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Heterogeneity of Carbohydrate Fragments Isolated from Human Blood Group H and Le^a Active Glycoproteins by Base–Borohydride Degradation†

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ABSTRACT: Degradation of two blood group substances possessing H and Le^a activity by 0.05 N NaOH and 1.0 M $NaBH_4$ gave a mixture of oligosaccharide chains all ending with *N*-acetyl-D-galactosaminitol with minimal amounts of degradation products. Passage of the dialyzable material on Bio-Gel P-2 revealed substantial heterogeneity evident primarily from the presence of several peaks and from the distribution of fucose. In the H active substance this sugar was present in several included peaks while it was restricted almost exclusively to the excluded carbohydrate fraction of the Le^a active glycoprotein. Although the gel filtration profiles of the two dialysates were generally similar, both showing a large excluded portion containing the more complete megalosaccharide chains and several additional included peaks, the H oligosaccharides had more material of larger size. All peaks were heterogeneous on analytical paper and on charcoal chromatography, and contained incomplete oligosaccharide chains decreasing in size and composition down to *N*-acetyl-D-galactosaminitol. Evidence is presented that some of the nondialyzable fraction had also undergone alkaline β elimination from the protein with the formation of large reduced oligosaccharides terminated by *N*-acetyl-D-galactosaminitol.

The series of oligosaccharides isolated in this and in other laboratories from blood group active glycoproteins by various degradation procedures such as mild acid hydrolysis and alkaline or alkaline borohydride degradation have been fundamental for establishing the structure of the blood group specific determinants and for the elucidation of the general structure of blood group active glycoproteins (Painter and Morgan, 1961; Schiffman *et al.*, 1964; Marr *et al.*, 1967; *cf.* Kabat, 1970, 1973, for reviews). It has also become possible to assemble them into a proposed overall composite structure (Lloyd *et al.*, 1968; Lloyd and Kabat, 1968; Vicari and Kabat, 1970; Lundblad *et al.*, 1972), accounting for almost all of the carbohydrate chains isolated from blood group A, B, H, Le^a, and from a precursor substance, shown to possess blood group I activity (Feizi *et al.*, 1971a,b) as well as for the products of alkaline degradation. This composite structure is thought to represent a relatively complete type of carbohydrate chain. Since experimental data always indicated that the carbohydrate moiety of blood group active glycoproteins showed substantial heterogeneity, individual chains could occur in the proposed more complete megalosaccharide, or in various stages of completion all the way down to single *N*-

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acetyl-D-galactosamine residues, linked to serine or threonine in the polypeptide backbone. Such a phenomenon appears to be characteristic of most glycoproteins (Spiro, 1970). Under the conditions of degradation used thus far (1% $NaBH_4$ in 0.2 N NaOH or 1% $NaBD_4$ in 0.2 N NaOD at room temperature) it has been difficult to obtain entire oligosaccharide chains because of the types of linkages of the sugars which make up the chains (Ballou, 1954; Whistler and BeMiller, 1958). Thus, usually oligosaccharides were obtained which were degraded to different extents by peeling from the reducing end after β elimination of the *N*-acetyl-D-galactosamine from its *O*-glycoside linkage to serine and threonine (Lloyd *et al.*, 1966, 1968; Lundblad *et al.*, 1972). The earlier studies were thus done with largely or partially degraded dialyzable fragments which made a precise evaluation of heterogeneity difficult and have left some uncertainties as to the structure and the biosynthesis of the blood group active glycoproteins; several fucose residues also remained unaccounted for in the composite structure (*cf.* Kabat, 1970, 1973).

Iyer and Carlson (1971) modified the above procedure and treated human ovarian cyst H material with 0.05 N NaOH and 1.0 M $NaBH_4$ at 50° and found that these conditions allowed the breaking of the protein–sugar linkage with release of entire oligosaccharide chains and reduction of the reducing end without detectable degradation. Applying the same conditions to several different blood group active glycoproteins, in a small-scale experiment, we found that no substantial amounts of degradation products could be seen by gas chromatography (Anderson *et al.*, 1972). These findings challenged us to treat larger amounts of cyst blood group substances to obtain undegraded oligosaccharide chains in amounts sufficient for isolation and chemical characterization.

