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Multiple Specificities of Mammalian Blood Group Substances Comparatively Studied with Human Isoagglutinins and Fractionated Anti-H Lectins

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Summary. Purified blood group-active substances derived from different pig, horse, baboon, Rhesus monkey and human tissues were quantitatively studied for their haemagglutination inhibiting potency with: (1) human IgM anti-A and anti-B; (2) human anti-Le^a and anti-Le^b; (3) *Ulex europaeus* extracts separated into lectin fractions with respective L-fucose-inhibitable ('anti-H^F') and chitobiose–cellobiose-inhibitable ('anti-H^C') combining sites. Irrespective of species origin, A and B blood group activity per milligram of purified material tended to be strikingly higher in substances low in, or devoid of, Lewis blood group activity. Most of the blood group substances displayed variable but about equally balanced amounts of *Ulex* anti-H^F and anti-H^C inhibiting activity. In contrast, pig submaxillary gland mucins displayed strikingly high levels of *Ulex* anti-H^C inhibiting activity, even in the complete absence of *Ulex* anti-H^F inhibiting activity. These serological findings are consistent with current biochemical concepts regarding the heterosaccharide microheterogeneity of blood group-active glycoproteins.

INTRODUCTION

Blood group-active substances of human and animal origin have been described in comprehensive detail by Kabat (1956). Subsequent advances in our knowledge of the biosynthesis and haptenic fine-structures of blood group substances have recently been reviewed in depth by Watkins (1972). One of the more interesting questions that has evolved, is whether blood group-active preparations which display multiple specificities are 'mixtures of molecules, or whether individual molecules can carry more than one blood group specificity' (Watkins, 1972).

The present study was undertaken to establish the comparative multiple specificity profiles of purified blood group-active substances of diverse mammalian origin, many of

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which have not been previously tested with some of the agglutinating reagents included in our antibody–lectin panel. The ultimate aim of this report and other studies presently underway in our laboratories is to shed further light on the biochemical basis for variability in the multiple specificity profiles of blood group-active substances from different individuals and different species.

MATERIALS AND METHODS

Antisera

Human IgM anti-A was fractionated from a high-titre group B serum by ammonium sulphate fractionation and subsequent gel-filtration with Sephadex G-200 as described in detail by Chuba (1973). Human IgM anti-B isoagglutinins were similarly prepared from a high titre group A serum. The human anti-Lewis isoagglutinins (anti-Le^a and anti-Le^b) were selected for suitable titre from different lots of commercial reagents (Dade). The Lewis specificities were in all cases independently confirmed with several different red cell panels containing at least two samples each of Le(a+b−), Le(a−b+) and Le(a−b−) reference cells. Preparation of the catfish anti-Lewis heteroagglutinins and immune goat and catfish saliva-precipitating antibodies has been described (Chuba, 1973).

Lectins

Initial extracts from *Ulex europaeus*, *Laburnum alpinum* and *Lotus tetragonolobus* seeds were prepared as described by Chuba, Kuhns, Nigrelli, Vandenheede, Osuga and Feeney (1973). The *Ulex* extract was further separated by DEAE–Sephadex A-50 ion-exchange chromatography (compare Zschocke, Grieble, Bach and Anderson, 1969) into the respective chitobiose–cellobiose–inhibitable and L-fucose-inhibitable lectin fractions (Flory, 1966; Matsumoto and Osawa, 1969, 1970; Chuba, 1973). Following the suggestion of Chuba (1973), the chitobiose–cellobiose-inhibitable *Ulex* fraction, which was eluted from the DEAE–Sephadex A-50 bed with the low ionic strength starting buffers, will subsequently be referred to in this study as '*Ulex* anti-H^C' and the L-fucose-inhibitable fraction, which was released from the DEAE–Sephadex beads with 0·2 M phosphate buffer (pH 7·5), will be referred to as '*Ulex* anti-H^F'.

Wheat-germ agglutinin was purified by the method of Marchesi (1972). The *Helix pomatia* snail anti-A^{HP} agglutinin (Prokop, Uhlenbruck and Köhler, 1968) was kindly provided by Dr O. Prokop.

Blood group-active substances

Pig group A gastric substance and horse group B gastric substance were of commercial origin (Pfizer). Primate gastric substances from frozen stomachs were purified as described by Chuba (1973) with a procedure that involved initial saline extraction of acetone-dried and pulverized stomach mucosae, ammonium sulphate precipitation (80 per cent saturation), Sephadex G-200 gel-filtration and, finally, ethanol precipitation (67 per cent v/v). Stock solutions were prepared by dissolving the dried ethanol precipitate in 1 ml of 0·9 per cent saline (usually 4 mg/ml). Such stock solutions, with 0·02 per cent sodium azide added as a preservative, were stable for several months but not over 1 year of storage at 4–8°. Saliva substances from volunteer donors of different phenotypes were similarly purified after heating freshly collected saliva samples for 15 minutes in a boiling water-bath.

