IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS LXII
Fractionation of Hog and Human A, H, and AH Blood Group Active Substance on Insoluble Immunoadsorbents of *Dolichos* and *Lotus* Lectins*

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The fucose-binding lectins from *Lotus tetragonolobus* have been purified (1–3), characterized, and shown to be specific for oligosaccharides containing fucosyl residues on C-2 of DGalβ1→4DGlcNAc[1] (type 2 chains) but not for DGalβ1→3DGlcNAc (type 1 chains) similarly substituted (3, 4). In addition, the *Lotus* lectin precipitated with H, A_2 and Le^a, but failed to interact with A_1 or B blood group substances (3, 4). The purified lectin from *Dolichos biflorus* did not precipitate with H or B blood group glycoproteins, but reacted with A_1 and A_2 substances (5). Because of their blood group specificities and since these two lectins can easily be purified in good yields using polyleucyl hog blood group A + H substances as immunoadsorbents (6, 7), they represent important potential tools in purification and for structural studies of blood group substances and their oligosaccharides. Indeed, lectins have already proven invaluable in the elucidation of the structures of blood group oligosaccharides isolated from human ovarian cysts, hog and horse gastric mucins, as well as from malignant cells and tissues (8–13).

The hog gastric mucin blood group substances are known to have A, H, or AH serological activities (14, 15). In addition, a new determinant consisting of terminal nonreducing DGlcNAcα1→4DGal has been identified in blood group glycoproteins from hog and human stomachs. This determinant is antigenic in man (16) and goat (17), and is responsible for Con A reactivity (9, 18). The commercial preparation of hog gastric mucin (Wilson) is a mixture obtained by pooling hog stomachs and therefore has A molecules and H molecules and contains individual molecules having both A and H activities. The usual fractionation procedures using phenol extraction and fractional ethanol precipitation (19) yielded products possessing both activities, while precipitation with Con A resulted in two fractions with similar blood group activities but differing in Con A reactivity (18, 20). However, a blood group A active material was separated from H by precipitation of hog mucin with A hemagglutinin from *Vicia cracca*, dissolution of the precipitate with 0.1 M sodium acetate pH 5.0, and gel filtration on Sepharose 2B (21).

The present study describes the purification and fractionation of the hog gastric blood group A + H glycoproteins by affinity chromatography with the purified lectins isolated from *L. tetragonolobus* and from *D. biflorus* seeds

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[1] Abbreviations used in this paper: Fuc, Fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HGM, hog gastric mucin A + H blood group substance; PBS, phosphate-buffered saline.

attached to Sepharose as immunoadsorbents. Fractions showing only A, only H, and a small amount of material with both activities are readily obtained. Human blood group A_1 and A_2 substances were also studied by this method. A_2 substance was separated into two fractions, one reactive with *Lotus* and the other lacking *Lotus* reactivity; A_1 substance was not retained by the *Lotus* column.

Materials and Methods

Analytical Methods. Methylpentose (fucose), hexosamine, *N*-acetylhexosamine, hexose (galactose), and nitrogen were estimated by colorimetric methods previously described (22, 23). Galactosamine was determined by the method of Ludowieg and Benmaman (24) Specific optical rotations were measured in a Perkin-Elmer polarimeter model 141 (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.).

Immunological Methods. Quantitative precipitin reactions with the *Lotus* and *Dolichos* lectins and human anti-A (Chris) were carried out at 4°C (3, 22); those with Con A were set up at room temperature (18). Hemagglutination inhibition was performed at room temperature with the Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) using four hemagglutinating units of a crude extract of *Ulex europeus* seeds or of anti-A (3, 22).

Lectins and Antisera. The *L. tetragonolobus* and *D. biflorus* lectins were purified by applying a crude extract of the seeds on to polyleucyl hog blood group A + H substance and eluting with L-fucose and *N*-acetyl-D-galactosamine, respectively (3, 5). The *Lotus* lectin thus isolated contains three or more components having different assocation constants and isoelectric points (2, 3), but with the same sugar specificity (3); no fractionation was needed for the purpose of this study. Con A was prepared by the method of Agrawal and Goldstein (25). Human anti-A (Chris) has been described (7). The *U. europeus* lectin was isolated using an *O*-α-L-fucosyl polyacrylamide adsorbent (26).

Conjugation of Lectins to Sepharose 2B. The *Lotus* and *Dolichos* lectins were coupled to Sepharose by the cyanogen bromide technique (27, 28). The isolated proteins, 121 mg of *Lotus* lectin in 52 ml of 0.15 M NaCl-0.01 M $NaHCO_3$ and 20.1 mg of *Dolichos* lectin in 46 ml of 0.15 M NaCl-0.01 M $NaHCO_3$, containing either 0.1 M L-fucose for the *Lotus* lectin or 0.05 M *N*-acetyl-D-galactosamine for the *Dolichos* lectin to protect the sites (29) were each incubated with 40 ml of Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, N. J.) activated with 8 g of CNBr. The pH of the Sepharose-lectin mixtures was adjusted to 7.5 with 2 N HCl and the coupling reaction allowed to proceed for about 15 h at 4°C; the lectin-Sepharose gels were then filtered on a Buchner funnel, washed with 0.15 M NaCl-0.01 M $NaHCO_3$, resuspended in 2 M glycine, and stirred for an additional 5 h to destroy residual activated Sepharose. The affinity adsorbents were then washed with about 1 liter 0.15 M NaCl-0.01 M $NaHCO_3$, followed by 0.01 M Na acetate buffer, pH 4.0 and 0.01 M phosphate-buffered saline, pH 7.1 containing 0.02% Na azide (PBS), and poured into appropriate columns (1.6 x 27 cm). This washing procedure was repeated before each experiment. In this manner, 96 mg of *Lotus* lectin and 19 mg of *Dolichos* lectin were bound to 40 ml of Sepharose 2B, respectively. An attempt to couple the Lotus lectin to Sepharose without using L-fucose to block its receptors yielded a product which did not significantly bind H substance.

