings. Subsequent treatment with an Le^a destroying enzyme removed more fucose and left a residue that cross-reacted strongly with the anti-Type XIV pneumococcal reagent (Figure 1). The step leading from H activity to the exposure of Le^a occurred only when the original substance was derived from an individual carrying *Se* and *Le* genes. These results suggested that the specificities exposed were initially part of the same oligosaccharide chains and that these chains were being built up by the sequential addition of single sugar units which masked the preceding structures [67]. Although the precise structures of the A, B, H, Le^a and Le^b determinants had not yet been established, the enzyme degradation results, together with the other biochemical findings on the active macromolecules, enabled

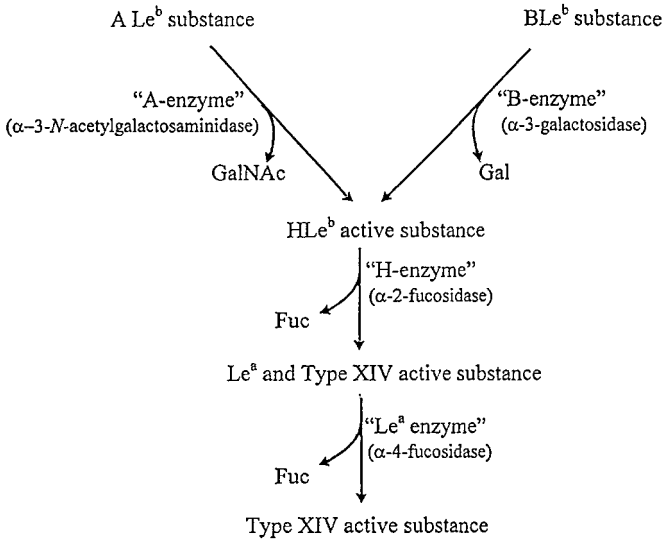


Figure 1. Sequence of specificity changes induced in blood-group ALe^b-and BLe^b-active glycoproteins by successive treatments with glycosidases from *Trichomonas foetus* (Watkins [61,62]). Abbreviations as in Table 3.

us in 1958/1959 [68,47] to put forward genetical pathways that explained the different combinations of the five determinants that appeared in secretions based on the inheritance and interactions of the *ABO*, *Hh*, *Sese* and *Lele* genes (Figure 2). An essentially similar scheme based on genetical analyses was advanced in 1959 by Ceppellini [69]. The ideas put forward in the schemes were thought to be possibly an over-simplification in view of our fragmentary knowledge at that time but, since the suggested steps were open to a degree of experimental proof, it was hoped they would provide a basis for further biochemical investigations [47]. In fact the suggested order in which the *H* and *Le* gene-encoded enzymes act has needed revision in the light of later knowledge on the precise specificity of the glycosyltransferases encoded by these genes, and the presence of both Type 1 and Type 2 chain endings in the macromolecules (see below), but the basic premise in the schemes that the determinants are built up by the sequential addition of sugar units under the control of the different blood-group genes, with each structure synthesised constituting the substrate for the next glycosyltransferase, has proved to be correct.

The apparently straightforward inheritance patterns of the ABO groups had earlier led to the view that the blood-group antigens were the direct products of the genes [70] although until the publication in 1953 of the double helix structure for DNA [71], and the subsequent breaking of the three letter nucleotide code, it was unclear how the information was carried or transcribed. However, as the genetical pathways for the biosynthesis of the blood group determinants were being outlined the idea that proteins are the only primary translated products of genes was just beginning to penetrate into general biochemical thought and therefore it became apparent that, since the antigenic structures were carbohydrate, they must be secondary products of the blood-group genes. The mechanism of biosynthesis of polysaccharides had also been unclear until the pioneering work in the 1950s of Leloir and his colleagues [72] on nucleotide sugars and their role as sugar donors in glycosylation reactions was described in plants and bacteria. It seemed to us that the most obvious candidates for the primary products of the blood-group genes were glycosyltransferase enzymes and, although at that time no mammalian enzymes belonging to this class had been identified, we proposed that the blood-group genes encoded glycosyltransferases that added the final sugar to complete the determinant structures [67,73].

Characterisation of blood-group active fragments isolated from ovarian cysts

At the same time as the biosynthetic pathways were being formulated from the knowledge of the immunodominant sugars and the results of the enzyme degradation experiments, other members of the group at the Lister Institute were making

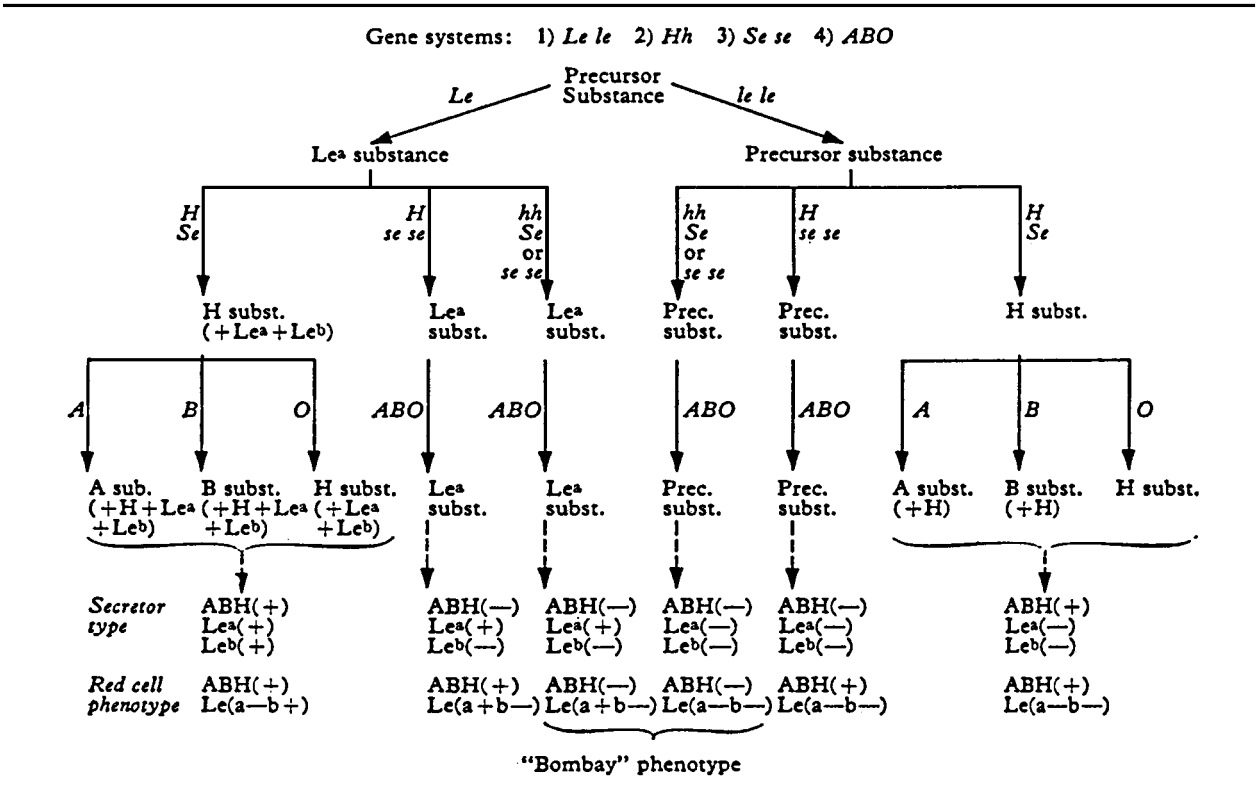


Figure 2. Genetical pathways for the biosynthesis of blood-group A, B, H Le^a and Le^b determinants based on the knowledge available in 1959 (Modified from Watkins and Morgan [47]).

different approaches to the structure of the blood-group determinants. The arrival in 1955 of a French Canadian worker, Raymond Côté, saw the beginnings of a serious chemical attack [74] applying the newly introduced techniques of charcoal column [75] and paper chromatography [76] to separate small oligosaccharides liberated from the purified ovarian cyst glycoproteins by mineral acid hydrolysis. In time others, including post-doctoral workers T.J. Painter and A.S.R. Donald, and students I.A.F.L. Cheese, V.P. Rege and A. Marr, joined the team and made substantial contributions to the isolation and identification of oligosaccharide fragments. Among the first five disaccharides released from the partial acid hydrolysis products of A-substance was the serologically A-active disaccharide, GalNAcα1-3Gal; the structure of this compound confirmed the role of a terminal *N*-acetylgalactosamine unit in A-specificity and the α-anomeric linkage of this terminal sugar. Moreover it showed that the *N*-acetylgalactosamine was joined to the OH-3-position of a subterminal galactose residue [74]. Two other disaccharides among these first identified fragments were Galβ1-3GlcNAc and Galβ1-4GlcNAc [74]: these were later to be designated Type 1 and Type 2, respectively, and have since been shown to occur as peripheral core structures in both *O*- and *N*-linked oligosaccharide chains. It is perhaps of interest that in part the identification of the structures of the disaccharides was based on reactions on paper chromatograms with benzidine-trichloracetic, a reagent giving colour reactions with compounds containing galactose joined to *N*-acetylglucosamine that differ markedly according to the positional linkage of the galactose residue; later it was found necessary to ban this reagent when its carcinogenic properties were recognised but its use contributed considerably to the early identification of the linkages. Further investigations on the products of mineral acid degradation of A-active glycoprotein yielded in 1961 two A-active trisaccharides (Table 4) which demonstrated that the terminal *N*-acetylgalactosamine residue could be linked αl,3 to both the Type 1 and Type 2 disaccharides [77]. In the following year the isolation of the Type 1 A-trisaccharide was reported from Elvin Kabat's laboratory in New York [78] where a programme to characterise oligosaccharides released from the acid hydrolysis products of ovarian cyst glycoproteins had been initiated. For a few years thereafter a friendly rivalry existed between the two laboratories. The two trisaccharides isolated at the Lister Institute were more active than the A-active disaccharide in haemagglutination inhibition tests, and the trisaccharide isolated in New York was more active in precipitation inhibition tests than the disaccharide: thus supporting the belief that the complete A-determinant was larger than a simple mono- or di-saccharide unit.