A purified, apparently H-negative, but strongly A-active preparation of pig submaxillary mucin was kindly provided by Dr Ward Pigman (compare Payza, Martinez and Pigman, 1970). Additional pig submaxillary mucin was purified by the method of De Salegui and Plonska (1969). Separate aliquots were then either treated with chymotrypsin by a modification of the method of Hashimoto, Tsuiki, Nisizawa and Pigman (1963), utilizing Enzite chymotrypsin (Miles–Seravac), or subjected to mild acid hydrolysis as described by Hagopian and Eylar (1968).

Crude pig submaxillary gland extracts from individual glands (Pel–Freeze) were prepared by extracting approximately 1 g of minced glandular tissue (carefully trimmed of depot fat and connective tissue) with 2 ml of 0·9 per cent saline. An additional millilitre of saline was added if after approximately 1 hour of extraction at room temperature the supernatant was too viscous to be readily expressed as a discrete drop from a capillary-tipped pipette.

Serological tests

Haemagglutination titrations to standardize the indicator reagents for each of the haemagglutination–inhibition systems were performed as described by Chuba, Kuhns and Nigrelli (1968); namely, by mixing two-drop aliquots of doubly diluted agglutinating reagent with one-drop aliquots of a 2 per cent suspension of indicator red cells. The master working dilutions were retitrated to confirm their titre (1:8) with the indicator red cells immediately prior to each series of tests. One drop of the appropriately diluted agglutinating reagent was incubated for 10 minutes at 21° with one drop of diluted test substance and then one drop of indicator red cell was added. Results were read after 20 minutes incubation at 21° and a light spin (e.g. 1000 ***g*** for 15 seconds) and reported as described in the footnotes to Tables 1 and 2.

For maximal reproducibility and sensitivity (compare Chuba, 1973), indicator red cells used with the anti-Lewis antibodies and the *Ulex*, *Laburnum*, *Lotus* and wheat-germ agglutinins were pretreated with the plant protease, ficin (Nutritional Biochemicals). Four volumes of an approximately 6 per cent suspension of thrice saline-washed erythrocytes were incubated with one volume of a 1·0 per cent crude ficin solution (in 0·9 per cent saline) for 15 minutes at 37°. The treated red cells were washed three times with saline. For agglutination tests the ficin-treated red cells were made up as uniform 2 per cent suspensions in 0·9 per cent saline.

Immunoelectrophoresis

Immunoelectrophoretograms were prepared in 0·75 per cent agarose on 50 × 75 mm glass slides by standard procedures adapted from Crowle (1961) and described in detail by Chuba (1973). Five microlitres of antigen in each round sample well were tested with 80 μl of precipitating antibody in each long trough. Staining of the dried slides prior to photography was done with Crowle's triple protein stain (Crowle, 1961).

RESULTS

The quantitative haemagglutination inhibiting activities of different pig, horse, baboon (*Papio hamadryas*, *P. ursinus*, *P. anubis* and *P. cynocephalus*), Rhesus monkey (*Macaca mulatta*) and human preparations when tested with human IgM anti-A and anti-B, human anti-Le^a and anti-Le^b and chitobiose–cellobiose-inhibitable *Ulex* anti-H^C and L-fucose-inhibitable

Ulex anti-H^F lectin fractions are given in Table 1. Irrespective of species origin, group A or B activity tended to be inversely related to Lewis blood group activity, as evidenced by the strikingly high specific activity of the Lewis-negative pig and horse A and B substances, as well as the primate substances that were comparatively low in, or altogether devoid of, Lewis activity (e.g. BabStm number 2, RhStm 164, HumStm number 4 and HumStm number 6).

Ulex anti-H^C and anti-H^F inhibiting activities were present in about equally balanced amounts except for the group-A substance from pig submaxillary glands, which was completely devoid of *Ulex* anti-H^F inhibiting activity, even though it was the strongest inhibitor of *Ulex* anti-H^C among the eighteen preparations recorded in Table 1. The *Ulex* anti-H^C and anti-H^F inhibiting activities of the infrahuman primate stomach substances, although present in about equally balanced amounts, were both some three- to four-fold lower than the *Ulex* anti-H^C and anti-H^F inhibiting activities of blood group substances derived from pig, horse and human stomachs. The specific activities of the human saliva substances were all comparatively low, except for the strikingly high Le^a activity of the salivary substances from the Lewis-positive, group-O non-secretor.