Affinity Chromatography. Solutions of hog gastric mucin or human ovarian cyst blood group substances were dissolved in PBS (Na azide) and applied on the *Lotus* or *Dolichos* lectin affinity adsorbents, previously equilibrated with the same buffer at 4°C, the effluent being monitored by N content (ninhydrin procedure) and by inhibition of hemagglutination. The columns were then extensively washed with buffer and eluted with 0.01 M L-fucose and with 0.01 M *N*-acetyl-D-galactosamine in the same buffer, respectively, collecting samples of 2–3 ml, at a flow rate of about 9.0 ml/h. After exhaustive dialysis of the individual fractions against distilled water the peaks were detected by chemical and serological assays. Preliminary experiments showed that the concentration of hapten used was optimum for elution of the bound blood group substance, a 10-fold increase in concentration or the use of 6 M $MgCl_2$ not eluting additional peaks. The materials obtained were pooled, lyophilized and analytical solutions prepared.

Blood Group Substances and Monosaccharides. Experiments were carried out using hog gastric mucin blood group A + H substances (HGM) isolated from hog gastric mucin powder

(Wilson Laboratories, Chicago, Ill.; lot no. 120764) by ethanol precipitation (30). The human A_1 (MSS 10% 2×) and A_2 (cyst 14 phenol insoluble) blood group substances used were those described previously (3, 31, 32). L-fucose and *N*-acetyl-D-galactosamine were purchased (Mann Research Laboratories, New York).

Results

Fractionation of Hog Gastric Blood Group A + H Glycoproteins on Dolichos-Sepharose Columns. The elution profile of 25.0 mg of HGM on the *Dolichos*-Sepharose immunoadsorbent is shown in Fig. 1. The A and H blood group activities are almost completely separated in a single step, the H-active material (13.0 mg) appearing in the effluent and the A-active substance (9.2 mg) being specifically bound and eluted by *N*-acetylgalactosamine. As calculated from hemagglutination inhibition, the effluent contained about 88% of the H activity and the GalNAc eluate 87% of the A activity; however, the effluent and GalNAc eluates showed 4% of the A and 6% of the H activity, respectively. Quantitative precipitin assays (Fig. 1, inset) clearly show the effluent to be more active than the original HGM in interacting with *Lotus* lectin. The GalNAc eluate is about twice as active as the unfractionated material in precipitating the *Dolichos* lectin and anti-A, but did not react significantly with the *Lotus* lectin.

It should be noted that the above fractionation was carried out with 19 mg lectin on 40 ml Sepharose 2B; when 246 mg of *Dolichos* lectin on 30 ml Sepharose 2B were used, no fractionation of A and H activities was obtained since the effluent and the GalNAc eluate reacted equally well with both the A and H specific lectins and with anti-A. Indeed, when 16 mg of a purified hog blood group substance possessing only H but no detectable A activity (hog 38 8% 2×, cf. ref. 19) was run on the column with the large amounts of *Dolichos* lectin, the nonretained fraction (8.6 mg) as well as the material bound and eluted with GalNAc (5.4 mg) did not differ from the original H substance. Both had the same H activity as assayed with *Lotus* and *U. europeus* and did not react with the Dolichos lectin or a human anti-A. Thus, when the *Dolichos* lectin is coupled to Sepharose 2B at high concentrations (ca. 8.2 mg lectin/ml gel as opposed to 0.48 mg lectin/ml gel), it apparently loses its ability to discriminate between A and H substances, thus rendering the affinity adsorbent ineffective. This could be due to short chains of DGalNAcα1→3DGalNAc linked to serine or threonine in hog gastric (33) and human (34) ovarian cyst blood group substances.

Fractionation on Lotus-Sepharose 2B. When 39.1 mg of HGM were chromatographed on the *Lotus*-Sepharose 2B adsorbent, (Fig. 2 *a*) 22.1 mg of the applied material emerged in the effluent containing 88% of the original blood group A activity while the 13.0 mg eluted with L-fucose comprised 91% of the original H activity. The quantitative precipitin tests show the A activity of the effluent to be higher than that of the original HGM with both *Dolichos* and anti-A, but only negligible activity in precipitating *Lotus* (Fig. 2 *a*, inset). The fucose eluate shows negligible A but H activity higher than the original.

