IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

VII. Chemical Changes Associated with Destruction of Blood Group Activity and Enhancement of the Type XIV Cross-Reactivity by Partial Hydrolysis of Hog and Human Blood Group A, B, and O Substances*

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(Received for publication, March 15, 1948)

In a previous study (1), it was reported that preparations of purified blood group A substances from individual hog stomachs varied in their capacity to precipitate with Type XIV antipneumococcal horse sera. Nevertheless these substances had the same blood group A activity (2, 3) as determined by quantitative immunochemical methods (*cf.* references 4–7). Blood group O substances from individual hog stomachs as well as preparations of human blood group substances also showed similar variations in their cross-reactivity with Type XIV antiserum. From these observations, it seemed that the two activities might be associated with different portions of the complex molecule and it was predicted that it might be possible to alter one of the two activities without influencing the other (1). The effect of heating the hog blood group A and O substances at various pH on cross-reactivity with Type XIV antiserum was therefore studied as had been done previously for the blood group A activity (2). It was found that exposure to 100°C. in dilute hydrochloric acid at a pH of 1.5–2.0 for 1 to 2 hours resulted in almost complete destruction of blood group A and O activity but strikingly increased the reactivity with Type XIV antiserum. Similar results were obtained on heating A and B substances from human saliva. Study of the chemical changes associated with this procedure showed that about 60 to 80 per cent of the fucose of the hog A and O substances (8) and some of the amino acid nitrogen together with small amounts of free galactose and glucosamine became dialyzable. In addition some dialyzable polysaccharide was also found. From the non-dialyzable portion, products were isolated by alcohol precipitation which had an enhanced reactivity with Type XIV antiserum and had a lower methylpentose content than the original material. These data, together with the findings of Bray, Henry,

* The work reported in this paper was carried out under a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service, and in part under the William J. Matheson Commission.

and Stacey (9) that the fucose was present as end groups, strongly indicate that both the blood group A and O substances are made up of long chains of *N*-acetylglucosamine and galactose residues with the fucose residues projecting outward as end groups at various undetermined points along the chain. These fucose residues reduce the reactivity of the blood group substances with Type XIV antiserum and their removal by heating at pH 1.5–2.0 results in the observed increased reactivity with Type XIV antibody. The methylpentose in blood group A substance from human saliva was identified as fucose by paper chromatography.

EXPERIMENTAL

The hog and human blood group substances employed were those described in earlier studies (3, 8, 10). Type XIV antipneumococcal horse serum H635 (*cf.* reference 1) supplied by Dr. Harold W. Lyall and Miss Jessie L. Hendry of the New York State Department of Health contained 0.87 mg. anti-SXIV N[1] per ml.

The stability of the Type XIV activity of blood group A and O substances after heating at various pH was studied as follows: To 1.50 ml. samples of a saline solution containing 2.00 mg. of blood group substance per ml., 1.50 ml. of solutions of appropriate pH (*cf.* reference 2) from about 1.5 to 10.8 were added; the pH was determined after mixing. The tubes were sealed and heated in a boiling water bath for 2 hours. After heating, 2.50 ml. aliquots were removed, neutralized with acid or alkali if necessary, and diluted to 5.00 ml. in a volumetric flask with saline to give a solution containing 500 μ g./ml. The cross-reactivity of each heated solution with Type XIV antiserum was determined at 0° by adding volumes containing 50, 100, 150, 250, and 500 μg. of blood group substance to 0.5 ml. portions of antiserum in a total volume of 2.0 ml. After 1 week in the refrigerator the precipitates were centrifuged off, washed twice in the cold with saline, and analyzed for nitrogen (11) by the Markham micro-Kjeldahl method (19). The blood group A activity was determined by comparing the minimum quantity of each heated solution of blood group A substance required for inhibition of hemagglutination of human A erythrocytes by human anti-A (2) with that of an unheated saline solution of the same preparation of A substance. Blood group O activity was assayed in a similar manner using O erythrocytes and a goat anti-Shiga serum (12) generously supplied by Dr. A. F. Coca of Lederle Laboratories. The serum contained anti-O and was used after absorption with A_1B cells.

RESULTS

Table I shows the effect of exposure to various pH at 100°C. for 2 hours on the Type XIV and blood group activity of solutions of two preparations of hog blood group A substance, on three samples of hog blood group O substance, and on one A preparation, hog 15 (8), which contained some O substance. It is apparent that, as compared with the corresponding unheated sample, the heated materials showed greatly enhanced capacity to precipitate Type XIV antibody after heating at 100° for 2 hours at pH ranging from 1.49 to 1.9 and that the blood group activity of these substances had been very extensively or almost completely destroyed. The resulting increase in the cross-reactivity

[1] SXIV the type-specific polysaccharide of Type XIV pneumococcus.

appeared to be greater for the blood group O substances than for the A substances. As a control, a sample of SXIV heated under similar conditions showed a slight decrease in reactivity with Type XIV antiserum.