TABLE 1

COMPARATIVE QUANTITATIVE HAEMAGGLUTINATION–INHIBITION POTENCY OF PURIFIED MAMMALIAN BLOOD GROUP-ACTIVE SUBSTANCES

Source of soluble substances	ABO group	Anti-A or anti-B isoantibodies with homologous human red cells		Anti-H^C or anti-H^F *Ulex* fractions with ficin-treated group O red cells		Anti-Le^a or anti-Le^b isoantibodies with homologous ficin-treated O cells	
		Group A activity	Group B activity	H^C activity	H^F activity	Le^a activity	Le^b activity
Non-primate							
PigSubMax*	A	+++++++†	0‡	++++	0	0	0
PigStm*	A	+++++	0	+++	++++	0	0
HorseStm	B	0	+++	+++	+++	0	0
Infrahuman primate							
BabStm number 2 (*P.h.*)	A	+++	0	(+)	(+)	+	0
RhStm number 164 (*M.m.*)	B	0	++++	+	(+)	+	+
BabStm number 7 (*P.u.*)	B	0	++++	+	++	+	++
BabStm number 5 (*P.a.*)	A	+	0	+	+	+	+++
BabStm number 3 (*P.a.*)	AB	+	+	+	(+)	+	+++
BabStm number 4 (*P.c.*)	O	0	0	+	(+)	+	+++
Human							
Stm number 4	A	+++	0	+++	++++	0	0
Stm number 6	B	0	++++	++	++++	0	0
Stm number 3	O	0	0	+++	+++	++	+++++++
Sec*, Le(a−b−)	O	0	0	+	+	0	0
Sec, Le(a+b+)	B	0	(+)	(+)	(+)	+	+
Sec, Le(a+b+)	A	+	0	+	+	+	++
ns*, Le(a+b−)	O	0	0	(+)	0	+++++	0

* SubMax = submaxillary glands; Stm = stomach mucosa; Sec = saliva from human ABH secretors; ns = saliva from human ABH non-secretor.

† Comparative quantitative haemagglutination–inhibition potency is graded from (+) (complete inhibition only at a minimum concentration of 1000 μg/ml) to +++++++ (complete inhibition with as little as 0·1 μg/ml concentration). Quantification is based on the concentration of soluble substance in the aliquot mixed with an equal volume of agglutinating reagent (titre 1:8) prior to the introduction of one volume of a 2 per cent suspension of homologous indicator red cells.

‡ 0 = no detectable haemagglutination–inhibition at 4000 μg/ml starting concentration.

Table 2 shows the haemagglutination–inhibition profiles of selectively pooled crude extracts of pig submaxillary glands which were tested with a number of agglutinating reagents including chitobiose–cellobiose-inhibitable *Laburnum alpinum* and L-fucose-inhibitable *Lotus tetragonolobus* anti-H lectins (compare Watkins and Morgan, 1962). Pig submaxillary gland pools I, II, and III show strikingly strong inhibiting activity with *Ulex* anti-H^C, but only weak inhibition with the similarly chitobiose–cellobiose-inhibitable *Laburnum alpinum* 'anti-H^C' lectin. The comparatively weak inhibition of *Ulex* anti-H^C by the most strongly A-active pig submaxillary, gland extract (pool IV, Table 2) suggests a possible inverse relationship between group A activity and H^C activity in pig submaxillary gland mucin. In contrast, the *Helix* anti-A^{HP} inhibiting activity of strongly

TABLE 2

HAEMAGGLUTINATION–INHIBITION PROFILE OF CRUDE EXTRACTS OF PIG SUBMAXILLARY GLANDS

Agglutinating reagent	Indicator red cells	Pooled PigSubMax extracts* mixed with an equal volume of agglutinating reagent (titre 1:8)			
		Pool I (4/25)	Pool II (2/25)	Pool III (5/25)	Pool IV (7/25)
Human anti-A	A_1	< 1†	< 1	128	512
Helix anti-A^{HP}	A_1	8	32	8	64
Human anti-B	B	< 1	< 1	< 1	< 1
Ulex anti-H^C	O‡	4000	16000	4000	32
Ulex anti-H^F	O‡	< 1	4	< 1	< 1
Laburnum alp.	O‡	1	2	4	< 1
Lotus tet.	O‡	1	2	< 1	< 1
Wheat germ	O‡	16	32	32	16
Presumptive phenotype		O,H+	O,H+	A,H+	A,H−

* Crude saline extracts from eighteen of twenty-five individual pig submaxillary glands (PigSubMax) were selectively pooled on the basis of the similarity of their initial serological profiles when screened undiluted with the eight agglutinating reagents listed above.

† Comparative haemagglutination–inhibition potency is expressed as the reciprocal of the maximum dilution of pooled PigSubMax which still completely inhibited macroscopic agglutination when mixed with an equal volume of agglutinating (titre 1:8) reagent prior to the introduction of one volume of a 2 per cent suspension of homologous indicator red cells.