This study describes gel filtration findings on Bio-Gel P-2 of the dialyzable oligosaccharide chains cleaved by 0.05 N NaOH and 1.0 M $NaBH_4$ at 50° from an H and from an Le^a active human ovarian cyst glycoprotein. This is the first step in the fractionation and purification of the oligosaccharides from the two blood group substances after alkaline borohydride degradation currently in use in this laboratory. The compositions and sizes of the fractions obtained from the H and Le^a substances are in good agreement with the immunochemical and genetic concepts of blood group structure. Findings on the purified oligosaccharides will be presented in a subsequent paper. In addition analytical data on the nondialyzable portion indicate that alkaline β elimination has also taken place and that it contains large oligosaccharide chains terminated by *N*-acetyl-D-galactosaminitol.

Materials and Methods

Blood Group Active Glycoproteins. The blood group substances used, JS (H and Le^b active) and N-1 (Le^a active), were purified from human ovarian cyst fluid by digestion with pepsin, precipitation with ethanol, extraction with 90% phenol and fractionation with ethanol (*cf.* Kabat, 1956).

JS phenol insoluble (2.56 g) described by Schiffman *et al.* (1964) were used. A second portion of N-1 was fractionated as described by Lloyd *et al.* (1968). The following fractions were pooled for a total of 4.56 g and used for this study: N-1-1 (2.20 g) isolated by two precipitations with 10% ethanol; N-1-2 (0.83 g) isolated by precipitation with 20% ethanol from the second precipitation with 10% ethanol; N-1-3 (1.52 g) isolated by subsequent precipitation with 10% ethanol from the first precipitation with 20% ethanol.

Colorimetric methods for the analysis of nitrogen, hexosamine, *N*-acetylhexosamine, methylpentose (fucose), and hexose (galactose) have been described previously (Kabat, 1961; Lloyd *et al.*, 1966; Ludowieg and Benmaman, 1967). Hexosamine was determined after hydrolysis with 2 N HCl for 2 hr at 100° and re-N-acetylation was performed for *N*-acetylhexosamine determination (Kabat, 1961).

The gas chromatographic assay for free or bound *N*-acetyl-D-galactosaminitol and degradation products after alkaline–borohydride treatment of blood group active glycoproteins, as well as standards and reference compounds, are described in Anderson *et al.* (1972). According to this method, bound *N*-acetyl-D-galactosaminitol is converted to its alditol acetate derivative by methanolysis of the carbohydrate chain followed by O-acetylation. Quantitative data are obtained by internal standardization with erythritol or inositol, or both, and are corrected for the detector response to the derivative by a molar response factor determined with standard *N*-acetyl-D-galactosaminitol. Variation in the molar response factors obtained using samples of unsubstituted or variously substituted *N*-acetyl-D-galactosaminitol derivatives was seen. This is a consequence of differences in number, location, and possibly nature of the substituents. It is not an erratic phenomenon in gas–liquid chromatography (glc), since a given structure always showed the same extent of degradation. Furthermore, the relation between alditol acetate concentration and the area under the curve in the chromatograms while linear did not pass through their origins and for accurate determination, a calibration curve was always necessary. These observations were made on pure oligosaccharides, with which one could estimate quite precisely the extent of degradation since several analytical criteria are determined independently in establishing their structures, and because it is often possible to use an appropriate standard for each. With complex mixtures of oligosaccharides such as those in the present study, the results in Tables I and II are based on an average molar response factor of free *N*-acetyl-D-galactosaminitol and some error can be expected. On the other hand, there does not appear to be a systematic study on the quantitation and extent of degradation of substituted *N*-acetyl-D-galactosaminitols so that this approximate method is the best as yet available. Attempts at acid hydrolysis of the oligosaccharides were not satisfactory because of the difficulty of hydrolyzing hexosaminide bonds after de-N-acetylation. If loss of an *N*-acetyl group occurs before complete hydrolysis of its glycoside bond, the bond becomes very resistant to further acid hydrolysis.

For paper chromatography the following solvent systems were used: 1-butanol–pyridine–water (6:4:3), solvent 1; 1-butanol–pyridine–water (35:39:26), solvent 2. Galactose, lactose, isomaltopentaose, and isomaltooctaose were reference compounds.