Blood group H glycoproteins devoid of any detectable A activity could readily be isolated by loading the Lotus column with a large excess of hog mucin, while pure A-active blood group substance was obtained by repeated rechromatography of the effluent as shown in Figs. 2 *b–f*. A protocol of the experiments is

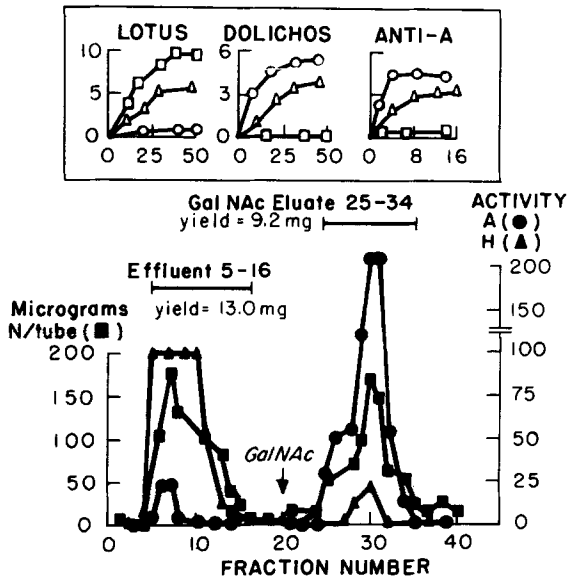

FIG. 1. *Dolichos* lectin-Sepharose 2B, 19 mg protein/40 ml gel. Affinity chromatography of hog gastric mucin A + H substance (25 mg) on *Dolichos*-Sepharose 2B. The column was developed as described in the text. A and H activities were calculated by multiplying the hemagglutination inhibition titer of anti-A and of *U. europeus*, by the volume of each fraction. The inset shows quantitative precipitin curves of the effluent (□), GalNAc eluate (○) and unfractionated hog mucin (△) with *Lotus* (9.9 μg N) and *Dolichos* lectins (7.5 μg N), and with anti-A (150 μl of serum Chris (7); the ordinate and abscissa are micrograms N precipitated and micrograms of blood group substance added respectively.

schematized in Fig. 3. Thus, when 200 mg of HGM was applied to the Lotus column, the eluted material (fucose eluate 1, 56.6 mg) was very potent in inhibiting *Ulex* hemagglutination (Fig. 2 *b*) and in precipitating *Lotus* lectin, while failing to inhibit A anti-A hemagglutination or to precipitate with *Dolichos* lectin or with human anti-A. This pure H substance represents 52% of the original H activity (cf. Fig. 3). It should be noted that by overloading the column not only was the purity of the fucose eluate improved but this was the procedure of choice for preparing large quantities of highly purified H substance. The nonadsorbed material (effluent 1, 123 mg) containing 92 and 52% of the original A and H activities, respectively, was rerun on the same column (Figs. 2 *c* and 3). Again the bulk of the A activity appears in effluent 2 (85 mg) but still contains some H activity; fucose eluate 2 (32.4 mg) showed slight A activity. This is seen in Fig. 2 *c* by the inhibition of hemagglutination profile and by the quantitative precipitin assay (Fig. 2 *c* inset). If fucose eluate 2 was again run on the *Lotus* column, fucose eluate 2a (Figs. 2 *d* and 3) free of A activity resulted. When effluent 2 was passed on the same column, the nonadsorbed peak (effluent 3, 68 mg) reacted only with anti-A and *Dolichos* lectin, (Fig. 2 *e*). Fucose eluate 3 (11.1 mg) possessed both A and H activities (Fig. 2 *e*). However, the A and H activities of the latter fraction could not be separated by rechromatography on the *Lotus*-Sepharose column (fucose eluate 3A, Figs. 2 *f*