‡ Normal adult group O, ficin-pretreated red cells.

A-active pig submaxillary gland pool IV was not notably stronger than the *Helix* anti-A^{HP} inhibiting activity of A-negative pool II. The wheat germ agglutinin was in turn moderately inhibitable by all four of the pooled pig submaxillary gland preparation (Table 2).

The high degree of variability in the comparative haemagglutination–inhibition profiles of individual crude extracts from ten additional randomly selected pig submaxillary glands (not included among the pooled samples in Table 2) is shown in Table 3. Arranged from left to right in their approximate order of increasing group A activity, the individual preparations in Table 3 confirm the inverse serological relationship of group A and *Ulex* anti-H^C inhibiting activity of pig submaxillary gland extracts suggested by the pooled sample (PigSubMax pool IV) given in Table 2. Sample number 3 (Table 3) appears to be the only pig submaxillary gland preparation possessing the 'inactive' blood group phenotype (Carlson, Iyler and Mayo, 1970).

TABLE 3

HAEMAGGLUTINATION–INHIBITION ACTIVITY OF INDIVIDUAL CRUDE EXTRACTS OF PIG SUBMAXILLARY GLANDS

Agglutinating reagent	Indicator red cells	Individual PigSubMax crude extracts* mixed with equal volume o agglutinating reagent (titre 1:8) number:									
		3	7	9	5	1	4	8	10	6	2
Human anti-A	A_1	< 1†	< 1	< 1	< 1	2	8	128	256	512	1000
Helix anti-A^{HP}	A_1	1	8	32	128	32	128	32	32	128	128
Ulex anti-H^C	O‡	2	8000	8000	2000	2000	32	64	32	128	32
Ulex anti-H^F	O‡	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Wheat germ	O‡	2	32	128	32	32	128	128	32	32	128

* Arranged in approximate order of increasing group A activity.
† Comparative haemagglutination–inhibition potency is expressed as the reciprocal of the maximum dilution of pooled PigSubMax which still completely inhibited macroscopic agglutination when mixed with an equal volume of agglutinating (titre 1:8) reagent prior to the introduction of one volume of a 2 per cent suspension of homologous indicator red cells.
‡ Normal adult group O, ficin-pretreated red cells.

Sample number 5 (Table 3) shows that an individual pig submaxillary gland preparation can have maximal *Helix* anti-A^{HP} inhibiting activity without concurrent group A activity. As in the case of the four pooled samples given in Table 2, the individual pig submaxillary gland preparations, except for 'inactive' sample number 3, all show moderately strong inhibitory activity with wheat-germ agglutinin.

The changes in haemagglutination inhibiting activities of purified pig submaxillary mucins (prepared from ten randomly pooled glands) before and after degradation with chymotrypsin or by mild acid hydrolysis are given in Table 4. The principal change after degradation with chymotrypsin is loss of group A activity. In the case of mild acid hydrolysis, the principal change is a loss of both group A and *Ulex* anti-H^C inhibitory activity. Undegraded pig gastric A substance (see Table 1), included for comparison, shows

TABLE 4

CHANGES IN HAEMAGGLUTINATION–INHIBITION ACTIVITY OF VARIOUSLY DEGRADED PIG SUBMAXILLARY GLAND MUCINS

Agglutinating reagent	Indicator red cells	Pooled purified PigSubMax*			Pig gastric A substance
		Undegraded control	Chymotrypsin-treated	Acid hydrolyzed	
Human anti-A	A_1	2000†	2	1	1000
Helix anti-A^{HP}	A_1	128	64	64	128
Ulex anti-H^C	O‡	4000	8000	2	256
Ulex anti-H^F	O‡	< 1	< 1	< 1	8
Wheat germ	O‡	4	< 1	4	2000
Principal change			Loss of A activity	Loss of A and H^C activity	

* All preparations tested at a starting concentration of 1 mg/ml.
† Comparative haemagglutination–inhibition potency is expressed as the reciprocal of the maximum dilution of pooled PigSubMax which still completely inhibited macroscopic agglutination when mixed with an equal volume of agglutinating (titre 1:8) reagent prior to the introduction of one volume of a 2 per cent suspension of homologous indicator red cells.
‡ Normal adult group O, ficin-pretreated red cells.

strikingly strong inhibitory activity with the wheat-germ agglutinin. Human stomach substances number 3 and number 4 (Table 1) also showed strikingly strong inhibitory activity with the wheat-germ agglutinin.

Lewis blood group activity was completely absent (Table 5) from all four of the selectively pooled pig submaxillary gland samples (pools I–IV, Table 2). This is in contrast to the high degree of variability in Lewis blood group activity found for the three human substances included for comparative purposes (Table 5).