Experimental Section and Results

Alkaline Borohydride Degradation. The two blood group active glycoproteins were degraded for 16 hr at 50° with 0.05 N NaOH and 1.0 M $NaBH_4$ at a concentration of 10 mg/ml as described by Iyer and Carlson (1971) (*cf.* Anderson *et al.*, 1972). The reactions were stopped by cooling and neutralizing to pH 7.0 with concentrated HCl and the materials were dialyzed against ten volumes of distilled H_2O. The dialysate was collected in two consecutive fractions: dial I, a pool of five successive dialysates obtained each after 30 min, and dial II, a pool of four successive dialysates, three obtained each after 24

TABLE I: Composition of Dialyzable and Nondialyzable Fractions of Blood Group H and Le[a] Substances after Alkaline Borohydride Degradation.

	Amount in Original Glycoprotein (mg)	Recov after Base–Borohydride Degradation			Total Recovery	
		Dial I (mg)	Dial II (mg)	Non-dial (mg)	mg	%
JS (2.56 g)						
Methylpentose (fucose)	493	43.6	295	154.5	493	100
Hexose (galactose)	731	55.8	321	197	574	79
N-Acetylhexosamines	649	38.1	299	174.5	512	79
Hexosamines[a]	800	48.4	424	212	684	86
Total N	118	6.3	67.2	24.4	98	83
Non-hexosamine N[b]	67.4	3.2	40.3	10.9	54	81
Galactosamine[a,c]	264	3.6	63	32	99	38
N-Acetyl-D-galactosaminitol[d]		23.5	122.9	48.5	195	74[e]
Peptide N[f]	67.4	1.8	33	7.9	42.7	63[g]
N-1 (4.56 g)						
Methylpentose (fucose)	352	6.4	109	213	328	93
Hexose (galactose)	1397	51	706	570	1327	95
N-Acetylhexosamines	1581	34.5	598	589	1221	77
Hexosamines[a]	1916	40	885	756	1681	88
Total N	252	7	132	63	202	80
Non-hexosamine N[b]	130	4.3	76	15.6	96	74
Galactosamine[a,c]	585	2.7	83	68	154	26
N-Acetyl-D-galactosaminitol[d]		39.2	267	57	363	62[e]
Peptide N[f]	130	2	59	12	73	56[g]

[a] Calculated as *N*-acetylhexosamines. [b] Calculated as difference between micromoles of total N and micromoles of hexosamine N. [c] Calculated colorimetrically (Ludowieg and Benmaman, 1967). [d] Calculated by glc method (Anderson *et al.*, 1972). [e] Per cent of original galactosamine. [f] Calculated as difference between micromoles of total N and micromoles of hexosamine N + *N*-acetyl-D-galactosaminitol N. [g] Per cent of original non-hexosamine N.

hr and the fourth after 48-hr dialysis. The two fractions and the nondialyzable portion were concentrated to dryness. Dial I was passed through a column of mixed-bed resin (Amberlite MB-3) and the column was washed with water. Borate was removed from both the dialyzable fractions by repeated additions and evaporations with about 100 ml of absolute methanol. Yields and analytical data for these products and for the nondialyzable fraction in comparison with the original blood group substances are given in Table I. The data show generally good recovery of all the sugars, with the exception of *N*-acetyl-D-galactosamine. After degradation, only 26 and 38% of this amino sugar remained in the N-1 and JS materials, respectively. This was expected because under the degradation conditions used most of the *N*-acetyl-D-galactosamine is converted to *N*-acetyl-D-galactosaminitol, as the oligosaccharide chains are released from the protein before further degradation due to the base-catalyzed peeling reaction could occur. From the recovery of *N*-acetyl-D-galactosaminitol, about 74 and 62% of the carbohydrate chains with internal *N*-acetyl-D-galactosamine linked to the protein were accessible to the degradation conditions used, and were split off from the JS and N-1 glycoproteins respectively. In JS only 79% of the galactose and in N-1 88% of the *N*-acetyl-D-galactosamine as the sum of *N*-acetyl-D-galactosaminitol and *N*-acetyl-D-galactosamine were recovered; in JS, however, the latter recovery was 112%.