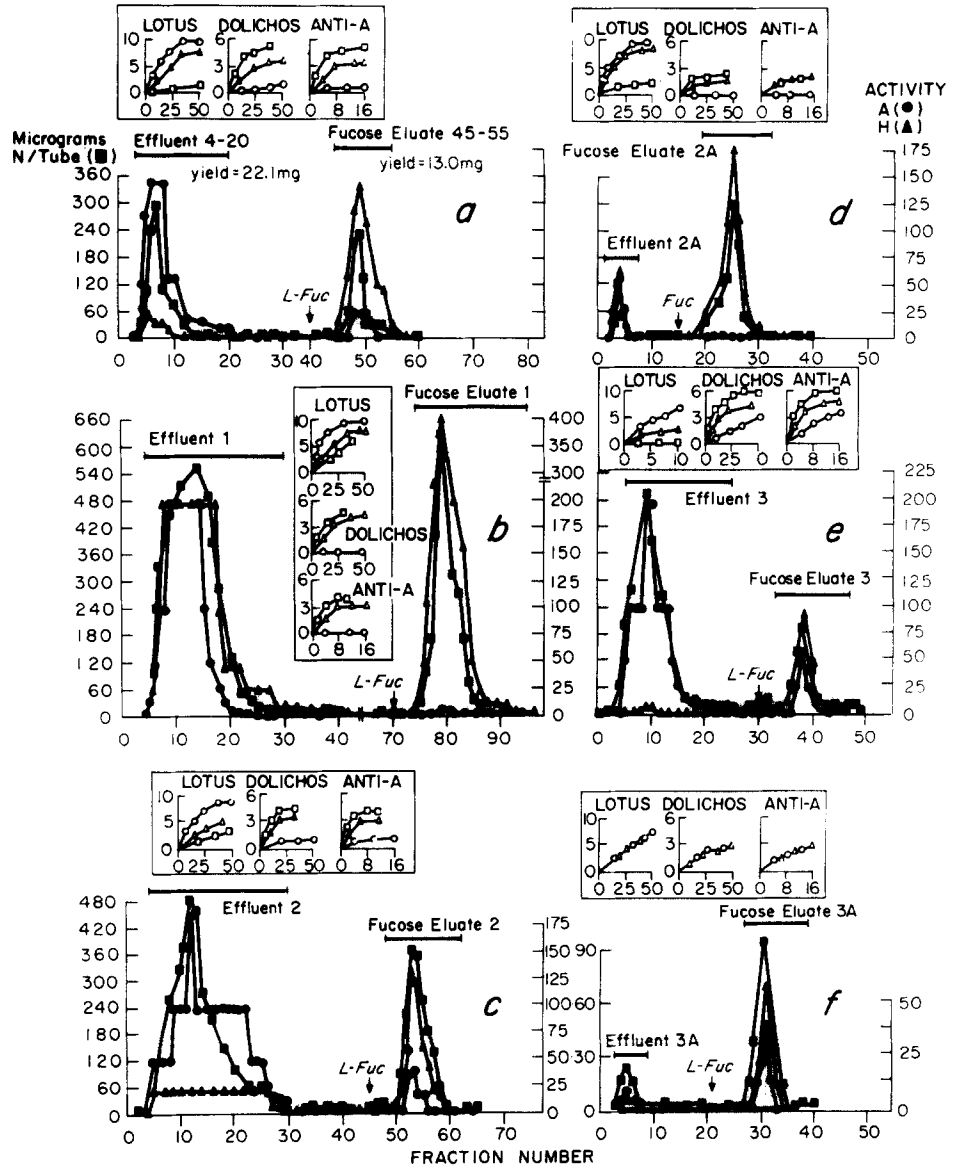

FIG. 2. *Lotus* lectin-Sepharose 2B, 96 mg protein/40 ml gel. Isolation of hog gastric mucin A, H, and AH blood substances by repeated affinity chromatography on *Lotus*-Sepharose 2B column. For details see text. Insets show quantitative precipitin curves of the applied materials (△), effluents (□) and fucose eluates (○). Abscissa and ordinates as in Fig. 1.

and 3) nor on the *Dolichos*-Sepharose column (not shown), suggesting that it might represent a component of HGM having A and H activities on the same molecule. This fraction (AH) comprises at least 4.5% of the HGM.

The A, H, and AH substances isolated all precipitated with Con A (not shown) indicating that the terminal nonreducing αDGlcNAc determinant is distributed

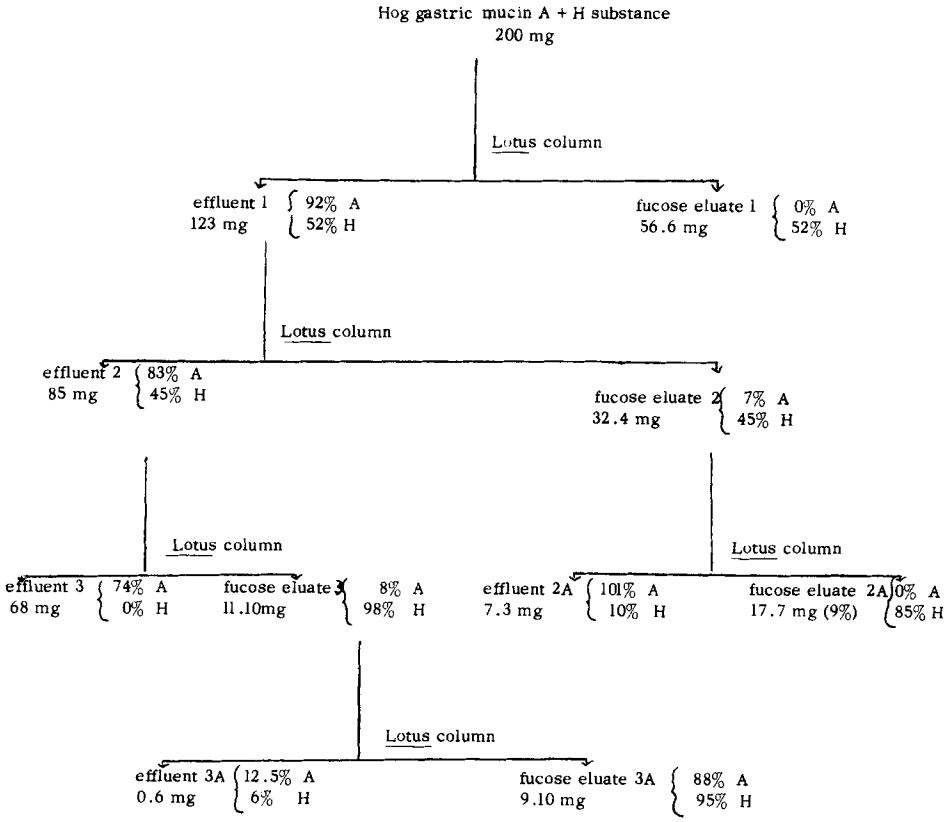

FIG. 3. Flow diagram showing procedure for isolation of A, H and AH substances by repeated affinity chromatography of hog gastric mucin A + H substance on a *Lotus*-Sepharose 2B column. The percent activity of each fraction is calculated from hemagglutination inhibition assays, the A and H activities of the applied material being taken as 100% in each step.