TABLE 5

COMPARATIVE LEWIS BLOOD GROUP ACTIVITY OF PIG SUBMAXILLARY GLAND MUCIN AND SELECTED HUMAN SUBSTANCES

Agglutinating reagent	Indicator red cells‡	Substances tested as haemagglutination inhibitors with equal volume of agglutinating reagent (titre 1:8)						
		PigSubMax*				Human†		
		Pool I	Pool II	Pool III	Pool IV	ns, Le (a+b−)	Stm number 3	Stm number 4
Human anti-Le^a	O, Le(a+b−)	<1§	<1	<1	<1	128	2	<1
Human anti-Le^b	O, Le(a−b+)	<1	<1	<1	<1	<1	512	<1
ChnCfh'anti-Le'¶	O, Le(a+b−)	<1	<1	<1	<1	1000	1000	2
ChnCfh'antiLe^b'¶	O, Le(a−b+)	<1	<1	<1	<1	256	512	<1
Lewis phenotype		All pig Lewis-negative				Le(a+b−)	Le(a±b+)	Le(a−b−)

* Compare with Table 2.
† Compare with Table 1.
‡ All red cells used for Lewis testing were pretreated with ficin for maximal sensitivity and titre reproducibility.
§ Comparative haemagglutination–inhibition potency is expressed as the reciprocal of the maximum dilution of pooled PigSubMax which still completely inhibited macroscopic agglutination when mixed with an equal volume of agglutinating (titre 1:8) reagent prior to the introduction of one volume of a 2 per cent suspension of homologous indicator red cells.
¶ Channel catfish anti-Lewis antibodies described by Chuba (1973); the ChnCfh 'anti-Le^b' was ChnCfh anti-Le diluted beyond the agglutination endpoint with ficin-treated group O, Le(a+b−) red cells to provide a agglutinating reagent (titre 1:8) selectively reactive with ficinated group O, Le(a−b+) red cells.

On immunoelectrophoresis the purified group A substance from the saliva of an ABH secretor and the group A substance from pig submaxillary gland gave a single pair of lines with slight anodal mobility to the right of the origin point (beta–alpha$_2$ region) (Fig. 1). The group A baboon Stm number 2 substance gave a single pair of precipitin lines at the origin point, and the group A human Stm number 4 substance a single pair of precipitin lines toward the cathodal side to the left of the origin point (gamma region).

Fig. 2 shows the immunoelectrophoretic pattern of the whole, boiled ABH-secretor salivas from which the purified secretor–saliva substances (Table 1) were derived. Plasma from a flathead catfish (number 59), immunized with A saliva, formed precipitin lines in the gamma region to the left of the origin point with all three of the ABH-secretor salivas, as well as with the ABH-non-secretor saliva. In contrast, serum from a goat (number 248), similarly immunized with A saliva, formed precipitin lines in the gamma region only with the group A and group B secretor salivas.

It may be noted that no gamma region precipitin lines were formed either with the human serum sample (bottom well), the purified blood group substances, or the whole, boiled A saliva sample (unlabelled top well) that had been preconcentrated 5-fold by dialysis against 10 per cent polyethylene glycol 20000 (Union Carbide) dissolved in 0·9 per cent saline (Fig. 1).

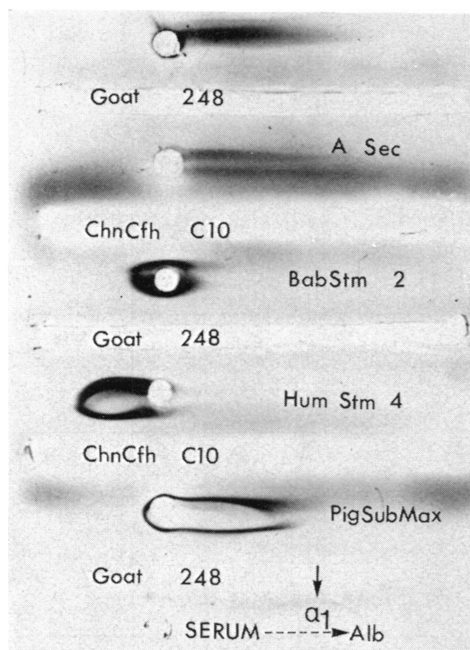

FIG. 1. Unlabelled top well: whole, boiled group A saliva preconcentrated 5-fold by dialysis against 10 per cent polyethylene glycol (mol. wt 20,000). Four interior wells: immunoelectrophoretograms of four different purified group A substances (1 mg/ml) from Table 1. Bottom well: human serum sample with $alpha_1$ component for which goat number 248 had 'naturally occurring' antibodies prior to immunization with whole, boiled group A saliva.