The two dialyzable and the nondialyzable fractions were analyzed by the gas chromatographic method of Anderson *et al.* (1972), for the detection of degradation products and for the quantitation of free and bound *N*-acetyl-D-galactosaminitol, which is not detectable by colorimetric analysis. This experiment presented some difficulties since a large amount of salt was present in the analytical solutions of the three fractions, especially in dial II, in spite of several desalting procedures tried, and seemed to reduce the precision of the method. However, the quantitative results for *N*-acetyl-D-galactosaminitol given in Table I are in fairly good agreement with the loss of hexosamines and of *N*-acetyl-D-galactosamine calculated from colorimetric analysis. Degradation products expected from a base-catalyzed peeling reaction, chromogens, free or bound galactitol, hexenetetrol(s), and 3-deoxygalactitol were only barely detected qualitatively in dial I, especially with N-1, and in both they were present in amounts too small to be quantitated exactly. These findings confirmed that the majority of the oligosaccharides obtained were intact and had *N*-acetyl-D-galactosaminitol at the original reducing end. Most were in dial II, while dial I contained largely free *N*-acetyl-D-galactosaminitol plus small amounts of degradation products and some salt from the base–borohydride treatment.

The analytical data on the two intact glycoproteins are representative and show that the two blood group preparations were fairly uniform except that the H substance (JS) has more fucose as expected. This is confirmed by the remarkable similarity in sugar composition found in the dial II fractions of the two substances, again except for the higher fucose content of JS. The ratio between fucose contents of N-1 and JS,

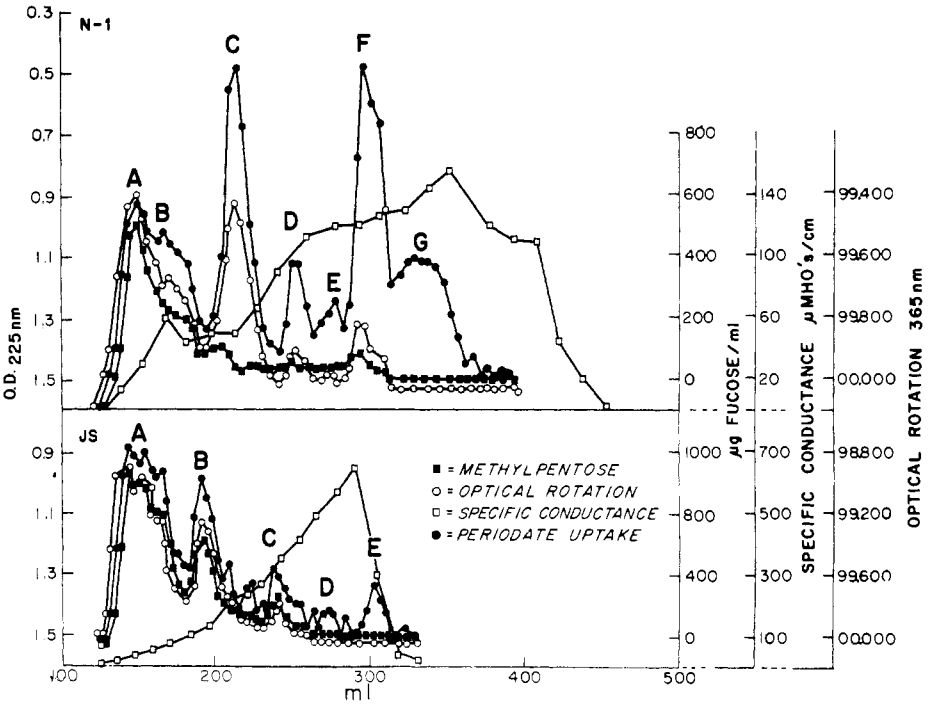

FIGURE 1: Chromatography on Bio-Gel P-2 of the dialyzable materials obtained by degradation of N-1 (Le^a) and JS (H) blood group substances by 0.05 N NaOH in 1.0 M $NaBH_4$ at 50°. Dextran elutes between 132 and 154 ml, isomaltohexaose at 209 ml, isomaltotetraose at 240 ml, and lactose at 280 ml.

which was about 1:3 in the original glycoproteins, is about 1:6 for the two dial II fractions. A larger portion of the fucose of N-1 than of JS remained nondialyzable.