independently of the blood group activity, as established earlier (18). However, some heterogeneity was observed since fucose eluate 2 was the best reagent in precipitating Con A, effluent 3 was only 60% as active, with the other fractions showing intermediate potency.

Tandem Fractionation of HGM on Lotus-Sepharose and on Dolichos-Sepharose. To obtain pure preparations of A and H substances in a single run, 39.3 mg of HGM was chromatographed on the *Lotus* adsorbent column, which was connected directly to the *Dolichos*-Sepharose column so that the effluent emerging from the *Lotus*-Sepharose (showing high A and low H activity, cf. effluent tubes 4–20, Fig. 2 *a*) then passed through the *Dolichos* column from which fractions were collected. As seen in Fig. 4, 13% of the starting material was not retained by either adsorbent (effluent tubes 7–14, 5.2 mg), which showed lower A and H activities than the original HGM. After 90 ml the columns were disconnected and 0.01 M *N*-acetyl-D-galactosamine applied on top of the *Dolichos* adsorbent, giving a peak (GalNAc eluate tubes 35–44, 10.9 mg) having

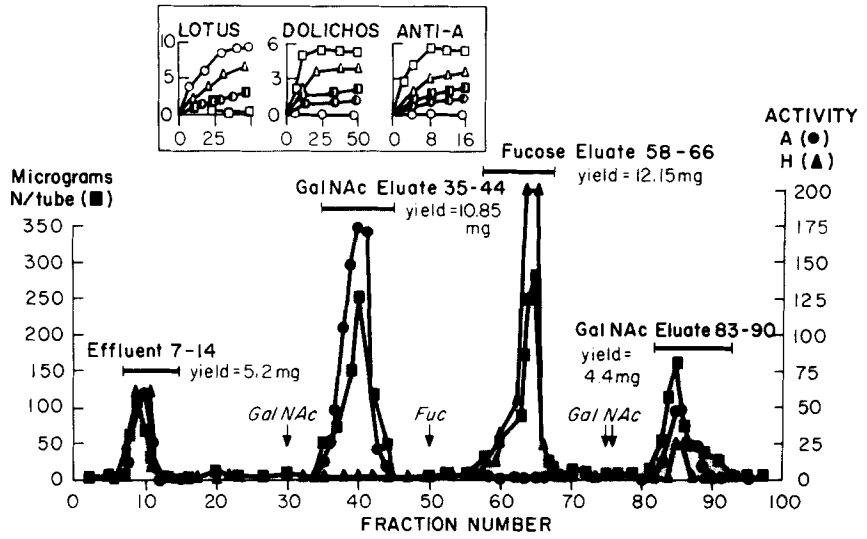

FIG. 4. Tandem fractionation of hog gastric mucin A + H substance on *Lotus*-Sepharose and *Dolichos*-Sepharose columns. Columns developed as described in the text. Inset shows quantitative precipitin curves of the original HGM (△), effluent tubes 7–14 (○), GalNAc eluate 35–44 (□), fucose eluate 58–66 (○) and GalNAc elute 83–90 (□) with *Lotus* and *Dolichos* lectins and with anti-A; abscissa and ordinate as in Fig. 1.

strong A but no detectable H activity (Fig. 4 and inset). The column was then washed with buffer to remove the GalNAc and reconnected to the *Lotus* adsorbent as before; elution was now performed by adding 0.01 M L-fucose to the *Lotus*-Sepharose, yielding a fraction after passing through both columns (fucose eluate 58–66, 12.2 mg) having only H activity (Fig. 4 inset). The A-contaminating activity usually present in the fucose eluate when similar amounts of HGM are passed on the *Lotus* adsorbent (cf. Fig. 2 *a*) was, therefore, retained by the *Dolichos* column and could be eluted with *N*-acetyl-D-galactosamine (GalNAc eluate tubes 83–90, 4.4 mg, Fig. 4). This fraction as well as the effluent tubes 7–14 showed some H activity in addition to A and might contain the HGM component having both A and H activities on the same molecule (cf. fucose eluate 3A, Figs. 2 *f* and 3). However, identification of these fractions as AH by rechromatography was not done in view of the low yields.

Analytical data on GalNAc eluate 35–44 (A-active) and fucose eluate 58–66 (H-active), are given in Table I. In general, the sugar compositions of these two fractions resembled one another as well as the original material. However, the galactosamine content was higher in the GalNAc eluate than in the fucose eluate, while methylpentose (fucose) tended to be lower as expected from their A and H activities respectively. The specific optical rotation was positive in the GalNAc eluate and negative in the fucose eluate, as compared with no significant rotation for the original. Analytical data and optical rotations were similar for the other fractionations.