With mineral acid as the hydrolytic agent it had been found necessary to use several grams of ovarian cyst glycoprotein in order to isolate milligram quantities of oligosaccharides and hence, in order to improve the yield, water-soluble non-dialysable polystyrene sulphonic acid was introduced in our laboratory by T.J. Painter as a catalyst in place of mineral acid [79,80]. The procedure that was devised for use with this reagent allowed continuous dialysis throughout the process of degradation and the continual removal of the liberated fragments from the catalyst gave better yields of oligosaccharides in which the amino-sugars largely retained their *N*-acetyl groups intact. Application of this method to the partial hydrolysis of B substance resulted in the identification of a disaccharide Galα1-3Gal [81] and two serologically-active trisaccharides Galα1-3Galβ1-3GlcNAc and Galα1-3Galβ1-4GlcNAc [82] (Table 4). The α1,3-linkage of the terminal sugar in the B-active oligosaccharides was thus shown to be the same as in the A-active structures and the isolation of the two trisaccharides demonstrated that the Type 1 and Type 2 chain endings were present in both specific substances; thus favouring the idea of common precursor oligosaccharide chains on which the active determinants were built. Further support for this view was derived from the isolation of the same three new trisaccharides [83] namely, Galβ1-3GlcNAcβ1-3Gal, Galβ1-4GlcNAcβ1-3Gal and GlcNAcβ1-3Galβ1-3GalNAc, from blood group A, B, H and Le^a substances. From these fragments, and others isolated earlier, the simplest sequences of the two types of chains bearing the blood group active groupings in the glycoproteins were proposed in which the reducing GalNAc was thought to be the sugar linking the chains to the peptide moiety [83] (Table 5).

The proposed structures did not contain fucose because, owing to its acid lability, it was lost from the hydrolysis products and we realised that if the complete structures of A, B, H and Lewis determinants were to be established, methods other than acid hydrolysis would have to be used. One exception was an H-active trisaccharide released from acid hydrolysis of H-active ovarian cyst glycoprotein which had the structure Fucα1-2Galβ1-3GlcNAc [84]. Serological inhibition tests had suggested that the fucose concerned in H specificity was probably α1,2-linked but the identified trisaccharide gave the first confirmation of this linkage. However, the survival of

Table 4. Oligosaccharides isolated from the acid hydrolysis products of human blood group A, B, H and Le^a ovarian cyst glycoproteins.

(1) Common to hydrolysates of A, B, H and Le^a substances
Galβ(1 → 3)GlcNAc Galβ(1 → 4)GlcNAc
GlcNAcβ(1 → 3)Gal Galβ(1 → 3)GalNAc
Galβ(1 → 3)GlcNAcβ(1 → 3)Gal
Galβ(1 → 4)GlcNAcβ(1 → 3)Gal
GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc
(2) Occurring only in hydrolysates of A substance
GalNAcα(1 → 3)Gal
GalNAcα(1 → 3)Galβ(1 → 3)GlcNAc
GalNAcα(1 → 3)Galβ(1 → 4)GlcNAc
(3) Occurring only in hydrolysates of B substance
Galα(1 → 3)Gal
Galα(1 → 3)Galβ(1 → 3)GlcNAc
Galα(1 → 3)Galβ(1 → 4)GlcNAc

Compiled from [74,77,81,82,83]. Abbreviations as in Table 3.

Table 5. Partial structures proposed for the oligosaccharide chains in the ovarian cyst glycoproteins.

(1) 1963: Rege *et al.* [83]

A-substance: Type 1 chain GalNAcα(1 → 3)Galβ(1 → 3)GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc

Type 2 chain GalNAcα(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc

B-substance: Type 1 chain Galα(1 → 3)Galβ(1 → 3)GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc

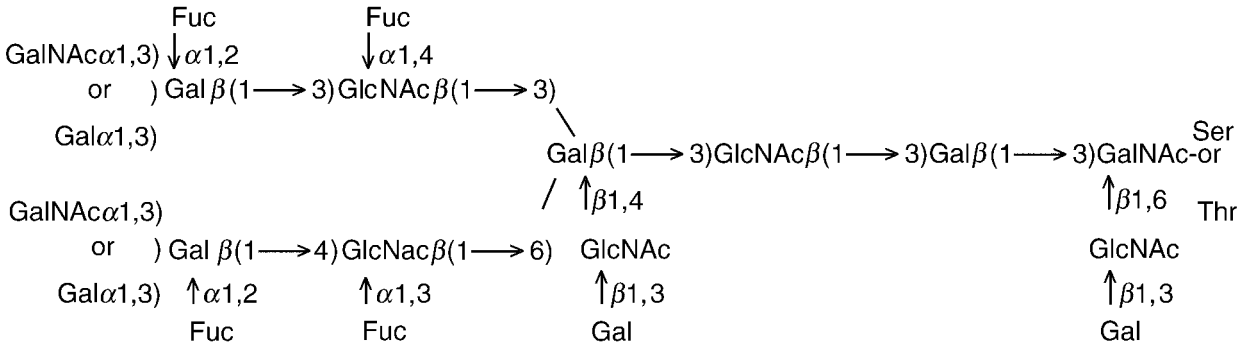
Type 2 chain Galα(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc

H, Le^a and precursor substances

Type 1 chain Galβ(1 → 3)GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc

Type 2 chain Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 3)GalNAc

(2) 1968: Lloyd and Kabat [92]



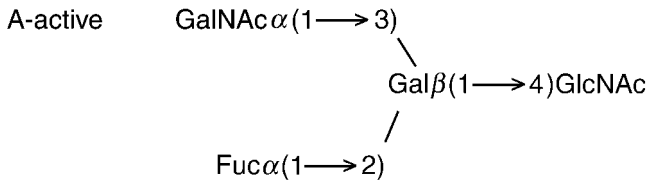
Abbreviations: Ser, Serine; Thr, threonine; others as in Table 3.

this fragment in the acid hydrolysis products was considered to be fortuitous and alkaline degradation was then introduced in attempts to isolate the intact determinant structures. Alkaline cleavage of oligosaccharide chains in glycoproteins was known to proceed by a β-elimination reaction with subsequent peeling of the chains unless they were immediately stabilised by reduction with sodium borohydride. This method theoretically yields intact oligosaccharides but, because at that stage we did not know the length or complexity of the oligosaccharide chains, and were convinced that the determinant structures were at their non-reducing terminals, we used alkaline conditions that allowed "peeling" to occur in order to obtain fragments of a size that could be more easily identified by the analytical methods then available. Degradation with aqueous methanolic triethylamine, followed by removal of the volatile base under reduced pressure and dialysis of the residual solution was the method selected. The indiffusible residue was repeatedly treated in the same way until 70% of the starting material was converted into diffusible fragments. The oligosaccharides were separated from this residue by charcoal and paper chromatography as before. By applying this procedure to an H-active glycoprotein a second trisaccharide, the Type 2 Fucα1-2Galβ1-4GlcNAc, was identified [84]; thus confirming that the H-structure can be built on both Type 1 and Type 2 chain endings (Table 6). We were gratified to find that an Le^a-active trisaccharide, Galβ1-3[Fucα1-4]GlcNAc, identified in the triethylamine degradation products of an Le^a glycoprotein [85] had a structure that was identical (Table 3) with that deduced for the Le^a determinant from the earlier serological inhibition tests with milk oligosaccharides [60]. Application of the same alkaline degradation procedure to A and B-active glycoproteins [86] finally led to the isolation in 1965 of Type 2 A and B-active tetrasaccharides, complete with fucose attached α1,2- to the sub-terminal galactose unit (Table 6). The earlier identification of the straight chain Type 1 and 2 trisaccharides from both A and B substances pointed to the presence of similar fucosylated structures occurring on Type 1 chains and we now felt confident that we had a firm grasp of the structures of the terminal sequences responsible for A and B specificity in the ovarian cyst substances.

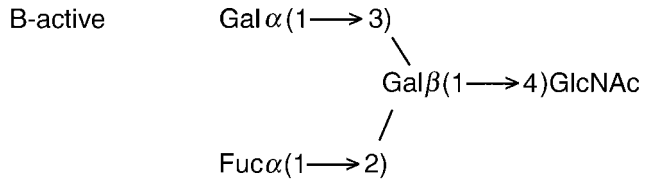
The last phase in the isolation of blood-group active fragments from acid and alkaline degradation products of the ovarian cyst substances at the Lister Institute came in 1967, with the identification of an Le^b-active pentasaccharide, Fucα1-2Galα1-3[Fucα1-4]GlcNAcβ1-3Gal [87] (Table 6). Many other fragments had been isolated during the course of these investigations but the characterization of the Le^b oligosaccharide completed our goal to chemically identify the five serologically-active blood-group determinants found in secreted glycoproteins. The Le^b structure was also of particular interest because it was a clear example of a hybrid sequence compounded of the H and Le^a active groupings that had neither H nor Le^a specificity, but a new specificity Le^b. It was evident from the structures of the Le^a and Le^b determinants that, although the A and B determinants can be attached to both Type 1 and Type 2 chain endings, only Type 1 chains can carry Le^a and Le^b sequences. The interrelationships between ABH and Lewis determinants became clear from the structures of these isolated fragments. In addition, the knowledge that there were two types of chain endings bearing the determinants enabled the results of the exo-glycosidase degradation experiments to be more clearly understood. Later studies

Table 6. A, B, H, Le^a and Le^b active fragments isolated from the alkaline degradation products of ovarian cyst glycoproteins.

(1) Fragments from HLe^b -active glycoprotein
H-active Fucα(1 → 2)Galβ(1 → 4)GlcNAc
Leb-active i. Fucα(1 → 2)Galβ(1 → 4)[Fucα(1 → 4)]GlcNAc
ii. Fucα(1 → 2)Galβ(1 → 4)[Fucα(1 → 4)]GlcNAcβ(1 → 3)Gal
(2) Fragments from Le^a-active glycoprotein
Le^a-active Galβ(1 → 3)[Fucα(1 → 4)]GlcNAc
Le^x-active Galβ(1 → 4)[Fucα(1 → 3)]GlcNAc
(3) Fragment from A-active glycoprotein



(4) Fragment from B-active glycoprotein



Compiled from [84,85,86,87,96]. Abbreviations as in Table 3.

showed the occurrence of other core structures bearing the determinants in different tissues (reviewed in [88]); another shown to be present in the glycoproteins in ovarian cyst fluids, and in saliva, is the Type 3 structure, Galβ1-3GalNAc-α, which carries short ABH sequences joined *via* the *N*-acetylgalactosamine unit to serine or threonine residues in the peptide backbone [89].

A few more oligosaccharide fragments were identified among the alkaline degradation products of the cyst substances at this stage in our laboratory but once we knew the structures of the determinants attention was largely turned to finding out how the antigenic structures were synthesised and their relationship to the blood-group genes. In Elvin Kabat's laboratory application of the alkaline borohydride procedure by K.O. Lloyd and colleagues led to the isolation from A, B and H substances of a series of larger fucose-containing oligosaccharides which confirmed the Type 2 structures and identified a similar series of Type 1 structures [90,91]. Subsequently, Lloyd *et al.* [92] confirmed the structure of the Le^a determinant and isolated from the same degradation products a reduced branched oligosaccharide Galβ1-3GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Gal-ol. This was an important discovery because it demonstrated that in the cyst glycoproteins the Type 1 and Type 2 structures could be present as branches on a single oligosaccharide chain. They also identified reduced oligosaccharides from Type 2 A, B or H structures which were further substituted with α1,3 linked fucose on the *N*-acetylglucosamine residue [90]; this was the first report of this linkage which has since been found to play such an important role in tumour antigens [93], developmental antigens [94] and in ligands for adhesion molecules involved in inflammatory reactions [95]. At the Lister Institute the unsubstituted trisaccharide, Galβ1-4[Fucα1-3]GlcNAc, was one of the last fragments identified in the alkaline degradation products of an Le^a active preparation, demonstrating that the structure exists as a terminal sequence on some of the Type 2 chains of the ovarian cyst glycoprotein [96]. This structure has more recently become known as SSEA1, CD15 or Le^x, and now tends to be included by glycobiologists in the Lewis blood-group system, although it does not occur on the red cell and may be synthesised by the enzymic products of genes other than *Le*. The recent use of the term Le^x has caused some confusion among blood-group serologists because the designation anti-Le^x had much earlier been coined [97] to describe an antibody that agglutinated both Le(a+) and Le(b+) red cells but not Le(a− b−)cells: this antibody was almost certainly not detecting the structure now called Le^x.