TABLE I

Effect of pH on Cross-Reaction of Blood Group Substances with Type XIV Antipneumococcal Serum. Tubes Heated 2 Hours at 37° at Indicated pH, Neutralized

Total N precipitated from 0.5 ml. antiserum H635

Amount of substance added	pH										pH				
	1.52	1.71	2.79	4.69	6.71	7.40	8.8	9.15	9.49	Unheated	1.9	4.72	7.5		Unheated
μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
	Hogl 6 (A)										Hog 13 (O)				
50	24	29	6	10	9	2	0	2	1	12	26	15	6		16
100	46	43	10	16	14		2	0	2	18	45	26	12		27
150	54	59	14	23	18	4	1	1		24	57	36	18		39
250	61	75	27	24	22	9	3	2	3	30	69	43	25		43
500	70	95	46	38	32	14	0	2	5	39	97	64	36		64
Agglutination-inhibiting power*...	>10‡	>50	1	1	1	2	10	50	50	1	>25	>25	10		2
	Hog 15 (AO)										Hog 25 (O)				
	1.49	1.51	2.90	4.75	6.91	7.75	8.8	9.62	10.05	Unheated	1.61	4.70	7.71	9.05	Unheated
50	24	20	17	7	6	7	2	0	1	8	50	12	8	2	11
100	43	29	26	12	9	9	2	1	1	13	69	18	11	2	14
150	54	43	34	14	14	16	4	0	1	17	86	21	11	4	18
250	67	57	47	22	21	24	3	2	3	23	107	25	14	4	20
500	84		74	39	35	32	3	4	1	35	128	37	20	7	27
Agglutination-inhibiting power....	>50		1.5	1	1	2	50	50	50	1	>50	1–2	5–10		1
	Hog 10 (A)										Hog 27 (O)				
	1.59		2.95	4.59	6.55	7.50				Unheated	1.52	4.71	7.71	8.9	Unheated
50	14		5	7	5	3				13	35	28	23	5	29
100	24		9	6	6	5				8	61	46	32	5	44
150	30		12	8	7	4				13	76	61	40	11	51
250	46		14	15	10	2				16	102	81	54	14	64
500	64		28	17	14	5				19	139	99	69	23	77
Agglutination-inhibiting power....	>50		0.2	0.1	0.1	0.1				0.1	>50	10	25		5

* Minimum quantity of A or O substances completely inhibiting hemagglutination of A cells by anti-A or of O cells by anti-O.

‡ Highest dilution tested showing no hemolysis.

With the three A products, heating at pH 2.79–2.95 seemed to result in a slight increase in cross-reactivity without significant loss of blood group A activity as measured by hemagglutination (*cf.* reference 2) but similar treatment at pH from 4.59–6.91 did not significantly affect either the cross-reaction or the

blood group A activity. At pH 7.4–7.5, 100°C. for similar periods produced some destruction of both cross-reacting and blood group activity in two instances, but with the hog 15 preparation, no significant change occurred; at pH 8.8 or above extensive loss in both activities resulted.

With the O substances heating at pH 7.5–7.71 resulted in a fivefold decrease in blood group O activity and in some diminution of cross-reacting potency. At pH 8.9–9.0 marked diminution of cross-reactivity occurred in the two instances studied. At pH 4.7 some decrease in blood group activity with no change or but a slight increase in cross-reactivity was noted.

Table II shows that the increase in cross-reactivity produced at pH 1.6 was greatest after 1 hour at 100° and decreased with further heating.

TABLE II

Effect of Time of Heating at pH 1.6 on Cross-Reaction with Type XIV Antipneumococcal Serum

Amount blood group substance* added	Total N precipitated from 0.5 ml. antiserum H635			
	Time of heating			
	1 hr.	2 hrs.	4 hrs.	6 hrs.
μg.	μg.	μg.	μg.	μg.
50	43	36	17	12
100	66	52	27	14
150	87	67	36	19
250	107	87	46	21
500	146	118	66	43

* Hog 13 O substance used.

Similar increases in cross-reactivity were obtained on heating three samples of A substance and one of B substance from human saliva (10) (Table III).

Chemical Changes Associated with Heating at pH 1.5–1.8.—

Chemical changes associated with the increase in reactivity with Type XIV antiserum and the destruction of blood group activity which resulted from heating at pH 1.5–1.9 were investigated using the hog 15 substance which was predominantly blood group A substance (8) and the hog 29 material a preparation of blood group O substance.