Fractionation of Crude Hog Gastric Mucin on the Lectin-Affinity Columns. Purification and fractionation of the hog gastric mucin blood group glycoproteins can be carried out by dissolving hog gastric mucin powder (Wilson

TABLE I

Analytical Compositions of Hog Gastric Mucin and Fractions from Dolichos-Sepharose in Tandem with Lotus-Sepharose Columns (Percent Composition by Weight)

Fraction	Total	Methyl-pentose (fucose)	Hexose (galactose)	Hexosamine*	*N*-acetyl-hexosamine	Galactosamine	Peptide N‡	Specific optical rotation (deg)	
								$[\alpha]_D^{37}$	$[\alpha]_{365}^{37}$
	%	%	%	%	%	%	%		
HGM	5.4	9.0	19.1	29.9	27.9	13.6	3.3	−1	−8
GalNAc eluate tubes 35–44	5.5	7.9	17.9	33.9	26.4	14.2	3.0	+47	+66
Fucose eluate tubes 58–66	5.5	8.6	18.2	25.9	23.1	6.7	3.5	−27	−51

* In the hexosamine assay *N*-acetylglucosamine and *N*-acetylgalactosamine give equal color intensities whereas in *N*-acetylhexosamine assay *N*-acetylgalactosamine gives one-third the color intensity of *N*-acetylglucosamine.
‡ Calculated as difference between total N and hexosamine N.

Laboratories) in PBS-azide, clearing the solution by ultracentrifugation at 25,000 rpm and then running on the *Lotus*-Sepharose and *Dolichos*-Sepharose columns, thus avoiding a preliminary purification on the HGM by ethanol precipitation. When 72 mg of crude hog gastric mucin powder was run through these adsorbents, 16.25 mg of fucose eluate and 10.85 mg of GalNAc eluate were isolated, respectively, their immunochemical properties as well as their analytical compositions being similar to the corresponding fractions obtained by chromatographing the ethanol purified HGM. However, pure A and pure H substances could not both be obtained using only one column because the effluent was largely contaminated with extraneous materials present in the gastric mucin, and thus only eluates would yield pure materials.

Attempts to Fractionate an Individual Hog Stomach Glycoprotein having Both A and H Activities. Earlier reports (14, 15) indicated that purified products from individual hog stomach linings can exhibit A or H blood group activity, and that some individual hog stomach linings from heterozygous hogs show both A and H activities. It was therefore of interest to find out if the A and H blood group activities from such a presumed heterozygous individual hog could be separated on the H and A lectin-affinity columns. Hog 32 possessed A and H activity (14); when 13 mg were passed on the *Lotus*-Sepharose column, a small amount of material (1.0 mg) was obtained in the effluent but 11.2 mg (85%) was eluted with L-fucose. In hemagglutination inhibition tests using *U. europeus* lectin and anti-A, the fucose eluate was active at 32 μg/ml and 64 μg/ml, respectively, as compared with 42 μg/ml and 84 μg/ml, respectively, for the original material; the 1.0 mg from the effluent behaved similarly. Thus there is no evidence of separation of the A and H activity. The *Dolichos* adsorbent also failed to fractionate the A and H activities; when 6.5 mg of the material eluted by fucose from the *Lotus* column was passed on the column containing 19 mg *Dolichos* lectin per 40 ml gel, almost all of the blood group glycoprotein was retained and 5.8 mg was eluted with *N*-acetyl-D-galactosamine. It showed A and H activity identical to that of the fucose eluate and the original hog 32. The effluent recovered (0.5 mg) was insufficient for characterization. Thus the A and H determinants of hog 32 are attached to the same molecules of glycoprotein since both are simultaneously bound and eluted from either the H (*Lotus*) or A

(*Dolichos*) specific lectins. In contrast, the A and H activities of the pooled hog gastric mucin A + H substance are separated one from the other in either affinity column (cf. Fig. 1 and 2 *a*).

Fractionation Studies of Human A_1 and A_2 Blood Group Glycoproteins on the Lotus-Sepharose Immunoadsorbent. The basis for the difference between A_1 and A_2 specificity is still unsettled, various investigators disagreeing as to whether the difference is qualitative or quantitative (31, 35). The chromatographic behavior of A_1 and A_2 substances on the *Lotus*-Sepharose adsorbent was examined in an effort to get additional information.