Many of the sera formerly designated anti-O are now known to belong to the group of anti-I blood-group reagents which react strongly with group O red cells. Ovarian cyst substances usually inhibit the agglutination of O cells by anti-I sera but increased activity is detectable in partially degraded cyst glycoproteins indicating that I determinants are present as cryptic internal sequences in chains bearing A, B and H determinants. The relationship in ovarian cyst glycoproteins between blood group I structures and A, B and H was

investigated by Elvin Kabat, Ten Feizi and colleagues [98,99]. Anti-I sera are heterogeneous and can be subdivided into several groups [98] but the structure Galβ1-4GlcNAcβ1-6-R was found to be inhibitory for one group [99]. This Type 2 structure, which also constitutes part of the branched pentasaccharide isolated from the Le^a-active ovarian cyst substance [92], is obviously a potential precursor of H and hence of A and B structures. An investigation of the relationship between the structure reacting with the anti-I sera and that recognised by Type XIV pneumococcal anti-sera showed that, whereas the anti-I sera require the subterminal β-*N*-acetylglucosaminyl residue to be linked β1-6 to the next sugar, the combining site of the anti-Type XIV sera is mainly directed to the terminal disaccharide unit [100]. This information therefore told us that the Type 2 structures earlier found to cross-react with the anti-Type-XIV reagent after enzymic degradation of the carbohydrate chains could have been present on either straight or branched oligosaccharide chains whereas the Type 2 sequences that react with anti-I sera after partial degradation must be present as part of more complex structures since the grouping attached by a β1-6 linkage only occurs as a branch unit.

The larger fragments isolated from the alkaline–borohydride degradation experiments, together with the demonstration of the branch structure carrying the Type 1 and Type 2 disaccharide sequences, showed that at least some of the complete oligosaccharide chains must be longer and more complex than the simple structures first suggested [83] and in 1968 Lloyd and Kabat [92] proposed a possible composite structure accommodating all the active and inactive oligosaccharides isolated at that time from ovarian cyst glycoproteins (Table 5). Although a convenient framework to demonstrate the determinants, and much quoted, such a megalosaccharide structure has yet to be isolated and was intended to be a statistical average and not a definitive structure of every carbohydrate chain [100]. The precise array of oligosaccharide structures present in the ovarian cyst glycoproteins remains to be elucidated but there is evidence of considerable heterogeneity, with chains decreasing in size and composition down to a single *N*-acetylgalactosamine residue [101].

The peptide moiety of the blood group active ovarian cyst glycoproteins

Although the major efforts of the group at the Lister Institute were directed toward elucidation of the blood-group determinant structures the analytical studies [38–41] carried out before we had determined which part of the molecules were involved in specificity had led to an interest in the peptide moiety of the ovarian cyst glycoproteins. Amino acid analyses carried out by hand before the introduction of automatic amino acid analysers indicated the presence of some 15 amino acids of which the predominant ones in all the preparations were threonine, serine and proline [102]; on a molar basis these three amino acids made up more than 50% of the total [102,103]. Pronase treatment of ovarian cyst glycoproteins led to a loss of some amino acids with the retention of all the carbohydrate and full blood group serological activity leading to the concept, now accepted for many mucin-type glycoproteins, of two regions in the peptide core, one rich in aspartic and glutamic acid residues and virtually free of carbohydrate chains and the other rich in serine and threonine that carries the majority of the densely packed carbohydrate chains [103]. The pronase products of B, H and Le^a substances, which contain *N*-acetylgalactosamine only in the carbohydrate-peptide linkage region, each had a near 1:1 ratio of *N*-acetylgalactosamine to hydroxyamino acids indicating that virtually all the serine and threonine residues are glycosylated. Partial deglycosylation of the pronase-treated glycoproteins by treatment with methanolic HCL removed 80% of the carbohydrate and left smaller glycopeptides that contained *N*-acetylgalactosamine as the predominant sugar [104,105]. These results confirmed the deductions from alkaline borohydride degradation that implicated this sugar in the oligosaccharide-peptide linkage region in the ovarian cyst glycoproteins [106]. The molar ratios for some of the amino acids in the pronase treated glycoproteins were fairly constant for all the samples, for example the threonine to serine ratio is close to 5:1 and the glycine to alanine ratio is close to 1: 2, but this was not true for other amino acids which suggested that there could be more than one peptide forming the scaffold for the oligosaccharide chains in these glycoproteins [103]. In view of what is now known about the many different peptide genes encoding mucin backbones [107] this would not be surprising but so far the genes for the peptide core(s) of the ovarian cyst glycoproteins have not been characterised.

ABH and Lewis antigens on red cells

In the 1950s the pioneering work of Yamakawa in Japan [108] suggested that blood-group activity was associated with glycolipid fractions isolated from red cell membranes but it was not until the 1970s that ABH antigens from red cells were purified and chemically characterised through the efforts of Koscielak in Poland [109] and Hakomori in the USA [110]. These first compounds demonstrated the occurrence of families of relatively short-chain glycosphingolipids that carried Type 2 A, B and H activity. In a simple experiment the specificity relationship between a glycolipid A preparation isolated from group A red cell membranes and A substance purified from ovarian cyst was examined by a double diffusion (Ouchterlony) test with a rabbit anti-A serum; the two substances gave a line of identity indicating that the same terminal structures were present in the two macromolecules despite their overall dissimilarity [111]. More complex glycolipids carrying ABH activities were subsequently identified in Hakomori's laboratory [112] and compounds with highly-branched carbohydrate moieties containing 20–60 sugar units were characterised by Koscielak and colleagues and termed polyglycosylceramides [113]. Although the characterisation of these glycolipid red cell preparations

appeared to fulfil the earlier predictions concerning the association of the ABH antigens with lipid on the red cell surface, later investigations revealed that, glycoproteins with *N*-linked glycans also bear ABH determinants [114,115] and probably carry the major part of the red cell blood-group ABH activity. In contrast to the Type 2 ABH determinants which are integral parts of the red cell membrane the Type 1 Le^a and Le^b determinants detected on the red cell surface are associated with glycosphingolipids that are taken up from the plasma in which the cells circulate [116].

Minimal ABH and Lewis determinant structures, distribution and chemical synthesis

The production of monoclonal antibodies against blood group structures carried on different carrier molecules has enabled the definition to be made of minimal structures necessary to give a particular blood group specificity [117] (Figure 3). Oligosaccharides bearing these terminal sequences can be found as cell surface structures in many different tissues and for this reason the determinants are now frequently referred to as histo-blood group antigens. The remarkable pioneering immunofluorescence studies of Szulman disclosed striking temporal and spatial changes in the expression of the ABH antigens in the course of normal embryonic development [118,119]. Changes of ABH expression occur in malignancy sometimes resulting in loss of expression and sometimes in gain of expression in tissues not normally expressing the blood-group antigens [120]. Loss of A or B expression in tumour cells can give a poor prognosis [121] despite the fact that no precise functions have as yet been assigned to the A and B antigens and that neither of these structures can be essential since absence of the antigens in group O individuals does not have any obvious deleterious effects.

Even the improved methods we had used for the fragmentation of the glycoproteins yielded barely sufficient material for structural characterisation and serological identification of the isolated oligosaccharides but in the mid-1970s Lemieux and colleagues introduced the halide ion catalysed α-glycosylation reaction which for the first time permitted chemical synthesis of complex carbohydrate structures containing both α- and β-linkages [122]. The use of this methodology has enabled quantities of the blood group determinants to be synthesised for practical purposes as artificial antigens and solid immunoadsorbents, and for use in fundamental studies concerned with molecular conformation and antibody and lectin binding [122,123].

Glycosyltransferase products of the blood group genes

The elucidation of the detailed structures of the determinants enabled more precise predictions to be made concerning the nature of the primary products of the blood-group genes and the additions to the Type 1 and Type 2 chains catalysed by these enzymes (Figure 4). Thus the *A* and *B* gene products were proposed to be an α1,3-*N*-acetylgalactosaminyl- and an α1,3-galactosyltransferase, respectively, the *H*-gene product to be an α1,2-fucosyltransferase and the *Le*-gene product to be an α1,4-fucosyltransferase [67]. A sabbatical spent in 1960/61 in the laboratory of Professor William (Zev) Hassid, University of Berkeley, a pioneer in the glycosyltransferase field, enabled W.M.W. to investigate the biosynthesis of lactose [124] and thereby to learn some of the properties of this newly characterised class of enzyme. On her return to the Lister Institute a small group was set up to begin the search for the enzymes we had predicted to be involved in biosynthesis of the blood-group determinants. UDP-galactose was the only nucleotide donor of the sugars present in the blood group-active structures that was comercially available at that time so that both unlabelled and radioactively labelled UDP-*N*-acetylgalactosamine and GDP-fucose had to be enzymically, or chemically synthesized, and purified, before experiments could be started. We were nevertheless fortunate to have a range of milk oligosaccharides [59] from Richard Kuhn that enabled us to test a series of closely related, well-defined, low-molecular-weight comounds as acceptor substrates for the glycosyltransferases. Initially, once the requisite radioactive nucleotide donor sugars were available, studies were carried out with post-mortem samples from human, baboon and rabbit stomachs and human submaxillary glands as a possible source of the transferases since epithelial tissues were known to be rich in ABH blood-group-activity. After suitable incubation times the reaction mixtures were serparated by paper chromatography or paper electrophoresis, the papers were scanned in a radiochromatogram scanner and incroporation of radioactivity into the acceptor was quantified by cutting out the peak areas and counting them in a scintillation scanner. Preliminary experiments revealed the presence of α-and β-galactosyltransferases and a β-*N*-acetylglucosaminyltransferase in particulate preparations from rabbit gastric mucosa [125] and, although caution prevented us voicing any firm conclusions at the time, the use of the Type 2 H-active trisaccharide as an acceptor for the α-galactosyltransferase suggested that we were in fact detecting the *B*-transferase in the rabbit tissue. Subsequently with colleagues Caroline Race, Veronica Hearn and Zeenat Gunja Smith the *A*-gene associated α1,3-N-acetylgalactosaminyltransferase was firmly identified in particulate preparations from human glands and stomachs from blood-group A and AB individuals [126] and the *B*-gene associated α1,3-galactosyltransferase in similar tissues of group B and AB individuals [127–129]. Neither of these enzymes was found in tissues from group O persons, but in individuals of the appropriate groups *A* and *B* transferases were expressed in epithelial tissues of both secretors and non-secretors; thereby confirming that absence of A and B activity in secretions is not due to failure of expression of the *A* and *B* genes. Both the transferases had a strict requirement for a terminal β-galactosyl residue substituted at the 2-OH position with L-fucose, that is, for an H-active structure. Whether the