Solutions containing about 5 mg. per ml. of the purified substances from hogs 15 and 29 were prepared and acidified to pH 1.5 and 1.8 respectively; total nitrogen, glucosamine, and reducing sugar before and after hydrolysis, methylpentose (13), cross-reactivity, blood group A or O activity, and specific optical rotation were measured. The solutions were then heated in sealed tubes in a boiling water bath for 2 hours and 1 hour respectively and the same analytical data obtained (Table IV). Measured volumes of the heated solutions (containing 260 mg. of hog 15 substance and 265 mg. of hog 29 substance) were then dialyzed against four and five 75 ml. portions of distilled water. The non-dialyzable portions were removed

from the cellophane sacs, 2 gm. of sodium acetate was added to each, and the material was fractionally precipitated with ethanol. Most of the material was precipitable by 2 volumes

TABLE III

Effect of Heating at 100° for 2 Hours at Acid pH on Cross-Reactivity of Human Blood Group Substances

Amount substance added	Total nitrogen precipitated from 0.5 ml. H635	
	Heated 2 hrs.	Unheated
μg.	μg.	μg.
A substance: W.H.$_2$ digest of water-insoluble, phenol-insoluble pH 2.18		
50	30	28
100	46	43
150	61	53
250	81	69
500	121	90
A substance: B.K. phenol-insoluble pH 1.99		
50	49	33
100	67	53
150	84	67
250	114	89
500	156	118
A substance: W.H.$_2$ digest of water-insoluble 10 per cent precipitate pH 1.72		
50	47	25
100	74	45
150	96	50
250	118	66
500	163	83
B substance: S.E. phenol-insoluble pH 1.90		
50	41	20
100	64	31
150	81	36
250	111	42
500	144	56

of ethanol (P1) and a small additional portion was obtained between 2 and 5 volumes (P2). In the case of hog 15 only an additional 3 mg. of substance was obtained by adding an equal volume of acetone to the ethanol supernatant. The yields, analytical properties, blood group, and cross-reactivity of these fractions are shown in Table IV.

Aliquots of the dialysates were analyzed and the remainder of the dialysates was concentrated *in vacuo* under CO_2 to a volume of 2.5 ml. and chromatographed on Whatman number 1 filter paper (14) using in separate experiments butanol-ethanol-water (40:10:50) and collidine-water as solvents as described by Partridge (15).

Qualitative identification of the constituents was made on the same paper by simultaneous comparison of the R_F values of the constituents of the concentrated hydrolysate with those of fucose, galactose, and glucosamine. With both solvents, fucose and galactose were found in the concentrated dialysates of both the hog 15 and hog 29 substances when chromatograms were developed by heating with alkaline copper or with ammoniacal silver. With collidine, glucosamine could also be identified but only in the hog 15 dialysate and in very small amounts.

TABLE IV

Properties of Unheated and Heated Hog Blood Group Substances and of the Non-Dialyzable Fractions Obtained after Heating at pH 1.5 and 1.8

	Ash as Na	Total N	On hydrolysis*		Unhydrolyzed		Methylpentose	$[\alpha]_D$	Acetyl	Minimum hemagglutination inhibiting dose	Total N precipitated from 0.5 ml. type XIV serum by					Amount of substance
			Reducing sugar	Glucosamine	Reducing sugar	Glucosamine					50 µg. substance	100 µg. substance	150 µg. substance	250 µg. substance	500 µg. substance	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	µg.	µg.	µg.	µg.	µg.	µg.	mg.
Hog 15		5.5	59	29	11	1.2	9.2	+10°		0.5	8	13	17	23	35	260‡
Hog 15 heated			57	27	22	3.2		+23°		>150	29	45	60	82	130	
P1	0.2	6.0	56	31	12	1.7	1.7	+30°	11.2	>100	39	56	68	81	100	120
P2		6.8	59	33		2.0	1.5	+25°		>100	27	40	50	68	92	12§
Hog 29		6.0	58	27	10	1.8	9.5	− 5°		1	21	32	41	57	88	265‡
Hog 29 heated			60	29	17	2.4		+ 7°		>100	66	104	131	161	200	
P1	0.2	5.8	56	29	15	1.2	3.7	+10°	10.3	>150	73	105	128	148	173	213
P2			48	26	16	1.2	3.0			>150	42	61	76	96	120	8

* With 2 N HCl for 2 hours at 100°C.

‡ Amount initially used, other figures in column indicate yield.