The elution profile of 15.5 mg of cyst 14 phenol insoluble (A_2 substance) on the *Lotus* column is shown in Fig. 5, and it is evident that two fractions were obtained, one in the effluent (3.8 mg) and the other eluted with L-fucose (7.05 mg). The inset in Fig. 5 shows the ability of these fractions to precipitate H (*L. tetragonolobus* and *U. europeus* lectins) and A (*Dolichos* lectin and anti-A) reagents. Thus, the effluent completely failed to interact with the *Lotus* lectin while precipitating the *Ulex* and *Dolichos* lectins as well as anti-A antibodies, even though exhibiting only 33, 40, and 54% respectively of the original activity of the cyst 14. Inasmuch as *Lotus* lectin is specific for fucosyl residues on C-2 of DGalβ1→4DGlcNAc (type 2 chains) but not for DGalβ1→3DGlcNAc (type 1 chains) similarly substituted (3), *Dolichos* lectin is specific for α-linked DGalNAc (5) and anti-A reacts with A determinants on type 1 and 2 chains (31), it can be concluded that, in the effluent, all type 2 chains are terminated by DGalNAcα1→3 thus accounting for their inactivity with *Lotus* and for the *Dolichos* and anti-A activity, while at least some type 1 chains must be unsubstituted with αDGalNAc, to explain the *Ulex* precipitation. However, since the effluent is less active than the original cyst 14 in precipitating the *Ulex* lectin, one would infer that this hemagglutinin is less active in binding type 1 than type 2 chains; this was found to be so by inhibition of precipitation JSR_L 0.75, LFucα1→2DGalβ1→4DGlcNAcβ1→6R being over 400 times more active than Lacto-*N*-fucopentaose I, LFucα1→2DGalβ1→3DGlcNAcβ1→3DGalβ1→4DGlc.[2]

Since *Lotus* lectin does not react with nonfucose-containing type 2 oligosaccharides (3), one could assume that the inability of the effluent to precipitate the *Lotus* lectin might be due to such chains; it should then however be a very good reagent in precipitating type XIV antipneumococcus antibodies, which react with the DGalβ1→4DGlcNAc structure (36, 37). Since there was no precipitation, it is reasonable to assume that all or almost all of the type 2 chains are substituted with both αDGalNAc and fucose.

The fucose eluate was twice as active in precipitating *Lotus* lectin as the unfractionated cyst 14, while showing approximately the same activity toward the purified *Ulex* and *Dolichos* lectins, and also to anti-A as compared to the unfractionated cyst 14. Therefore, the fraction is enriched in type 2 chains without αDGalNAc substitution. The distribution of unsubstituted type 1 chains between effluent and eluate cannot be estimated.

The effluent and fucose eluate did not differ significantly in sugar composition (Table II); however, colorimetric values are, in general, slightly lower than

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[2] Pereira, M. E. A., E. A. Kabat, and F. Gruezo. Manuscript in preparation.

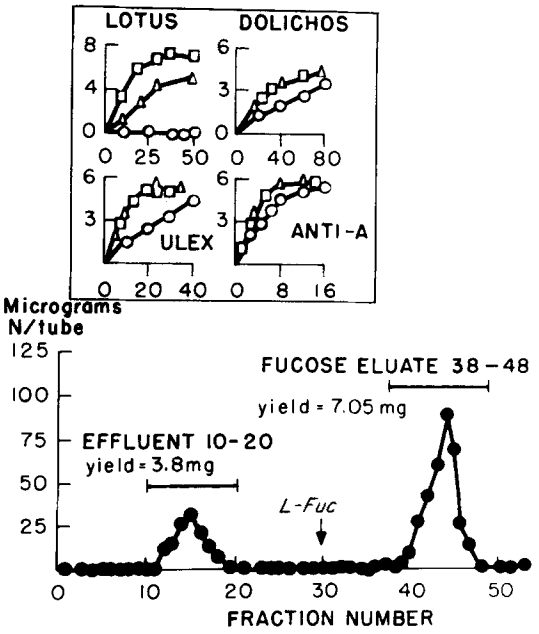

FIG. 5. *Lotus*-Sepharose 2B column, 96 mg lectin/40 ml gel. Fractionation of human ovarian cyst A_2 substance [cyst 14 phenol insoluble (31)] on a *Lotus*-Sepharose 2B column. Inset shows quantitative precipitin curves of unfractionated A_2 substance (△), effluent tubes 10–20 (○) and fucose eluate 38–48 (□) with *Lotus*, *Ulex* and *Dolichos* lectins and with anti-A; abscissa and ordinate as in Fig. 1.

TABLE II

Analytical Composition of Cyst 14 Phenol Insoluble and Its Fractions Separated on the Lotus-Sepharose Column (Percent Composition by Weight)

Fraction	Total N	Methyl-pentose (fucose)	Hexose (galac-tose)	Hexosa-mine	*N*-ace-tyl-hexos-amine	Galac-tosa-mine	Pep-tide N
	%	%	%	%	%	%	%
Cyst 14 phenol insoluble	4.5	22.6	31.4	29.5	25.9	10.9	2.2
Effluent tubes 12–18	3.9	18.7	25.3	25.3	23.0	7.8	1.9
Fucose eluate tubes 40–47	4.4	15.4	22.1	22.5	17.5	9.5	2.6

those for the original A_2 substance, perhaps due to contamination with materials coming from the immunoadsorbent column and the dialysis bags.

Attempts to fractionate 26.6 mg A_1 substance (MSS 10% 2×) on the *Lotus*-Sepharose column were unsuccessful, as the A_1 substance was not retained, 24.7 mg (93%) appearing in the effluent; only trace amounts were obtained by elution with fucose. A_1 substances do not react with *Lotus* lectin in the precipitin assays.