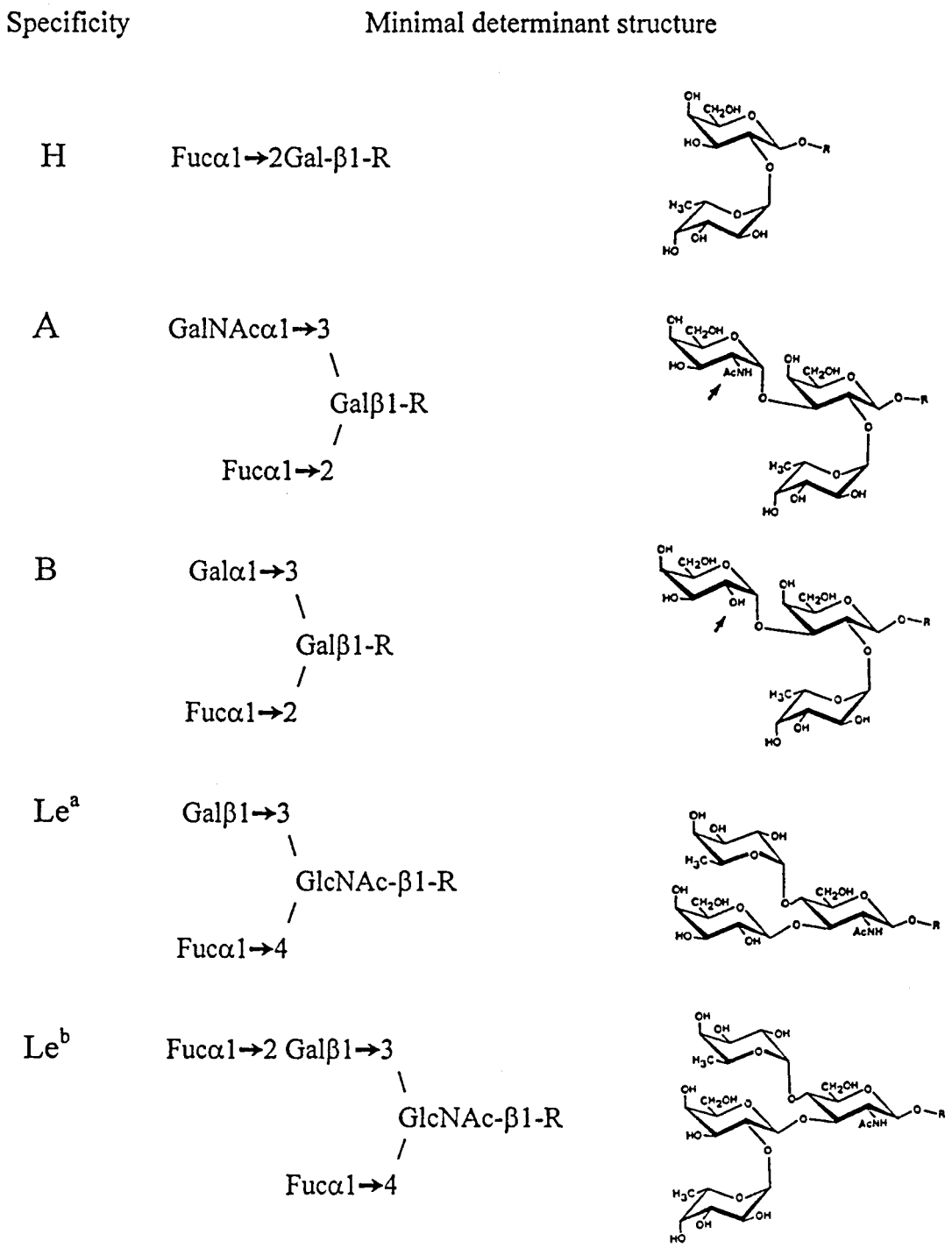


Figure 3. Minimal structures necessary for the expression of blood-group H, A, B, Le^a and Le^b specificity on glycoproteins or glycolipids. The arrows denote the only position where differences occur between the blood group A and B structures. AcNH, *N*-acetylaminogroup; other abbreviations as in Table 3.

sugar adjacent to the galactosyl residue is *N*-acetylglucosamine, (linked β1,3 or 1,4) or glucose (linked β1,4) did not influence the capacity of the oligosaccharides to function as acceptors and, because of its ready availability in human milk [59] the H-active trisaccharide, 2′-fucosyllactose (Fucα1-2Galβ1-4Glc), was selected as the low-molecular weight substrate of choice for routine assays for the *A* and *B* transferases. In contrast, compounds which have a fucose residue attached to the subterminal *N*-acetylglucosamine or glucose residue, whether in a mono-fucosyl compound as in

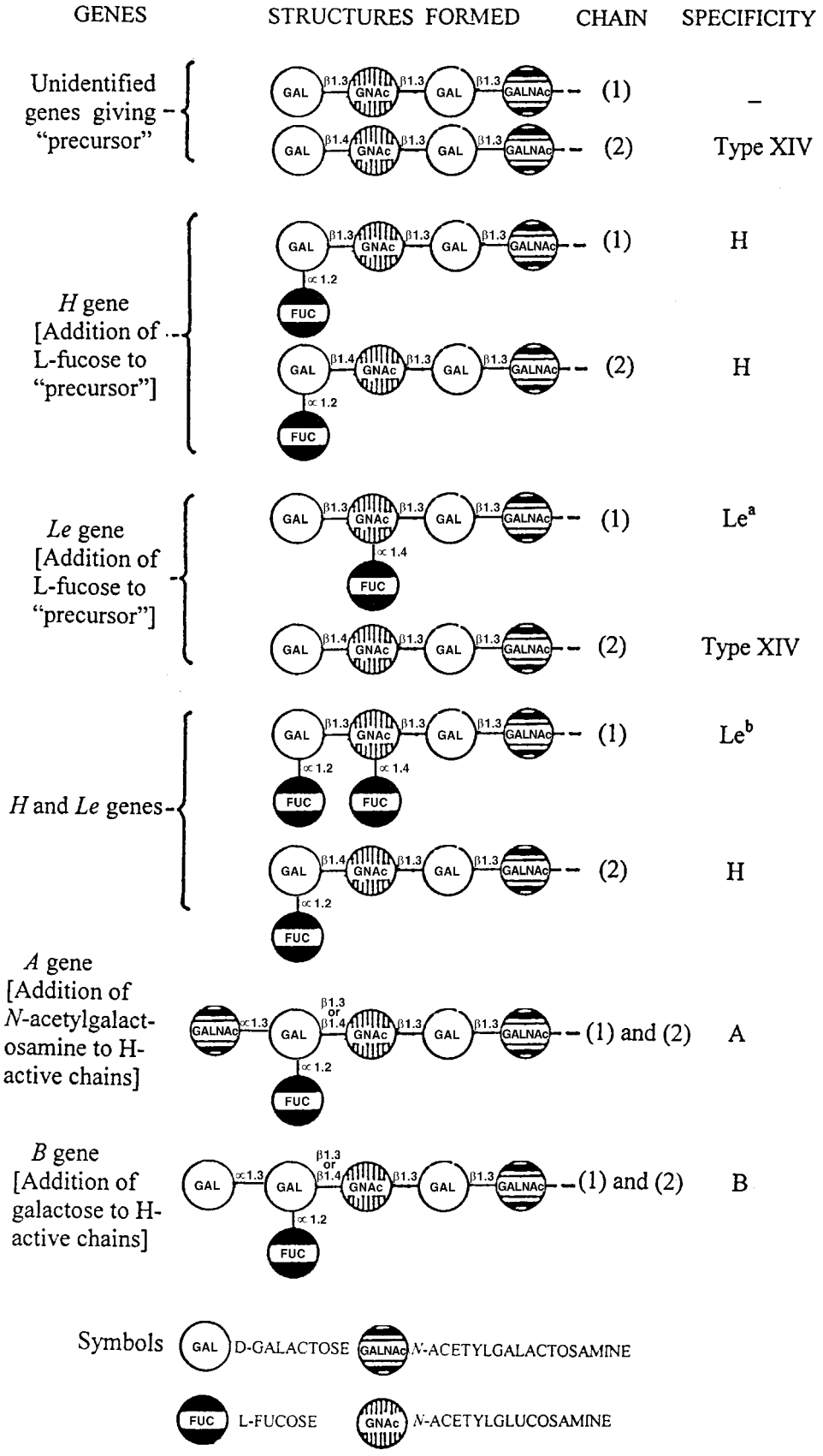


Figure 4. Proposed biosynthetic pathways for H, Le[a], Le[b], A and B determinants based on knowledge available in 1966 (Watkins [67]).

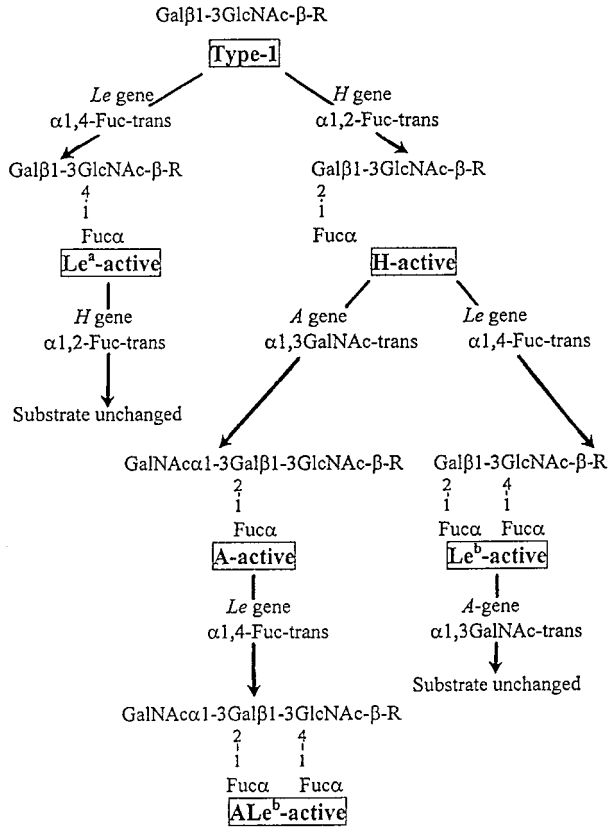


Figure 5. Biosynthetic pathway for the formation of Le^a, H, A, Le^b and ALe^b determinants on Type 1 (Galβ1-3GlcNAc-β-R) oligosaccharide chains. Abbreviations as in Table 3.

lacto-*N*-fucopentaose II (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc; Le^a-active) or in a di-fucosyl compound as in lacto-*N*-difucohexaose I (Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc; Le^b-active) were shown not to be substrates for the *A*- and *B*-transferases [126,128] (Figure 5).