The plasma from the catfish immunized with A saliva and the goat immune serum, however, both contained populations of precipitins specific for A substance that were able to diffuse through the group O saliva substances precipitated in parallel in the beta–$alpha_2$ region by the other immune antibody fractions. The precipitins unreactive with O saliva finally formed precipitin 'loops of identity' (emphasized with arrows in Fig. 2) with homologous A saliva substances diffusing toward the O saliva side of the plate in the beta–$alpha_2$ region.

DISCUSSION

An inverse relationship between human A or B and Lewis blood group activity was noted by Brown, Glynn and Holborow (1959) who, after demonstrating the ability of anti-A-precipitated saliva substances to neutralize anti-Le^a serum, hypothesized that at least in some ABH secretors 'part of the surface of the macromolecule not devoted to ABH activity has the configuration of Le^a specificity'. Brown *et al.* (1959) also noted the high Le^a activity of most non-secretor saliva substances, which they attributed to 'the greater proportion of the surface of blood-group macromolecules (of ABH non-secretors) occupied by groups with Le^a specificity'.

The present study further takes into account the amount of Le^a activity relative to Le^b activity in individual preparations of ABH-active purified substances from various primates. In Table 1, in the case of the most strongly A or B active primate substances (e.g.

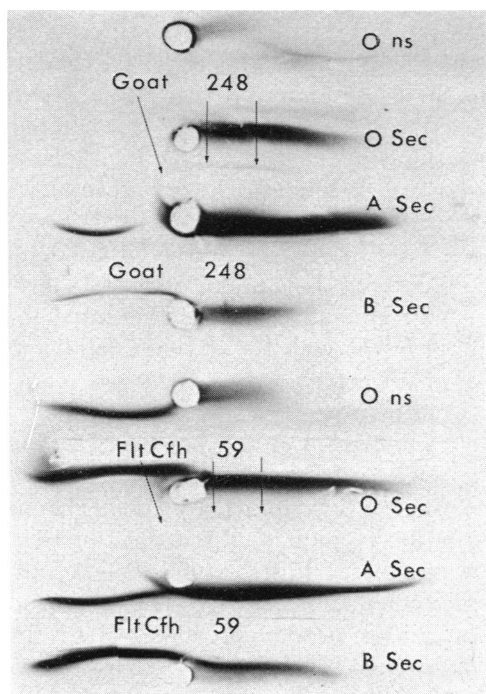

FIG. 2. Immunoelectrophoretograms of whole, boiled saliva samples from which purified saliva substances in Table 1 were derived. Goat number 248 and flathead catfish number 59 were similarly immunized with whole, boiled group A saliva.

BabStm number 2, RhStm 164, human Stm number 4 and human Stm number 6) both Le^a and Le^b activity are either absent altogether or present only at very low levels compared with the especially strong Le^b activity of individual preparations displaying comparatively weak A or B activity (e.g. BabStm number 5, BabStm number 3 and BabStm number 4).

Too few samples from each of the four different baboon species represented in Table 1 have been studied to draw any definite conclusions regarding possible species-associated tendencies to be strongly or weakly Lewis positive. Downing, Moores, Bolstridge, Schleyer, Klomfass and Davidson (1974), however, tested twenty-one different baboons of a single species (*Papio ursinus*) and found all specimens to be apparently devoid of Le^a activity but very strong in Le^b activity. In the present study, on the other hand, BabStm number 2 (*Papio hamadryas*), with the strongest baboon group A activity, was weak in Le^a activity and was altogether devoid of Le^b activity. BabStm number 5 (*Papio anubis*), in contrast, was strongly Le^b active and was also one of the most weakly A-active substances among all of the mammalian group A substances studied. The possibility of species-associated variability in Lewis versus A or B activity should obviously be taken into account in selecting primate materials for immunochemical studies.

The foregoing Lewis versus A or B profiles are remarkably consistent with *in vitro* biosynthetic studies (Shen, Grollman and Ginsburg, 1968; Kobata, Grollman and Ginsburg, 1968a, b; Grollman, Kobata and Ginsburg, 1969; Hearn, Smith and Watkins,

1968; Race, Ziderman and Watkins, 1968; Chester and Watkins, 1969). These investigators, using potentially blood group-active oligosaccharides as *in vitro* acceptor substrates, found that enzymic conversion to A or B chains required H-active monofucosyl acceptors for the respective attachment of A determinant α-*N*-acetyl-D-galactosaminyl or α-D-galactosyl end groups. H-active acceptors derived from 'type-1-like' precursor chains, however, could alternatively be converted to Le^b-active difucosyl chains by the enzymic attachment of an α-1,4-L-fucosyl residue to the penultimate monosaccharide residues. Le^b-active chains, however, could not be converted further to A or B active chains. No experiments appear to have in turn been conducted to demonstrate whether Lewis-associated α-1,4-fucosyl residues can be added to type-1-derived H chains that have already been converted to A or B chains. This is presumed to be possible, however, in order to account for the presence of such A- or B-active oligosaccharides with difucosyl structures among the alkaline degradation products of A and B substances (compare Watkins, 1972). In the case of a 'type-1-like' precursor chain first being enzymatically converted to an Le^a-active monofucosyl chain, however, no further enzymic conversion to Le^b or A or B activity appears to be possible.