Discussion

The data presented above show that A- and H-active substances were readily isolated from pooled hog gastric mucin A + H blood group glycoproteins by

affinity chromatography on lectin-Sepharose. The best results were obtained when the *Lotus* or *Dolichos* columns were overloaded with HGM (Fig. 2 *b–f*) or, even better, when they were connected in tandem (Fig. 4). The method also proved effective in isolating A and H substances from commercial preparations of crude hog gastric mucin that had not been subjected to prior purification. Recovery of materials generally ranged from 70 (Fig. 5) to 93% (Fig. 2 *e*). Analytical data on these materials usually were comparable to those purified by phenol-ethanol (19) although analytical values sometimes were slightly lower probably due to materials leaking from the immunoadsorbent and dialysis bags since about 0.5 mg of dried material could be recovered from 10 ml dialyzed and lyophilized effluent even after the immunoadsorbent column or plain Sepharose 2B were washed intensively with buffer. Lower sugar content of purified materials has also been observed previously following affinity chromatography (38, 39) and is a problem especially with small amounts (39).

The findings with hog 32 indicate that the A and H activity of this preparation cannot be separated either on Dolichos or *Lotus* adsorbents, thus strongly suggesting that both specificities of this presumed heterozygous hog are associated with the same macromolecular structure and are not a mixture of molecules possessing either A or H activity. It is interesting that a fraction obtained from hog gastric mucin A + H substance (fucose eluate 3 A, Fig. 2 *f*) behaved like hog 32 chromatographically on both immunoadsorbent columns, in that A and H activity were not separable and thus both were carried by single molecules. Since commercial hog gastric mucin is a pool of hog stomachs, including those from heterozygous hogs, the 4.5% yield of this material is a minimum estimate since all effluents were not examined. The suggestion that heterozygous human blood group substances possess multiple specificities on the same macromolecule was put forward by Morgan and Watkins (40, 41); they showed that when saliva or ovarian cyst fluid from A_1B or A_2B individuals were precipitated with rabbit anti-A or rabbit anti-B, the A and B activities were recovered in the precipitates in the same ratio as in the original solution, while with an artificial mixture of A and B substances, the rabbit anti-A or anti-B removed only the homologous substance, leaving the other in the supernatant. Brown et al. (42) provided serological evidence that Le^a and A specificities from a saliva sample are associated on the same macromolecule. That heterozygous individuals possess multiple blood group determinants on individual molecules is of considerable structural and genetic importance (cf. 12).

The results in Fig. 5 show that the two fractions obtained from an A_2 substance (cyst 14 phenol insoluble) on the *Lotus* affinity column differ remarkably in their specificities; material not retained lacking the ability to precipitate *Lotus* while showing 33, 40 and 54% of the original activity with *U. europeus* and *Dolichos* lectins and with anti-A respectively, implying that the H determinants of the effluent have some unsubstituted type 1 chains with all of their type 2 chains blocked by αDGalNAc.

The reverse is seen with fucose eluate which is a more potent reagent for *Lotus* than is the unfractionated A_2 substance but has about the same capacity as unfractionated A_2 substance for precipitating *Ulex*, *Dolichos*, and anti-A. The eluate therefore has some free type 2 H determinants since it reacts with the

Lotus and also has A determinants responsible for *Dolichos* and anti-A activities. Although these two fractions are both made up of molecules showing both A and H specificities, the H determinants of the effluent are qualitatively different from those of fucose eluate. Inability to determine the proportion of unsubstituted type 1 determinants makes it impossible to establish whether type 1 as well as type 2 determinants in A_2 substance are substituted by DGalNAcα1→3 residues.

Affinity chromatography on lectin-Sepharose columns has thus proven very satisfactory for fractionating and isolating A and H blood glycoproteins from the commercially available hog gastric mucin and, as a consequence, one can obtain pure preparations of hog A or H substances without the use of individual hog stomachs. The method also permits identification of A and H activities on the same molecule. In principle, these cultures may also be useful for fractionating erythrocytes and other cells based on their A and H receptors.

Summary

The purified lectins from *Lotus tetragonolobus* and *Dolichos biflorus* were coupled to Sepharose 2B to make insoluble adsorbents for purification and fractionation of blood group A and H active glycoproteins. With both adsorbents, hog gastric mucin A + H blood substance (HGM), purified by phenol-ethanol precipitation, yielded fractions showing only A, only H, or AH activities. The AH fraction was obtained when the adsorbent column was overloaded with HGM and its A and H specificities seem to be carried on the same molecules since they were not separable by chromatography on either column. However A and H specificities of blood group substance from the stomach of a presumably heterozygous individual hog were both on the same molecules as they too could not be fractionated on either column. Analytical properties of the isolated fractions were generally similar to those of the unfractionated material, the purified A substances had a higher galactosamine/fucose ratio than did the H substances. Although the original A + H showed very little specific optical rotation, the separated A and H substances rotated positively and negatively, respectively. The lectin-Sepharose adsorbents have also proven useful in isolating A or H substances directly from the crude commercial hog gastric mucin. Blood group A_2 substance from a human ovarian cyst yielded two fractions on the *Lotus*-Sepharose column; the effluent did not interact with the *Lotus* lectin but precipitated the *Ulex* and *Dolichos* lectins and anti-A, and appears to contain type 1 H determinants. The other fraction reacted with *Lotus* and *Ulex* lectin as well as with *Dolichos* and anti-A.

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