Meanwhile in the United States a different approach had been taken by Victor Ginsburg and his colleagues. His interest arose from earlier studies on the isolation of GDP-fucose and its formation from GDP-mannose [130] and, since the enzyme involved in lactose synthesis had been demonstrated to occur in soluble form in human milk [131], he investigated the possibility that this secretion might be a source of a fucosyltransferase that could utilise GDP-fucose to convert lactose into 2′-fucosyllactose. The discovery that an α1,2-fucosyltransferase acting on lactose could be detected only in the milk from women who were classified as ABH secretors [132] demonstrated that the α1,2-fucosyltransferase in this secretion was under the control of the secretor gene *Se* and thus confirmed the prediction that the secretion-non-secretion phenomenon is dependent on the expression of H structures. When Akira Kobata joined Victor Ginsburg's group they went on to investigate enzymes giving rise to Lewis phenotypes and identified an α1,4-fucosyltransferase in the milk of Le(a+) or Le(b+) donors that was absent from milk of donors grouped as Le(a− b−) [133]. They also showed the presence of soluble *A*- and *B*-transferases in this secretion from donors of the appropriate blood-groups [134,135]; these enzymes had specificity requirements that were identical with those of the *A* and *B* transferases in particulate preparations from epithelial tissues. At the Lister Institute M.A. Chester, having successfully made a preparation of GDP-fucose, confirmed that α1,2-fucosyltransferase activity was present only in human submaxillary glands and mucosal tissue of ABH secretor individuals and that α1,4-fucosyltransferase activity was detectable only in the tissues of Lewis-positive individuals [136]. Thus by the late 1960s the two laboratories had identified the four predicted transferases encoded by the *A*, *B*, *H* and *Le* genes in both particulate and soluble forms, and new tools were thus available to investigate the genetics of these blood-group systems at a level in the biosynthetic pathway closer to the genes than the antigens on red cells and in secretions.

Studies on the distribution of the glycosyltransferases in different human tissues revealed more convenient sources for investigations on rare ABO variants than mucosal tissue or milk. When in our laboratory we came to test for transferase activity in plasma it transpired that the use of particulate preparations from epithelial tissues had so raised our expectations of the level of activity at which the blood-group-gene encoded glycosyltransferases are expressed that

we concluded that this tissue fluid was not a potential source. However, others using more sensitive methods, or with lower expectations, recorded that plasma was indeed a source of *A*, and *B* transferase activities [137,138] and subsequently this tissue fluid proved invaluable for carrying out routine studies on these enzymes (reviewed in [139]). A visiting worker in our laboratory in 1972, Helmut Schenkel-Brunner, who, while working in Vienna with H. Tuppy, had shown that it was possible to convert group O erythrocytes into A-active cells by treatment with the *N*-acetylgalactosaminyltransferase in human gastric mucosa [140] undertook with M.A. Chester an investigation of the fucosyltransferases in plasma [141]. In contrast to the α1,2-fucosyltransferases in human milk, submaxillary glands and stomach tissue that are found only in ABH secretors, an enzyme that transferred fucose to the 2-OH position of non-reducing *β*-galactosyl residues was found to be present irrespective of secretor status in the plasma of all normal ABO donors. It was reassuring to find, in accordance with earlier predictions that the Bombay O_h phenotype results from the genotype *hh* [29,30], that the only plasmas from which α1,2-fucosyltransferase activity was missing were the samples from Bombay O_h donors. In contrast to the missing *H* transferase, the *A* and *B* transferases appropriate to their true *ABO* groups are readily detectable in plasma of O_h individuals [142] and can thus be used to establish the true ABO phenotype of persons belonging to this rare blood group. Measurement of the levels of α1,2-fucosyltransferase activity in plasma from donors of different ABO groups revealed that, while there is a spread of activity for each ABO group, the average levels in the plasma from donors carrying an *A* gene is higher than in other groups [139,143,144]; no explanation has yet been found for this observation. No *Le* gene associated α1,4-fucosyltransferase activity was detectable in plasma but all samples, including those from Bombay O_h donors, contained an α1,3-fucosyltransferase that conveyed fucose to the 3-OH position of *N*-acetylglucosamine [141]. The demonstration that phenyl *β*-D-galactoside is specifically fucosylated in the OH-2 position by the enzymes in plasma suggested this compound as a useful acceptor substrate for the assay of the blood-group *H*-gene specified α1,2-fucosyltransferase [143].

Change of venue

1975 saw the closure of the Lister Institute and the transfer of our laboratory to the Medical Research Council's Clinical Research Centre on the outskirts of London where work continued on the blood-group-related glycosyltransferases. W.T.J.M. had retired from his University chair in 1968 and, although he continued with research both at the Lister Institute until 1975 and at the MRC Clinical Research Centre until 1989, following the completion of the structures of the ABH and Lewis determinants he turned his attention to structural studies on other carbohydrate blood-group antigens, firstly to investigations on the P_1 determinant in the P blood-group system [145] and subsequently to the Sd^a determinant in the Sid system [146].

Weak and rare subgroups of ABO

The availability of the glycosyltransferase products of the blood-group genes provided new reagents for examination of weak ABO subgroups and rare anomalies uncovered by serological studies. Schachter *et al.* [147] demonstrated that, although individuals grouped as A_1 and A_2 both express *N*-acetylgalactosaminyltransferases in sera, the enzymes from individuals of the A_2 subgroup differed in the level of activity and pH optimum from those in sera of A_1 individuals. We also found differences in the isoelectric points of the A^1 and A^2 transferases that enabled the two transferases to be preparatively separated from the serum of a donor of the genotype A^1A^2 by isoelectric focusing [148], thus demonstrating that the enzymes encoded by the two genes are expressed independently in this genotype. These differences for the first time permitted the genotype A^1A^2 to be clearly distinguished from A^1A^1 and A^1O. In addition, measurements of activity and kinetic properties of the enzymes in rare weak subgroups of A and B, both in our laboratory and in C. Salmon and J-P Cartron's laboratory in Paris, indicated that there were probably a large number of other mutant alleles at the *ABO* locus expressing modified forms of the *A* and *B* transferases (reviewed in [139]). An interesting application of transferase assays was that of determining the true ABO genotype of chimeras resulting from placental cross circulation in dizygotic twins. In these twins the cells with different zygotic lineages are confined to the haemopoietic tissue and if the original zygotes differ in their ABO groups each twin may carry two red cell populations carrying different antigenic determinants. In these individuals assays of the *A* and *B* transferases in their plasma can identify their true ABO phenotype [149,150]. In the second type of human chimera, the dispermic or tetragametic chimeras, the presence of two genetically different cell populations in different tissues is believed to arise from the fertilisation of two maternal nuclei by two sperm and the subsequent fusion of the two zygotes to form one organism. Most examples have been detected initially because of an unusual appearance, such as eyes of a different colour or patchy skin pigmentation, or because of abnormalities of the external genitalia. However, in a case referred to us for transferase assays because of the absence of anti-A in the serum of an apparently normal group B blood donor the findings of a strong A^1 transferase in his serum initiated a study which eventually showed the propositus to be a chimera with two cell populations expressed in different tissues, one female A^1A^1 or A^1O/XX and the other male BO/XY [151]. In another unusual case which appeared to combine aspects of twin and dispermic chimerism [152] apparently identical twin boys both had *XX* and *XY* lymphocytes and two red cell populations, A_1 and B, in their peripheral blood. However, whereas twin I appeared from transferase assays to be

genetically A^1 and to have B cells only his blood, hence to be a normal twin chimera, twin II had a strong *B* transferase and evidence of B expression in tissues other than blood, suggesting that he is a dispermic chimera. The results of cytogenetic tests combined with the ABO grouping of blood and saliva, together with the transferase studies, suggest the most probable explanation for this pair is that they originated as an A^1O/XY zygote, which became monozygotic foetuses, and that a third female embryo, BO/XX fused with one of the twins to give the dispermic chimera. We examined blood from quite a few other dispermic chimeras kindly sent to us by our blood grouping colleagues but no others turned out to be quite so fascinating, or were enlightened to the same degree by transferase assays, as these two cases.

Studies on certain AB phenotypes revealed how apparent gene domination can arise through competition of the enzymic products for the common H substrate. AB individuals who had inherited a *B* gene encoding a strong *B* transferase and an A^2 gene express both *B* and A^2 enzymes in their plasma at levels appropriate to their subgroup, but the strong *B* transferase appears to assume dominance over, and largely suppress, the weaker *A* transferase at the level of expression of the red cell surface antigens [153,154]. Similarly a number of bloods grouped on red cells as A_2B could be shown to be of the genotype A^1B when the enzymes were assayed. Solving anomalies of this kind proved of value in paternity cases where serological evidence alone appeared to preclude parenthood and contributed to the argument as to whether the A_1-A_2 subgroup difference is qualitative or quantitative. This debate has been vigorously pursued for many years and the question is still not completely resolved. In 1957 we proposed that the difference was quantitative and that the A_2 cells had fewer A sites and more H antigenic sites than A_1 cells because fewer of the H structures are converted into A determinants [155] and the demonstration that the A^2 gene encoded enzyme is less effective in transfering *N*-acetylgalactosamine to H structures than the enzyme encoded by the A^1 allele [147] lent support to this idea. The opponents of this view, however, have assumed there are qualitative differences between the A antigens on the red cells of the two subgroups based on the existence of antibodies that agglutinate A_1 cells and not A_2 cells. The identification of extended Type 2 A determinants on A_1 cells that do not occur on A_2 cells [156], and the demonstration that these structures give rise to specific antibodies that agglutinate only A_1 cells, has shown that some qualitative differences do indeed exist between the determinants on A_1 and A_2 cells. However, conversion of A_2 cells into A_1 cells can be achieved by the transferase in A_2 serum after prolonged incubation [157] and this finding together with the suppression of an A^1 transferase by a strong B transferase so that the cells react as A_2B cells, indicates that the presence of the extended A structures are not the only differences that make for distinctions between A_1 and A_2 reactivity. Both quantitative differences in the total number and density of A sites as well as qualitative differences in the nature of some of the A determinants appear to contribute to the overall phenotypes of the A_1 and A_2 subgroups. It is of interest to note that A_2 cells have extended H structures in which Type 2 A determinants are masked [156] and therefore numbers of A sites on both A_1 and A_2 cells measured by conventional serological methods reveal only the structures exposed on the surface and not the total number of A sites that are synthesised.