If one subtracts from the total N, the sum of the hexosamine N and the *N*-acetyl-D-galactosaminitol N, one can estimate the N associated with peptide in the dialysates and in the nondialyzable fraction. This is listed as peptide N in Table I. Several important inferences can be made. The total peptide in the nondialyzable fractions of N-1 and JS is but a small proportion of the weight. Most of the peptide N is found in dial II indicating that the borohydride had probably produced considerable hydrolysis of peptide bonds (Kabat *et al.*, 1965). On hydrolysis amino acids were detected in both dialysates of N-1. Moreover the substantial proportion of *N*-acetyl-D-galactosaminitol in the nondialyzable fractions indicates that the β elimination from the protein had taken place in this portion as well and therefore that larger oligosaccharides than those in the A fractions are present. It is of significance in this connection that fucose in the nondialyzable portions of N-1 accounts for 61% of the original fucose in the glycoprotein while in JS it was but 31% although the latter had a much higher fucose content; this is consistent with the finding that most of the dialyzable fucose of N-1 was present in the A fraction.

Galactosamine in dial I, dial II, and nondialyzable fractions of JS and N-1 makes up about 38 and 26%, respectively, of the total galactosamine. These values may indicate the maximum numbers of chains resistant to alkaline elimination as would obtain with chains linked to N-terminal or C-terminal serine or threonine (Carubelli *et al.*, 1965) or attached to the protein portion by an *N*-glycosylamine linkage to asparagine (Marks *et al.*, 1963; *cf.* Gottschalk, 1966).

Fractionation of the Dialyzable Products. A Bio-Gel P-2 (−400 mesh) 2.8 × 88 cm column was prepared as described by the manufacturers (Bio-Rad laboratories) and calibrated with a mixture of Dextran NRC 3 (mol wt 51,000) (Kabat 1961), isomaltohexaose, isomaltotetraose, lactose, and NaCl. The same column was used for the dialyzable material from both N-1 and JS. It was washed with distilled H_2O between each run, and kept in 1% sodium azide when not used for long periods. The two dialyzable fractions, dial I and dial II, of N-1 and of JS were pooled and 700–2000 mg of sample dissolved in the minimum amount of water were chromatographed at a time eluting with distilled water. Routinely 400 tubes of 1.2 or 1.9 ml were collected, and small aliquot portions of every fifth tube were analyzed for periodate consumption by the decrease in its absorption at 225 nm (Kabat, 1961) and for electric conductance. Within the carbohydrate-containing region, every fifth tube was then analyzed for fucose and for optical rotatory activity.

Typical elution curves for N-1 (Le^a) and for JS (H) are shown in Figure 1. Because of the large amount of material, it was necessary to make four runs on the column to fractionate the JS dialysate and six runs for the N-1 dialysate. The diagrams in Figure 1 are representative and the profiles of the elution patterns for repeated runs on each substance were the same. As shown in Figure 1 the elution diagrams of the dialyzable fractions of N-1 and JS gave an excluded peak and a number of included peaks, indicating that the dialysates were mixtures of oligosaccharides of different molecular weights.

Peaks in Figure 1 were pooled for N-1 and for JS as follows:

	N-1 (ml)		JS (ml)
A	124–154	A	124–172
B	155–194	B	173–224
C	195–242	C	225–257
D	243–265	D	258–276
E	266–284	E	277–324
F	285–316		
G	317–372		

Analytical paper chromatography of the fractions on solvents 1 or 2 showed that they were all complex mixtures reflecting the lack of complete fractionation. A large portion of the JS and N-1 dialysates was excluded by Bio-Gel P-2 (the excluded volume of the column was 154 ml). This fraction is designated A for the N-1 dialysate, and comprises most of the A fraction of JS. The entire A fraction of JS represents about 60% of the dialyzable carbohydrate, and consists largely of the excluded portion and the first material which is included in Bio-Gel P-2, but is not clearly separated from the excluded fraction. The included as well as the excluded portions showed a very high fucose content (Figure 1). This way of pooling was suggested by theoretical and practical considerations. Cyst substance JS possessed both H and Le^b activity and is therefore very rich in fucose (Table I). In accord with the proposed composite structure of the blood group determinants, its largest chains should carry up to four fucoses, and possibly even more if the structure carries three branches as postulated (Lloyd and Kabat, 1968). In addition, the megalosaccharide chains would be expected to show extensive heterogeneity, as is seen on analytical paper chromatography, probably due to different amounts and/or to different locations of the fucose residues. On the basis of these observations, we anticipated the same difficulties of fractionating the large heterogeneous fragments from this high fucose-containing region, both in the Bio-Gel excluded, as well as in the included portion, and the entire peak A was pooled.