§ An additional 3 mg. of material containing 2.1 per cent methylpentose and 27 per cent glucosamine and which also reacted with Type XIV antiserum was isolated from the P2 supernatant by addition of an equal volume of acetone.

However, in both solvents a definite spot remained at the starting point with the two concentrated dialysates; this was designated as the slow spot. In addition, when chromatograms were developed with ammoniacal silver a very fast moving spot was noted with each solvent in both the hog 15 and hog 29 dialysates. The R_F values of this spot ranged from about 0.40 to 0.44 in butanol and 0.61 to 0.64 in collidine. No such spot was obtained when chromatograms were developed with alkaline copper. This spot was shown to be due to glycerol, and control experiments showed it to come from the dialyzing membranes and not from the blood group substances. Its identification as glycerol was made by measuring its R_F values and preparing the triphenylurethane on material isolated from the fast spot.

To obtain more precise information about the composition of these concentrated dialysates, the technic of paper chromatography was adapted to quantitative estimation of the various sugars, modified from qualitative and

quantitative procedures for sugars and amino acids used by other workers (14–18).

Sheets of Whatman number 1 filter paper measuring 19 × 46 cm. were used. A pencil line, perpendicular to the length was drawn 10 cm. from one end of the paper and exactly 0.10 ml. of the concentrated dialysate to be examined was distributed from a calibrated 0.10 ml. pipette as a series of small spots along this line allowing margins of about 2 cm. at each end. In each margin, a small additional drop of the dialysate was placed. When the spots were dry the end of the paper was placed in the trough containing the desired solvent and the chromatograms developed at room temperature for about 20 hours when butanol was used and 24 to 26 hours with collidine. Duplicate papers were chromatographed simultaneously in each solvent. The papers were then removed, the position of the solvent boundary marked, and the papers dried at room temperature in a chemical hood in a strong current of air. The marginal strips were cut off and the spots on one strip were developed by spraying with alkaline copper and those on the other with ammoniacal silver and heating in an oven at 110°C. Using these developed margins as a guide, strips of measured width were cut at right angles to the marginal strips from the main pieces of paper so as to include each spot as completely as possible. When inadequate separation of spots was obtained a single strip containing two spots was cut. In this manner, strips containing the fast component, the fucose, the galactose plus glucosamine, and the slow component were obtained. Blank strips of measured width were cut from areas where no spots were evident but which had become wet with solvent. The strips containing the various constituents were marked with pencil and residual solvent was removed under vacuum in a desiccator containing vessels of concentrated sulfuric acid, phosphorus pentoxide, and pellets of sodium hydroxide; the desiccator was placed in an incubator at 37°C. for from 2 to 4 days. This procedure was found necessary to reduce blank values to a minimum in the subsequent analytical procedures; the longer drying period is preferable for chromatograms developed with collidine. The papers were then removed and strips containing a given component from the duplicate papers were placed in a test tube containing an analytically measured volume of distilled water (usually 10 or 15 ml.), stoppered with rubber stoppers, and allowed to stand overnight at room temperature to extract the various constituents from the paper; the blank strips of paper were extracted similarly. The solvent was decanted from the strips of filter paper and as large a quantity as possible recovered by pressing the paper with a stirring rod. The extracts were then centrifuged at room temperature to remove lint. Aliquot portions of the extracts corresponding to the various spots and blanks were analyzed for reducing sugar and glucosamine, both before and after hydrolysis for 2 hours with 2 N HCl, for methylpentose by the method of Dische and Shettles (13), and in the case of papers developed with butanol-ethanol-water for nitrogen by the Markham (19) micro-Kjeldahl method. Values obtained were corrected for the blank by calculating the blank according to the width of paper taken for each individual strip. The amounts found in the 0.2 ml. of each concentrated dialysate placed on the two papers and the total amount of each constituent in the entire dialysate were calculated. The results obtained are given in Table V. Since blank values were appreciable in all instances and since differences were multiplied by very large factors, low values are not considered significant; such values are given in parentheses in Table V. It is also possible that some small quantities of a given constituent were accidentally included in the adjacent strip by inadequate separation in the chromatograms or by tailing (14, 16). It will be noted from Table V, that recoveries of total reducing sugar, nitrogen, and methylpentose are in close agreement with those found in the total dialysate; recoveries of total glucosamine were somewhat poorer. No values are included for reducing sugar and glucosamine after hydrolysis or for glucosamine before hydrolysis when collidine was used as solvent since reproducible values could not be obtained under these conditions with this solvent.