Allelic status of the A and B genes

The successful cloning of the *ABO* gene locus [7] has provided incontrovertible proof that *A* and *B* are allelic forms at a single genetic locus. However, until cloning had been achieved doubts were still expressed concerning the allelic status of the sequences encoding the blood-group associated glycosyltransferases. These doubts arose because of the qualitatively different sugar donor specificity of the *A*-gene associated α1,3-*N*-acetylgalactosaminyl- and the *B*-gene associated α1,3-galactosyl-transferases. It appeared to us that in view of the close structural relationship between D-galactose and *N*-acetyl-D-galactosamine, differing as they do only by the substituent OH or $NHCOCH_3$ at the OH-2 position of the basic galactose ring, and the fact that both sugars are transferred to the same acceptors in α1,3-linkage, that the amino acid differences in the enzyme proteins necessary to accommodate the alternative donor nucleotide sugar would not necessarily have to be very great [139]. However, in order to discover how much they had in common we decided to examine the properties of the two enzymes more closely. A polyclonal antibody raised in rabbits against a purified preparation of the plasma *A* transferase was found to cross-react with the plasma *B* transferase [158] suggesting protein homology between the two enzymes. Yoshida *et al*. [159] had also raised an antibody to the *A* transferase but in contrast to the one we had prepared this antibody was said to cross react not only with the *B*-transferase but also with an enzymically inactive protein in O plasma. More rigorous tests on the donor substrate specificities of the transferases revealed that plasma samples from B individuals could under certain *in vitro* conditions transfer small amounts of *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine to H-precursor structures to form A determinants [160–162]. Unequivocal proof that an enzyme in B plasma is capable of synthesising A structures was provided by experiments in which group O cells were converted into cells readily agglutinable by mono-specific anti-A reagents [162] and by the structural characterisation of an A-active tetrasaccharide synthesised by the *B* transferase [161]. Subsequently plasma from group A individuals was found to have a weak capacity to utilise UDP-galactose to form a tetrasaccharide product with 2′-fucosyllactose that was identical with that synthesised by the *B* transferase [163]. These experiments therefore demonstrated that *in vitro* the two transferases had overlapping functions compatible with those to be expected of the products of allelic genes. Until this stage the A and B blood-group

status of red cells had appeared to be so distinct serologically that the two genes seemed not to have any overlapping functions *in vivo* but unexpectedly the advent of highly specific mouse monoclonal A antibodies revealed a subset of group B (now termed B(A)) blood donors whose red cells showed weak, but definite, A activity [162]. We had initially shown that *in vitro* the stronger the *B* transferase activity the greater the utilisation of UDP-*N*-acetylgalactosamine to make A structures [160] and the B(A) donors tested in our laboratory were found to have exceptionally high levels of *B* transferase activity [162]. Earlier Badet *et al*. [164] had noted a trimodal distribution when they studied the α1,3-galactosyltransferase levels in the Paris blood donors and it seems probable that the B(A)s belong to the third, small group with exceptionally high *B*-transferase activity.

Kinetic studies on the transferase activities when the "wrong" nucleotide was used showed that the *A* transferase has a greater capacity to accommodate the alternative donor substrate in its binding site than the *B* transferase, but once the substrate is bound the *B* enzyme has a greater capacity to catalyse the transfer of the sugar moiety [160]. When the *ABO* genes were cloned four amino acid substitutions were identified between human *A* and *B* transferases (Arg176-Gly, Gly235-Ser, Leu266-Met and Gly268-Ala) [7,165] and these changes were sufficient to switch the enzyme specificity from an enzyme transferring *N*-acetylgalactosamine to one transferring galactose. More recently the nature of the amino acid at codon 268 has been implicated as the residue most important in determining the *A* versus *B* nucleotide-sugar donor specificity [166]. To our knowledge the *A* and *B* genes are still the only recorded examples of allelic genes in mammals giving rise to glycosyltransferases with different donor sugar specificities although the phenomenon has been recorded in plants [167].

A puzzling observation for which no explanation has yet been found is that concentration of group O plasma many-fold sometimes reveals the presence of a glycosyltransferase that can synthesise A determinants [168]. Whether this is because an *O*-gene can express a small amount of *A*-transferase activity or whether some other enzyme, if suitably concentrated, can also transfer *N*-acetylgalactosamine to H acceptors has not been established although, as far as could be ascertained, the properties of the transferase in group O plasma are identical with those of the *A* transferase in group A plasma.

The secretor phenomenon

A question that was never far from our thoughts was the basis of the secretor phenomenon. The concept that was later to emerge for the sialyl- and fucosyl-transferases of families of tissue-specific transferase enzymes with closely related specificities encoded by different genes [169,170] had not been envisaged in the late 1950s when the biosynthetic pathways for the ABH antigens were put forward so that the idea that more than one enzyme could be involved in the addition of fucose to form H structures was not considered. Our first proposal was that the gene *Se* was a "transforming gene" controlling the addition of fucose units to a precursor glycoprotein to give H substance [68]. However, in order to have a unifying hypotheses that would take into account the differential appearances of H antigen on red cells and in secretions, we subsequently proposed that the *Se* gene was some form of regulator gene that controlled the expression of the *H*-gene in epithelial tissues [44]. Sustenance for the theory that *Se* regulated the expression of the *H* gene came from a much cited paper which was published in 1955. A child who was an ABH secretor appeared to have inherited an *H* and an *se* gene from his father and, since he was a secretor, the inference was that he had inherited from his O_h mother an *h* gene and an *Se* gene that she had been unable to express because of the absence of an *H* gene [171]. Twenty five years later, however, Oriol *et al*. [172] reported that analysis of 44 published pedigrees from Bombay O_h families failed to reveal any other examples with suppression of *Se*; suggesting doubts about the paternity of the child whose blood-group and secretor status had appeared to support the 'regulator' gene theory. Moreover, sporadic reports of a different Bombay phenotype, so-called "para-Bombay", in which an individual lacked, or had only very weak, ABH antigens on red cells but secreted normal amounts of these activities in saliva [173] led to doubts about the interpretation of the mode of action of the *Se* gene. From a different point of view, Lemieux's studies on the Type 1 and Type 2 disaccharides led him to suggest that their conformations were so different that it was unlikely that the same α1,2-fucosyltransferase could utilise both these disaccharides as substrates [122]. In view of these doubts Oriol *et al*. [172] returned to the idea that *Se* is a structural gene and proposed that it is a second gene encoding an α1,2-fucosyltransferase that is inherited independently of the *H* gene. The *Se* encoded enzyme was suggested to react preferentially with Type 1 chains, and to be expressed in tissues related to exocrine secretions, such as saliva, whereas the *H* gene encoded transferase was considered to react preferentially with Type 2 chains, and to be expressed in mesodermal tissues, such as red cells. This proposal did not challenge the predicted rôle of the *Se* gene in controlling the appearance of H structures in secretory tissues but it provided a different explanation for the function of this gene that could account for the para-Bombay phenotypes and moreover, stimulated a re-examination of the kinetics and fine specificity of previously identified α1,2-fucosyltransferases. We investigated preparations from submaxillary glands of secretors and found that the αl,2-fucosyltransferase in this tissue did indeed have a preference for Type 1 structures whereas this preference was not shown by the weak α1,2-fucosyltransferase activity detectable in the glands from non-secretors [174]. Other laboratories similarly demonstrated differences in the specificities between the αl,2-fucosyltransferases in milk and serum [175] and between the α1,2-fucosyltransferase gene in serum of a para-Bombay individual, in which the enzyme was

judged to be derived from secretory tissue, and that in normal serum where the greater part is derived from haemopoietic tissue [176]. In fact, the conformational differences between, Type 1 and Type 2 disaccharide structures [122] do not appear to be an inevitable barrier to use by the same α1,2-fucosyltransferase because both were good acceptors for a purified αl,2-fucosyltransferase isolated from normal human plasma [177] which is believed to be the product of the *H*-gene. Nevertheless, the proposal [172] that there is more than one α1,2-fucosyltransferase, with the one in secretory tissues of mesodermal origin expressed independently of the gene encoding the enzyme that is expressed in endothelial tissues, has proved to be correct. In 1990 Larsen *et al*. [178] successfully cloned the *H*-gene associated α1,2-fucosyltransferase (*FUT1*) and the cDNA was shown to correspond to a single 3.6 kb transcript in human cells and to encode a protein with 365 amino acids and a calculated Mr 41 249. The gene consists of two exons and the entire coding region is contained in exon 1. Since that time a large number of inactivating mutations have been reported in the *FUT1* gene in individuals with the Bombay O_h phenotype which lead to inactive, or weakly active, α1,2-fucosyltransferases and hence impair the expression of the red cell ABH antigens (reviewed in [179]). Subsequently a second α1,2-fucosyltransferase, believed to be the product of the *Se* gene, was cloned [180,181] and called *FUT2*. The cDNA predicts a 332 amino acid-long polypeptide that shares 68% sequence identity with the COOH-terminal 292 residues of the *H*-gene (*FUT1*). *FUT2* also consists of two exons with the entire coding region contained in exon 1 [180–182]. The *FUT1* and *FUT2* structural genes are closely linked on human chromosome 19 (19q13.3) [183]. Despite the difference in properties exhibited by the α1,2-fucosyltransferases in different tissues which seemed to be in agreement with the general hypothesis concerning the specificity of *H* and *Se* genes [172] when *FUT2* was expressed in COS cells the resultant α1,2-fucosyltransferase did not appear to show the expected preference for Type 1 substrates [181] and this point remains unresolved. Numerous inactivating mutations of *FUT2* genes leading to the non-secretion phenomenon, including deletions, single nucleotide changes and the occurrence of a fusion gene have been reported (reviewed in [179]).

The Lewis family of antigens and fucosyltransferases

Since the *Le*-gene encoded α1,4-fucosyltransferase, was not detectable in human plasma [141] we examined other secretions in order to find a convenient source of this genetic marker for family studies and routine investigations. After demonstrating the enzyme in saliva, Philip Johnson in 1981 studied the fucosyltransferase activities in this secretion from individuals of known Lewis phenotypes. Interpretation of the results was complicated by the occurrence of an α1,3-fucosyltransferase in all samples that was expressed independently of Lewis group or secretor status [184]. Nevertheless he was able to show that not only is the α1,4-fucosyltransferase expressed in saliva of individuals whose red cells group as Le(a+ b−) and Le(a− b+) but also that those carrying an *Le* gene can transfer fucose to the 3-OH position of glucose in lactose-based structures. At about the same time a report indicating that a purified fucosyltransferase preparation from human milk could transfer fucose to both Type 1 and Type 2 chains had been interpreted as indicating that the *Le* gene encodes an α1,3/1/4-fucosyltransferase [185]. Although in strict fact this has turned out to be true, separation of an independent α1,3-fucosyltransferase from the mixture in the milk preparation [186] leaves an α1,4-fucosyltransferase that had only very limited capacity to transfer fucose α1,3-to *N*-acetylglucosamine in Type 2 chains. The major activities exhibited by purified preparations of the *Le* enzyme from human milk [187] and from the human A431 cell line [188] is that of transferring fucose in α1,4-linkage to *N*-acetylglucosamine in Type 1 structures and adding fucose α1,3 to glucose residues. Philip Johnson also found that, contrary to previous reports stating that sialylated oligosaccharides were not substrates for the α1,3-fucosyltransferases [185], both the α1,3-and the α1-4-fucosyltransferases in human milk utilised 3′-sialylated oligosaccharides as acceptor substrates to synthesise sialyl-Le^x and sialyl-Le^a, respectively [189,187]. Mature granulocytes were found to express α1,3-, but not α1,4-fucosyltransferase activity [190] and subsequently we showed that the α1,3-fucosyltransferase in leukaemic cells arrested at an early developmental stage differed in its ability to transfer fucose to sialylated acceptors from the enzyme in normal granulocytes and thus provided the first indication that more than one α1,3-fucosyltransferase is expressed in developing myeloid cells [191]. This topic has since been developed further in our laboratory [192] but diverges from the ABO theme and so will not be discussed here.