N-1 glycoprotein has less fucose as compared with JS, as expected from its Le^a activity, and was already suggested as having a simpler structure, because it represents an earlier stage of biosynthesis (Watkins and Morgan, 1959; Ceppellini, 1959; Lloyd *et al.*, 1968). This was confirmed by its behavior on Bio-Gel P-2. The A fraction of N-1 is completely excluded by Bio-Gel P-2, but seems to be clearly separated from the first included peak, called B. It is clearly differentiated from B by its higher fucose content (Figure 1) and peaks A and B from N-1, representing about 35 and 23%, respectively, of the dialyzable carbohydrate were further fractionated separately.

In the included region, the Bio-Gel profile shows a well differentiated peak for both N-1 and JS accounting for about 24% of the carbohydrate of both dialysates and is called C for N-1 and B for JS. N-1 peak C elutes in the region at which material with molecular weight around 1000–600 would be found, based on the standardization with isomaltose oligosaccharides. JS peak B elutes somewhat earlier than N-1 peak C, suggesting a slightly larger average size in accord with its much higher fucose content. The entire peak B from JS contains large amounts of fucose, while only the early portion of peak C from N-1 shows a very small amount of fucose corresponding to the region at which peak B of JS is eluted. The bulk of peak C and all the other peaks of N-1 subsequently eluted contain no fucose. By contrast, fucose is seen in peak C of JS.

The peaks eluted later represent smaller incomplete oligosaccharide chains, of decreasing size down to *N*-acetyl-D-galactosaminitol, which has been isolated from peaks E and F of N-1. There was some difficulty in working with the material eluted in the region between 260 and 400 ml, since it represents only a small per cent of the dialyzable carbohydrate and because of the very large amount of salt and perhaps peptides which occur in the same region. However, some oligosaccharides eluted in this region have been purified. The borate would tend to produce higher values for periodate uptake than was found in the earlier fractions from the column giving deceptively higher amounts of carbohydrate than were actually present.

Fractionation of A Peaks. The A peaks from Bio-Gel P-2 of both JS (708 mg) and N-1 (664 mg) were chromatographed on a charcoal–Celite column (Whistler and Durso, 1950) and eluted with an ethanol gradient. The material eluted as a broad peak with indications of several subsidiary peaks, and various fractions were pooled on the basis of the ethanol concentration as shown in Table II; yields and analytical data on all the fractions for N-1 and on some fractions of JS are also given. Most of the data for JS are after attempts at further fractionation on Bio-Gel P-2 and P-6. Analytical paper chromatography in solvent 2 of the fractions in Table II showed them to be heterogeneous. Much of the material did not move after 5–6 days, and the small proportions of faster components moved in the region around isomaltooctoase. The number of sugar residues in these oligosaccharide mixtures estimated from the component ratios relative to 1 mol of terminal *N*-acetyl-D-galactosaminitol, ranged from 8 to 15 residues increasing with increasing ethanol concentration as expected (Table II). Despite the problems of obtaining accurate values for *N*-acetyl-D-galactosaminitol, the results based on total nitrogen agree well with the estimated number of hexosamine + *N*-acetyl-D-galactosaminitol residues. Extra nitrogen, possibly attributable to glycopeptides, was detected only in fraction 800–900 of N-1. Furthermore, an Le^a active oligosaccharide with a retention time relative to isomaltooctaose of 0.78 has been isolated from fraction 636–670 of N-1. Its structure is still uncertain, but it contains 3.2% nitrogen, 16.5% fucose, 39.2% galactose, 28.8% *N*-acetylhexosamine, and 12% *N*-acetyl-D-galactosaminitol. The molar ratios based on 1 mol of *N*-acetyl-D-galactosaminitol are: nitrogen 4.2, galactose 4, *N*-acetylhexosamine 2.5 (taken as 3), fucose 1.9, and the calculated molecular weight, as a reduced decasaccharide, is 1762. These findings suggest that the fractions of the N-1 and JS dialysates excluded by Bio-Gel P-2 contain mostly oligosaccharide chains with high molecular weights and represent the bulk of the complete megalosaccharide structure proposed as carrying the blood group determinants (Lloyd *et al.*, 1968; Lloyd and Kabat, 1968); similar estimates of size were made on charcoal fractions of H substance (Iyer and Carlson 1971).