Thus the α-1,4-fucosyltransferase determined by Lewis gene appears to 'compete' with the α-1,2-fucosyltransferase of the H gene for the utilization of type-1 precursor chains as acceptor substrates. In the case of the initial attachment of an Le^a determinant fucosyl residue, any further enzymic conversion to Le^b activity or subsequent A or B activity appears to be blocked. The initial attachment of an H determinant L-fucosyl residue, however, apparently does not in turn block further enzymic conversion of a type-1-derived H chain to an Le^b-active chain. The enzymic conversion from monofucosyl-associated H activity to difucosyl-associated Le^b activity, on the other hand, does apparently obstruct further enzymic conversion to either A or B active chains.

If the *in vitro* studies discussed above are applicable to the sequential transglycosylations that take place during the *in vivo* biosynthesis of the multiple heterosaccharide side chains of blood group-active macromolecules, then Lewis activity should tend to be inversely related to A or B activity. The results shown in Table 1 indicate that this indeed appears to be the case when a large number of blood group-active substances of diverse origin are comparatively tested on a quantitative basis. This study thus provides cogent serological evidence supporting the *in vivo* applicability of the *in vitro* biosynthetic studies.

That the chitobiose–cellobiose-inhabitable *Ulex* anti-H^C and L- fucose-inhibitable *Ulex* anti-H^F fractions do indeed represent two distinctly different lectin specificities was first reported by Flory (1966) and subsequently confirmed by Matsumoto and Osawa (1969, 1970) and Chuba (1973). Flory (1966) showed that non-secretor buccal cells possessed only H^C activity, but that secretor buccal cells, as well as both secretor and non-secretor red cells possessed H^C and H^F activity. Flory (1966) also showed that the presence of H^C activity in some non-secretor salivas could largely be attributed to the release of H^C antigen from buccal cells present in fresh saliva samples subjected to boiling water-bath inactivation without prior centrifugation to remove cellular debris.

Chuba (1973) subsequently discovered that apparently H-negative, group A pig submaxillary mucin purified in the laboratory of Dr Ward Pigman (compare Payza *et al.*, 1970), although completely devoid of H^F activity, was, on the other hand, the strongest H^C-active preparation found among nineteen different mammalian blood-group substances tested.

The results given in Tables 2 and 3 confirm the presence of strikingly high levels of H^C

activity in many pig submaxillary gland preparations, even in the complete absence of H^F activity. Further, the chitobiose–cellobiose-inhibitable anti-H lectin derived from *Laburnum alpinum* is only weakly reactive with pig submaxillary gland preparations (Table 2). Flory (1967) also noted a distinct difference in the respective specificities of *Ulex* anti-H^C and *Laburnum* anti-H, which in her studies could be demonstrated by the failure of *Laburnum* anti-H to react with the H^C-positive buccal cells of non-secretors.

The inverse relationship between pig submaxillary mucin group A and H^C activity is particularly well illustrated when individual preparations are tested separately (Table 3), rather than pooled according to the similarity of their profiles when screened initially (Table 2). Whether or not this relationship is primarily a serological phenomenon rather than an indication that pig H^C receptors can be enzymically converted into group A receptors remains to be determined.

Whether maximal *Helix* anti-A^{HP} reactivity of some pig submaxillary mucin preparations which do not react with IgM anti-A (e.g. extract number 5, Table 3) in turn indicates the presence of 'cryptic' A receptors selectively accessible to *Helix* anti-A^{HP} or, alternatively, a cross-reaction of *Helix* anti-A^{HP} (Hammarström and Kabat, 1971) with porcine α-*N*-acetylglucosaminyl end groups (Marcus and Cass, 1967; Lloyd, Kabat and Beychok, 1969) also remains to be determined. In the case of pig red cells, Uhlenbruck, Reifenberg and Heggen (1970) reported a close correlation in most samples between *Helix* anti-A^{HP} and *Dolichos biflorus* 'anti-A' titres. They also observed, however, that some pig red cell samples altogether devoid of *Dolichos* reactivity were, nevertheless, strongly reactive with *Helix* anti-A^{HP}, especially after pronase treatment.

Treatment with chymotrypsin was attempted in order to reduce the viscosity of purified, concentrated pig submaxillary mucin. Such treatment resulted in a selective loss of group A activity without a concomitant loss of H^C activity (Table 4). At present we do not have an explanation for this observation.