The cloning of the *Le* gene (*FUT3*) by John Lowe's group in Ann Arbor [193] made available a further tool for exploring the specificity of the enzyme encoded by this gene. Transfection of recombinant *FUT3* into mammalian COS [194] or BHK [195] cells has resulted in expression of enzymes that have little, if any, activity with Type 2 acceptors. It thus seems improbable that *FUT3* makes much contribution to the appearance of the Type 2 Le^x, Le^y or sialyl-Le^x structures on normal cell surfaces *in vivo* and its major function appears to be the synthesis of Le^a, Le^b and sialyl-Le^a sequences. A number of missense mutations in *FUT3* have been described which account for the Lewis negative phenotype on red cells and in secretions (reviewed in [196]).

Purification of the A and B transferases and attempts to clone *ABO*

As stated earlier our original aim was to find the relationship of the blood-group antigens to the genes that encoded their formation. After the identification of the transferases as the probable primary products of the blood-group genes these

became the obvious targets to pursue and with the rapid advances in molecular genetic techniques the possibility of cloning the genes became a reality. For conventional cloning by screening cDNA libraries with nucleotide or antibody probes the enzymes needed to be purified to homogeneity in order to obtain amino acid sequence analysis or to raise specific antibodies free of other contaminants. However, the low abundance of the transferases in readily available human tissues made for considerable difficulties in achieving this aim. Over the years we made many attempts to purify the *A* and *B* transferases, as did workers in other laboratories (e.g. [197,198]). We first tried an affinity procedure involving adsorption on to an acceptor substrate in an attempt to purify the *B* transferase in plasma. The enzyme was adsorbed onto H determinants on group O erythrocyte membranes, and eluted with the H-specific trisaccharide, 2′-fucosyllactose [199]; this method yielded over 100 000-fold purification of the *B* enzyme but the preparation was not homogeneous. From the specific activities quoted in the literature it is doubtful whether any of the preparations described up until the late 1980s were anywhere near homogeneous. A purification method based on the fortuitous affinity of the *A* transferase for Sepharose 4B (agarose) introduced by Whitehead *et al.* [200]) proved most useful for obtaining preparations of *A* transferase free from other glycosyltransferases in plasma. However, attempts by Pamela Greenwell and colleagues in our lab [201] to clone the *A* gene by screening a human jejunum λgt11 library with a polyclonal antibody raised against an *A* transferase preparation purified by adsorption on Sepharose 4B were unsuccessful, probably because contaminating antibodies were present despite rigorous attempts to remove them. Further attempts by Naveenam Navaratnam to purify the *A* transferase from human lung using adsorption first on UDP-hexanolamine and then on octyl-Sepharose CL-4B gave a purification of 100 000-fold and showed single band on SDS-PAGE corresponding to a M_r 40 000 [202]. The yield was, however, poor and in order to obtain greater quantities of purified material for sequence studies attempts were made to purify the enzyme from pooled human gut mucosal tissue [203] removed from post-mortem stomach and duodenal samples from group A subjects. The tissue was homogenised, treated with Triton-X-100 to solubilise the enzyme and then purified in the same way as the lung enzyme. An active *A*-transferase purified to homogeneity was recovered in 44% yield with a M_r 40 000 and a specific activity approximately 7 μmol/min per mg. To date, this remains the only *A* transferase from natural sources to have been purified to homogeneity and to have retained sufficient activity for some of its enzymic properties to be examined. Specificity studies confirmed that the transferase had the capacity to transfer *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine only to substrates containing a subterminal β-galactosyl residue substituted at the 2-OH position with L-fucose and also had a limited capacity to utilise UDP-galactose as donor substrate. A short amino-acid-sequence was obtained for the *N*-terminal region of the soluble α-3-*N*-acetylgalactosaminyltransferase. However, time was no longer on the side of our laboratory! The MRC decided to close the Division on W.M.W.s official "retirement" in 1989 and members of the group had to find positions elsewhere; further attempts to clone the *ABO* genes had therefore to be abandoned. Meanwhile in Sen Hakomori's laboratory in Seattle, Henrik Clausen and his colleagues were also purifying the *A* transferase from human lung tissue utilising a Sepharose 4B (agarose) affinity step, followed by cation exchange chromatography and reverse phase chromatography [204]. During this last step enzyme activity was lost but, on the basis of the partial amino acid sequence of the inactive protein, cDNA encoding the A transferase was cloned by Yamamoto and colleagues from a human stomach cancer cell line [7]. Although, naturally, we were disappointed that we had not completed the task of cloning the gene, a small consolation was that the short amino acid sequence recorded for the transferase purified from human mucosal tissue did correspond to part of the deduced amino acid sequence of the protein encoded by the *A* gene [204] proving that the correct protein had been purified to homogeneity.

The ABO locus 1990–2000 and future prospects

The years since the cloning of the *ABO* locus by Yamamoto *et al.* [7] in 1990 have seen remarkably rapid strides towards understanding the molecular genetics of this blood-group. Nucleotide sequence analysis of the *ABO* gene revealed a coding region of 1062 base pairs corresponding to a 41 kDa protein [205]. Subsequent studies showed the gene to consist of seven exons, with the coding region spanning 18–20 kb of the genomic DNA [206,207]. The largest exon is the seventh (688 bp) and most of the coding sequence lies within this region (Figure 6). Hydrophobicity analysis of the predicted amino acid sequence indicated that, as is common for many glycosyltransferases, the protein has a three domain structure, with a short hydrophilic *N*-terminal, a hydrophobic transmembrane region and a long C-terminal catalytic domain [205].

The existence of many variations of *A* and *B* genes had been predicted from serological, enzymic and family studies [139,208] but until the *ABO* gene was cloned there was no possibility of subdividing group O since it simply defined those without active *A* or *B* glycosyltransferases. Once the locus had been cloned the expectation that there could be many *O* alleles was signalled by an approach using denaturing gradient gel electrophoresis [209] that demonstrated 4 different *O* alleles in ninety five unrelated individuals; these alleles were not further investigated. The first characterised and most common *O* allele, O^1, differs from the A^1 allele by a deletion at nucleotide 261 in exon 6 which leads to a frame shift and a premature stop codon (7) (Figure 6). An interesting fact about this common *O* allele is that exon 7, which carries the consensus sequence for the catalytic region of the A^1 transferase, is intact in the gene but untranslatable because of the frameshift caused by the deletion in exon 6. A second, less

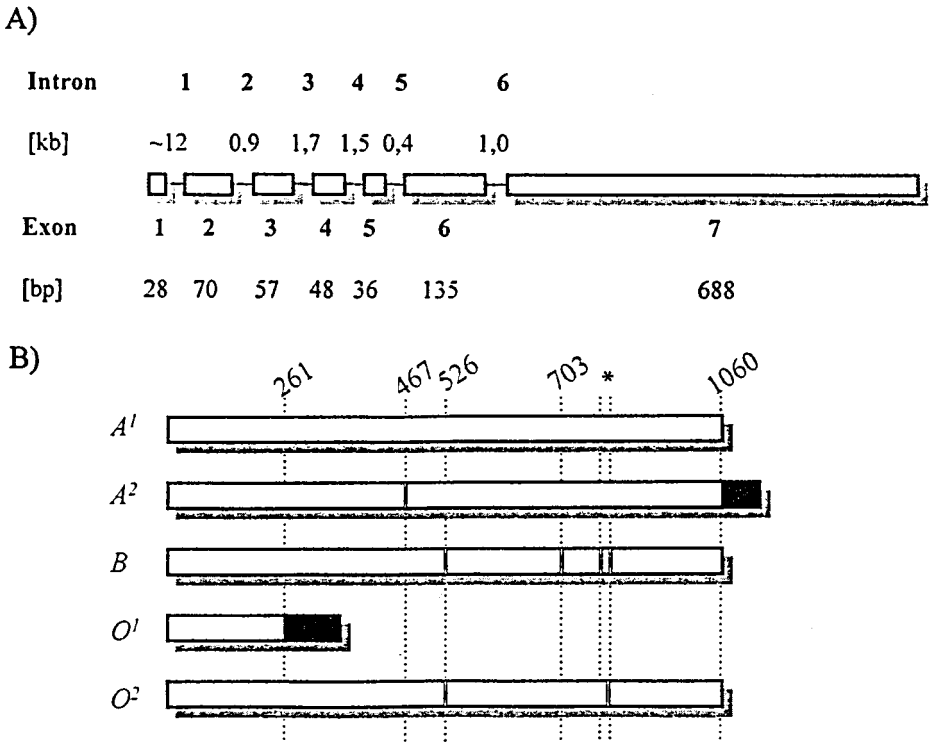


Figure 6. Schematic representation of (A) the genomic organisation of the *ABO* gene and (B) the predicted reading frame for some common *ABO* alleles. The white blocks represent the translated A^1 consensus sequence, the black blocks represent translated non-A^1 consensus (nonsense). The vertical bars indicate nucleotide (nt) positions of mutations leading to amino acid changes. Asterisk indicates position in which *B* allele is mutated at nt 796 and 803 and O^2 allele at nt 802. Reproduced from Olsson [235].

common, *O* allele has been described, O^2, which lacks this deletion and has a mutation in exon seven that presumably inactivates the enzyme [210,211]. A frequently occurring variation of the O^1 allele has an additional nine mutations and has been called O^{1v} [212], the frequency of this allele differs widely in different ethnic groups. Yet another type of *O* allele, O^3, lacking the 261 deletion but carrying a combination of mutations found in weak A subgroups has been described [213]. These polymorphisms should provide valuable additional markers for anthropological and forensic investigations. Studies on blood groups of peoples from different parts of the world, designed to investigate the origins, movements and evolution of human populations, occupied A.E. Mourant and colleagues [214] for a period of over twenty five years. This work has become a classic in its field and would be a mammoth task to repeat but much more could now be deduced from the more accurate genotyping methods, and immeasurably greater number of alleles, that are now available for studying the distribution and evolution of the ABO system.