Discussion

The data presented confirm the finding (Iyer and Carlson, 1971; Anderson *et al.*, 1972) that the degradation conditions used release entire carbohydrate chains containing *N*-acetyl-D-galactosaminitol from the blood group active glycoproteins studied, so that each reduced oligosaccharide reflects the actual structure of the side chain on the glycoprotein; much of the material is dialyzable. Thus the findings by gel filtration on the dialyzable materials become of greater significance, since they give the first direct experimental evidence of heterogeneity of the carbohydrate portion of blood group active substances and can lead to quantitative estimates of the extent of this heterogeneity.

As shown in Figure 1 resolution into a number of components of decreasing size was accomplished by fractionation of the dialysates of N-1 and JS on Bio-Gel P-2. The general similarity between the two patterns is striking and the differences are as expected from earlier structural, biosynthetic, genetic, and immunochemical considerations (Kabat, 1970, 1973). They show similar distribution of carbohydrate chains within the same molecular weight zones and both have a very

TABLE II: Composition of Oligosaccharide Mixtures on Charcoal–Celite Fractionation of Peak A from N-1 and JS.

	Fractions from Charcoal–Celite Column Eluted by Ethanol Gradient																			
	N-1												JS							
Tube numbers	636–670		671–710		711–760		761–800		800–900		516–547				548–580		581–610			
Ethanol concentration (%)	16–21		21–25		25–29		29–35		35–46		19–23				23–27		27–31			
Yield (mg)	77		171		141		48		42		124				162		85			
Additional fractionation											Charcoal–Celite Followed by Bio-Gel P-6				Charcoal–Celite then Bio-Gel P-2 and Bio-Gel P-6					
											100–140[c]		141–185[c]		141–185[d]					
	%[a]	Molar Ratio[b]	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio		
Nitrogen	3.4	3.7	3.0	5.4	3.6	4.8	3.6	6.8	3.7	10.3	2.9	3.5	3.3	5.3	2.6	4.2	2.7	4.9		
Methylpentose (fucose)	14.1	1.3	9.1	1.4	9.6	1.1	8.3	1.3	7.4	1.8	23.3	2.5	23.4	3.2	26.4	3.6	26.8	4.2		
Hexose (galactose)	31.7	2.7	30.8	4.3	38.3	4.0	36.0	5.2	28.0	6.1	24.4	2.3	25.3	3.2	27.5	2.7	29.6	4.2		
Hexosamines	27.4	2.4	29.9	4.2	35.2	3.6	36.3	5.3	27.7	6.1	24.5	2.3	25.4	3.2	27.4	3.4	27.0	3.9		
N-Acetylhexosamines	31.9	2.3	31.5	3.6	38.6	3.3	40.0	4.8	30.1	5.4	24.8	1.9	25.0	2.6	27.3	2.8	28.8	3.3		
N-Acetyl-D-galactosaminitol	14.5	1	8.9	1	11.9	1	8.4	1	5.7	1	13	1	9.8	1	9.9	1	8.7	1		
Estimated size per 1 *N*-acetyl-D-galactosaminitol	6–9		9–11		9–10		11–12		13–15		7–9		10–11		9–11		12–13			

[a] Per cent composition determined by colorimetric analyses for all components except *N*-acetyl-D-galactosaminitol, which was determined by glc on the alditol acetate. [b] Moles per mole of *N*-acetyl-D-galactosaminitol. [c] Tubes pooled after two Bio-Gel P-6 filtrations; fractions 100–140 yielded 24 mg and fractions 141–185, 21 mg. [d] Tubes pooled after Bio-Gel P-6-filtration; final yield 31 mg.

large excluded fraction comprising over half of the dialyzable carbohydrate.