The whole question of the fine structure of the H^C receptors is at present clouded by the reported lability of both H^C and H^F activity to treatment with highly purified α-L-fucosidase preparations (Matsumoto and Osawa, 1970). This is perplexing in view of the fact that *Ulex* anti-H^C is readily inhibitable with chitobiose or cellobiose, but not with L-fucose, even when L-fucose is used at very high concentrations. Possible explanations are: (1) that receptor-associated fucose acts as an allosteric mediator for anti-H^C binding; (2) that the presence of fucose on the receptor oligosaccharide is necessary for the correct steric orientation of those portions of the receptor molecule which bind to *Ulex* anti-H.

Another aspect of protein–carbohydrate interaction to be taken into account would be the possibility that the *Ulex* anti-H^C-combining site, like the saccharide-combing site of eggwhite lysozyme and certain anti-dextran antibodies (Kabat, 1968), can accommodate several monosaccharide residues. Selective binding of saccharides other than L-fucose in portions of the anti-H^C-combining site could thus conceivably distort the configurational complementarity necessary for subsequent binding with L-fucose-associated H^C receptors. Possibilities along these lines could also be offered to explain how *Ulex* anti-H^C and *Laburnum* anti-H can be selectively inhibited with essentially the same saccharides and still have different anti-H specificities, as discussed above.

Figs 1 and 2 illustrate the usefulness as well as the limitations of immunoelectrophoresis for analytical purposes. In Fig. 2, for example, the presence of saliva fractions with gamma mobility was detected in all of the whole saliva samples tested with plasma from flathead catfish number 59 which had been immunized with A saliva, but not with serum from goat

number 248, which had also been immunized with A saliva. As shown in Fig. 2, the goat number 248 serum detected saliva fractions with gamma mobility only in the case of the group A and group B salivas. The A saliva gamma fraction, however, was not detectable with the goat number 248 serum after the whole, boiled A saliva sample had been concentrated 5-fold by dialysis (unlabelled top well, Fig. 1). This was possibly due to the loss of the A saliva gamma fraction along with other material during the membrane-mediated concentration procedure.

The goat and catfish reagents prepared by immunization with A saliva both demonstrated the presence (compare 'loops of identity' indicated with arrows in Fig. 2) of A-specific determinants which were present on the A saliva macromolecules in addition to determinants present also on group O saliva macromolecules showing the same beta–alpha$_2$ mobility.

The immunoelectrophoretic homogeneity of the four purified group A substances tested with the goat number 248 serum and with the plasma from channel catfish C10 is seemingly impressive but does not necessarily preclude the presence in the purified preparations of other subfractions for which corresponding precipitins were fortuitously not present in either immunoprecipitating reagent.

This study as a whole is consistent with, but does not unequivocally confirm, the currently postulated heterosaccharide microheterogeneity of individual glycoprotein macromolecules with blood group activity (Watkins, 1972; Marchesi, Tillack, Jackson, Segrest and Scott, 1972; Jackson, Segrest, Kahane and Marchesi, 1973).

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ADDENDUM

Prior to, and in accord with this study, I. Matsumoto and T. Osawa ((1971). 'On the specificity of various heterologous anti-H hemagglutinins.' *Vox Sang.* (*Basel*), **21,** 548) observed that pig submaxillary mucin, not characterized for its group A activity, was a

potent inhibitor of highly purified *Ulex* anti-H^F but failed to inhibit highly purified *Ulex* anti-H^C.

Previously D. Aminoff and M. P. Morrow ((1970). 'Effect of sialidase on blood group specificity and hog submaxillary glycoproteins.' *Fed. Europ. Biochem. Soc. Lett.*, **8,** 353) showed that *Ulex* anti-H reagent (apparently with predominantly anti-H^C activity) was strikingly less inhibitable with group A-active hog submaxillary glycoproteins than with preparations totally devoid of A activity. Moreover, they found that the potency with which A-inactive hog submaxillary glycoproteins inhibited their *Ulex* anti-H reagent was not correlated with the ability or inability of individual A-inactive preparations to inhibit eel anti-H serum. After treatment with sialidase, however, all their A-inactive preparations became potent inhibitors of eel anti-H.

Aminoff and Morrow (1970) therefore postulated that, in the case of phenotypically A-inactive hog submaxillary glycoproteins that failed to inhibit eel anti-H prior to sialidase treatment, L-fucose-associated H activity was 'masked' by the proximity of sialic acid residues.

Cogent immunochemical evidence supporting this hypothesis has subsequently been presented by M. M. Baig and D. Aminoff ((1972). 'Glycoproteins and blood group activity. I. Oligosaccharides of serologically inactive hog submaxillary glycoproteins.' *J. biol. Chem.*, **247,** 6111).

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