The molecular basis of the A_2 phenotype was revealed in 1992 when a single point deletion near the 3′ end of the A^2 allele was found to cause a frame shift that leads to an extra domain of 21 amino acids [215] (Figure 6): this change presumably in some way alters the conformation of the transferase leading to less activity. Yamamoto and colleagues also identified mutations in A^3, A^x and B^3 alleles and clarified the molecular basis of the *cis-AB* and *B(A)* allelles (reviewed in [216]). Not a great deal is yet known about the regulatory elements controlling the expression of the *ABO* genes but a beginning has been made. In 1997 Kominato *et al.* [217] investigating blood-group A expression in a cancer cell line described an enhancer element located −3.7 kb upstream from the transcription start site that was composed of four tandem repeats of a 43 bp unit and required binding of transcription factors CBF/NF-Y. Examination of this minisatellite region in blood samples from 160 random Swedish donors surprisingly revealed an allele-related variation in the number of tandem repeats: the A^2, B and O^1 alleles had four repeats whilst the A^1 and the infrequent O^2 allele had only one of the four repeat units [218]; moreover, this one unit contained a substitution. No intermediate forms have been detected so far and no clear explanation for these observations has been advanced although it was suggested that uncoupling of allele correlation may occur as a result of crossing over [218]. The regulatory role of DNA methylation of the promotor region of *ABO* genes has also been examined by Kominato *et al.* [219]. The *ABO* promoter region contains a CpG island whose methylation correlates well with expression of *ABO* in certain cell lines whereas this region was hypermethylated in other cell lines that did not express *ABO*.

However, Iwamoto *et al.* [220] found that, although deletion of A-antigen in a human cancer cell line is associated with reduced promotor activity of the CBF/NF-Y binding region, there is possibly enhanced DNA methylation of the *A* transferase promotor. The work in progress will doubtless soon clarify these anomalies and identification of the genetic elements involved should yield valuable insights into the tissue specific, and developmentally regulated, expression of the *ABO* genes. The isolation of blood group A active glycolipids from the cancer tissues of blood group O patients [221] has confirmed the earlier reports [120] of aberrant expression of A and B antigens incompatible with the individuals ABO blood group but although various suggestions have been advanced to account for this phenomenon (see [88,222]) none have yet provided completely satisfactory explanations.

An interesting and unexpected outcome of the sequencing of the genomic *ABO* DNA has been the demonstration of recombination events; these were unexpected because they appear to conflict with the apparently clear cut Mendelian inheritance patterns of the genes. Sequencing of the intron between exon 6 and 7 revealed evidence of crossing-over points of hybrid alleles formed from an A^1 gene and an O^{1v} allele leading to an A^x allele [223] and to the production of new *O* alleles formed by crossing-over events between known alleles in the ABO system [224]. One very fascinating example of a *de novo* recombination event was described by Susuki *et al.* [225]. In a paternity case in which the mother was group B and the child group A_1, the putative father (group O) was excluded only on the *ABO* system, since all other polymorphic markers were consistent with paternity. Sequencing revealed that one of the child's alleles had a hybrid nature comprising exon 6 of a *B* allele (which would not have had the nucleotide 261 deletion which occurs in normal O^1 genes) and exon 7 of an O^1 allele (which would carry the A^1 consensus sequence); together these exons had formed a new A^1 allele which the authors assumed had arisen *de novo* from recombination between the *B* and O^1 alleles of the mother during meiosis. Although recombination such as this must be relatively rare, it does show that inheritance in the ABO system is not as straightforward as previously thought and also opens to doubt forensic decisions made in the past on the basis of blood-group data when far fewer polymorphic markers were available. Olsson and Chester [223] have commented on the Chi and Chi-like sequences near the 3′-end of intron 6 and have suggested, since such sequences in *Escherichia coli* are considered hot-spots for breakpoints in double stranded DNA, that this sequence may function as an initiator of intragene crossing over in the *ABO* gene.

The interest in, and use of, the glycosyltransferase gene products as genetic markers has understandably waned since the *ABO* gene was cloned. Attempts to establish heterozygous and homozygous (*AA* or *AO*/*BB* or *BO*) genotypes by dosage measurements of the transferase levels in plasma were never entirely satisfactory in our laboratory and we concluded that other factors were influencing the level of expression of the enzymes. In the last ten years a large number of papers have appeared describing rapid *ABO* genotype screening methods based on PCR products amplified by allele-specific primers directed against exons 6 and 7 of the *ABO* gene or PCR of genomic DNA followed by specific restriction enzyme digest of products (reviewed in [216]). Although some caution is required in interpretation of genotype screening methods because of the possibility of recombination of known alleles [223,225] and the occurrence of, as yet, unidentified alleles, on the whole the screening procedures are much more informative with regard to heterozygous or homozygous states, and are quicker and simpler to carry out, than the enzyme assays. Nevertheless the opportunity to obtain larger quantities of the transferases by expressing recombinant forms in *E. coli* have enabled more detailed studies to be carried out on the kinetic properties and specificities of the *A* and *B* transferases [226,227]. The overlapping specificities of the A and B transferases have been confirmed and the use of UDP-galactose by the *A* transferase was shown to proceed at only ~0.5% of the rate of its use of UDP-*N*-acetylgalactosamine whereas the use of UDP-*N*-acetylgalactosamine by the *B* transferase was ten times faster and amounted to approximately ~5% of the rate of its utilisation of UDP-galactose. A hybrid *A*/*B* transferase has been genetically engineered that efficiently utilizes both UDP-*N*-acetylgalactosamine and UDP-galactose as donor substrates and these hybrid enzymes have been employed for the chemo-enzymatic synthesis of A and B trisaccharides and their corresponding analogues [228].

What then does the future hold for research on ABO? Those entering the field at this stage must see many questions that still remain to be answered. The molecular basis of some weak forms of A and B antigens have yet to be deciphered and no explanation has been found for why the inheritance of some alleles, such as A^3, leads to mixed field agglutination, suggesting differential expression of the antigens in some members of the red cell population. The regulatory and modifying elements controlling the ABO genes have still to be fully elucidated and explanations found for the remarkable patterns of expression and regression of the antigens that are observed in embryological development [118]. The frequency of occurrence of recombinant forms in different populations also needs to be established and the genetic basis investigated. On a biochemical level the possibility of expressing recombinant *A* and *B* glycosyltransferases in reasonably large amounts *in vitro* raises the expectation that they may be crystallised in the not too distant future and their overall structure, conformation and substrate binding sites established.

An observation first made by Barbolla and colleagues [229] that probably deserves more attention than it has so far received is the development of strong antibodies to *A*- and *B*- transferases in patients receiving ABO incompatible bone marrow or liver transplants. In the case of group A recipients receiving bone marrow from group O donors the antibody reacted with both *A*- and *B*-transferases and some patients exhibited severe graft versus host reactions. Similar observations have since been

reported by others (see [88]) and the antibody nature of the transferase inhibitor has been confirmed [230].

If anyone could find a satisfactory answer to the vexed question of the function of the *ABO* and *H* genes that would indeed be a triumph [231]. The advantage of having either anti-A or anti-B antibodies in certain infections when related antigens are expressed on the surface of the infecting agents would seem the most likely explanation for the balanced polymorphism of the *ABO* alleles but if group O individuals represent the human equivalent of a "knock-out" mouse with A and B genes deleted, there is no apparent loss of function in those individuals, and indeed they comprise the largest group in many populations. Bombay O_h individuals, who lack H determinants, although rare, do not appear to suffer from this deficiency. Nevertheless loss of antigens that sometimes occurs in malignancy can mean a poor prognosis [121] and this fact combined with the complex pattern of switching on and off the *ABO* and *H* genes in development, and the re-expression of these genes in some tumours, suggest that they might have a function in relation to the immune system and recognition of "self".

Another puzzle relates to the lower levels in plasma of von Willebrand factor in normal group O individuals [232] than in other groups and the reason why the half-life of infused factor VIII is shorter in group O haemophiliac patients than in group A patients [233]. Von willebrands factor is a glycoprotein bearing A, B and H blood-group determinants that plays a key role in haemostasis. The higher levels of von Willebrand factor found in those not carrying an *O* gene presents a risk factor for venous thrombosis and ischaemic heart disease [232] but whether the lower level of the glycoprotein in group O individuals results from decreased production or from increased clearance has yet to be established. These and many other problems related to disease associations with the *ABO* locus show that the study of this system has not come to an end with the cloning of the gene and indeed has entered a new phase with the possibility of answering many of the questions that could not be approached until the molecular genetic information became available.

Reflections

When we entered this field nearly 60 years ago we knew that we were treading a little known path but we had no idea the direction the work would take or the techniques or skills we would be required to learn. At the outset we were immersed in serology, preparative biochemistry and chemical analytical techniques. Subsequently chemical procedures to achieve partial fragmentation of the blood-group active glycoproteins, together with degradation with glycosidases, led to the structural identification of the blood-group determinants. The next phase of the work was the proposal of the biosynthetic pathways for the formation of the antigens and the search for the glycosyltransferases that we predicted to be the primary products of the blood-group genes. The characterisation of these enzymes in individuals of the appropriate blood-groups eventually gave the window for the cloning by others of the *ABO* locus. This path from antigens of unknown nature to the genes controlling their appearance on the cell surface would doubtless be achieved in a fraction of the time with the techniques available today but no automated equipment for analysis, or precise protocols for determination of the structure of complex carbohydrates were in existence when the structural work on the antigens was carried out. In the 1960s groups working on bacterial antigens were in advance of those of us tackling mammalian antigens and, although the bacterial polysaccharides were structurally very different, we found inspiration and encouragement from the work on immunodominant sugars and biosynthetic enzymes that was emerging from that field (reviewed in [234]).

Safe blood transfusion can still be, and indeed is, carried out by clinicians who have little or no knowledge of the biochemical basis of blood-group antigenic specificity but we believe that the most significant contribution of the work has been the general understanding it has provided concerning the regulation and expression of cell surface carbohydrate antigens and the part they play in cell-cell recognition. The sequential exposure of new antigenic structures by the removal of single sugars with exo-glycosidases not only suggested the manner in which the determinants are built up but also revealed how antigenic structures could appear and disappear in the course of normal differentiation and how epitopes which are normally covered reappear when there is a failure of expression of glycosyltransferases in malignancy. The discoveries in the 1990s that adhesion molecules involved in the inflammatory response recognise carbohydrate ligands related to the Lewis blood-group antigens [95] broadened still further the interest in these structures and the genes encoding the enzymes that control their formation. Now that the *ABO* and *Lewis* genes are cloned new opportunities have opened up for obtaining a deeper understanding of the factors regulating the tissue specific expression of the antigens in normal tissues and the changes that take place in tumours. Despite the ups and downs of research it has been a wonderful experience to have played a part in unravelling the ABO and Lewis story and to be able to follow the exciting developments that are taking place today.

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