The most remarkable difference between the two profiles is in the distribution of the fucose which was found throughout many peaks from JS but was only in largest peaks of N-1 (Figure 1). This is in agreement with the qualitative differences among the fucosyl transferases involved in the assembly of the blood group H, Le^a, Le^b, and difucosyl type 2 determinants (Watkins, 1970; Kobata *et al.*, 1970; Jarkovsky *et al.*, 1970). In particular, the lack of the fucosyltransferase which produces H specificity seems to have a controlling effect on the pattern of oligosaccharides isolated from N-1. The finding of a very broad distribution of fucose-containing peaks throughout the JS Bio-Gel column suggests that at least some of the fucosyltransferases present as expression of the JS genotype can use as substrate any galactose capable of accepting fucose on C-2 regardless of the length and size of the oligosaccharide on which the galactose occurs.

About 85% of the sugar content of each dialysate is associated with molecular weights above 600, showing that carbohydrate chains containing less than three residues occur to a relatively small extent in both glycoproteins.

The portion excluded from Bio-Gel P-2 shown in both diagrams of Figure 1 is rich in fucose and is a heterogeneous mixture of many compounds of higher molecular weight (Table II) than any blood group oligosaccharides isolated to date and approaching in size the proposed composite megalosaccharide structure of Lloyd and Kabat (1968). This megalosaccharide is thought to represent a relatively complete expression of the biosynthetic potentialities as determined genetically, but within an individual glycoprotein, heterogeneity due to incomplete biosynthesis would be expected. The heterogeneity and large size of the excluded fraction have made it difficult, thus far, to isolate individual pure oligosaccharides. The finding of substantial amounts of nondialyzable *N*-acetyl-D-galactosaminitol in both substances (Table I) also indicates that reduced oligosaccharides of large size are present in this fraction as well.

The peaks from JS and N-1 included on Bio-Gel P-2 are susceptible to further fractionation and purified oligosaccharides are obtainable by the methods of purification used earlier (Lloyd *et al.*, 1968; Vicari and Kabat, 1970; Etzler *et al.*, 1970). A considerable number of oligosaccharides have been obtained from these fractions and are being characterized and should provide a quantitative picture of the extent of the heterogeneity.

Such heterogeneity, which appears to be characteristic of polysaccharides and glycoproteins, has special significance in the case of the blood group active substances since the incomplete chains often show antigenic specificities controlled by genes, acting prior to the A, B, H, Le^a, and Le^b genes but which are not necessarily expressed on the complete carbohydrate chain. At least one blood group I specificity could be produced by one stage of periodate oxidation and Smith degradation of human ovarian cyst blood group A and B substances indicating that such I determinant was covered by other sugar residues in the intact blood group substances (Feizi *et al.*, 1971a,b). Furthermore a precursor substance OG (Vicari and Kabat, 1970) from ovarian cyst fluid which was devoid of A, B, H, Le^a, and Le^b specificities showed many blood group I and i specificities and quantitative precipitin assays with several fractions of purified OG substance showed them to differ in their capacity to react with various anti-I and anti-i sera and with type XIV antipneumococcal horse sera and therefore to be heterogeneous with respect to the numbers

of the different antigenic determinants which they contain. Various fractions obtained from N-1 by phenol–ethanol fractionation also differed strikingly in their reactivities with these same antisera. Since the N-1 fractions were approximately equivalent in their reaction with anti-Le^a, the differences in reactivity could only be accounted for by variations in the minor population of incomplete chains of different kinds (Feizi *et al.*, 1971a,b).

The basis for the characteristic heterogeneity of glycoproteins and its biological importance is a major problem for further study. In the case of the blood group substances from human ovarian cyst fluid, enzymatic degradation while in the cyst cavity is always a possibility, but data on other glycoproteins tend to favor heterogeneity being due to incomplete biosynthesis (Spiro, 1970; Ginsburg and Neufeld, 1969; McGuire, 1970). Whether this is due to the location of certain acceptor sugar residues in sterically unfavorable positions, to errors in biosynthesis which block further addition of other sugars or for other reasons which are not apparent is not clear. The structures of the oligosaccharides included on Bio-Gel P-2 from N-1 and from JS may shed further light on this problem. While many of the shorter chains may be incomplete insofar as the genetic potential of the cell is concerned, they may nevertheless be complete in that some regulatory process has limited the total expression of certain blood group genes.

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