TABLE V

Composition of Dialysates Obtained after Partial Hydrolysis of Hog A and O Substances

Fraction	Reducing sugar			Glucosamine		Methyl pentose direct		Total nitrogen	Non-glucosamine nitrogen	R_F				
	Hy-drolyzed	Unhy-drolyzed		Hy-drolyzed	Unhy-drolyzed					Butanol			Collidine	
										Found		Known	Found	Known
	mg.	*mg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*					
Total dialysate hog 15	57	31		23	4.3	17.1		3.2	1.4					
Paper chromatography of hog 15 dialysate														
Spot	Bu-tanol	Bu-tanol	Colli-dine	Bu-tanol	Bu-tanol	Bu-tanol	Colli-dine	Bu-tanol						
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*						
Slow moving	18.9	12.4	6.9	8.0	1.3	(1.1)	(0.2)	1.5*		0.00–0.02			0.00–0.02	
Galactose-glucosamine	15.0	5.7	11.0	4.6	2.0	(1.2)		1.7*		0.11; 0.13	Galactose	0.12; 0.12	0.39; 0.36	0.37; 0.37
											Glucosamine		0.30; 0.28	0.30; 0.28
Fucose	22.4	14.7‡	15.3‡	5.2	1.8	16.4	15.2	(0.4)		0.28; 0.26; 0.26		0.28; 0.27; 0.26	0.50; 0.51	0.52; 0.50
Fast moving	(1.5)	(0.0)	(3.9)	(0.0)	(0.3)	(0.6)§	(1.8)	0.0		0.44; 0.43‖	Glycerol	0.41	0.61; 0.64‖	0.60
Total in dialysate calculated from paper	57.8	32.8	37.1	17.8	5.4	19.3	17.2	3.6						

TABLE V—*Concluded*

Total dialysate hog 29.	30.6	15.3		7.4	1.2	17.9		2.2	1.6					
Paper chromatography of hog 29 dialysate														
Slow moving	11.6	1.2	0.8	3.1	(0.6)	(0.6)	(0.2)	0.8¶		0.00			0.00	
Galactose-glucosamine	2.0	2.2	0.6	0.9	(0.4)	(0.2)	0.0	1.5¶		0.09	Galactose	0.08		
Fucose	14.4	11.3‡	11.1‡	1.1	(0.7)	14.2	14.0	0.0		0.22		0.22	0.50	0.48; 0.50
Fast moving	(0.0)	(0.0)	(0.2)	(0.0)	(0.1)	(0.1)	(0.7)	(0.0)		0.40‖	Glycerol	0.41	0.61‖	0.60
Total in dialysate calculated from paper	28.0	14.7	12.7	5.1	1.8	15.1	14.9	2.3						

Values in parenthesis may not be significant due to low value found variations in blanks and large conversion factors.

* Average of nitrogen determinations on three independent sets of paper chromatograms.

‡ To convert reducing sugar to fucose multiply x 3/2 giving 22.0 and 23.0 mg. for hog 15 and 17.0 and 16.6 mg. for hog 29.

§ In repeated runs, values of 1.1 and 1.4 mg. of methylpentose found; probably the differences are due to variations in the position at which the papers were cut to separate the components. Similar variations were found with the galactose-glucosamine and the slow spot with relation to the separation of glucosamine and reducing sugar.

‖ Spots demonstrable only by spraying with ammoniacal silver and not with alkaline copper. The glycerol was shown to come from the dialysis sac.

¶ Average of two independent sets of nitrogen determinations.

In the case of the hog 15 substance, heating at 100°C. for 2 hours at pH 1.5 resulted in an increase in the unhydrolyzed reducing sugar from 11 to 22 per cent and in the glucosamine from 1.2 to 3.2 per cent. For the 260 mg. of substance used, this amounted to 28.6 and 5.1 mg. respectively. This was accompanied by an increase in optical rotation from +10° to +23°, essentially complete loss of blood group A activity, and a marked increase in the capacity of the heated solution to precipitate Type XIV antibody (Table IV). On dialyzing the heated solution against repeated changes of distilled water, the dialysate was found to contain 31 mg. of reducing sugar and 4.3 mg. of glucosamine indicating that almost all of the reducing groups and glucosamine liberated by heating had become dialyzable. Part of this material was polysaccharide in nature since, on hydrolysis of the dialysate with 2 N HCl, the reducing sugar increased from 31 to 57 mg. and the glucosamine from 4.3 to 23 mg. Some of the non-glucosamine nitrogen had also become dialyzable since the 23 mg. of total glucosamine in the dialysate accounted for but 1.8 of the 3.2 mg. of nitrogen found. The most striking finding was that the dialysate contained 17 of the 24 mg. of fucose initially present. The concentrated dialysate showed some reactivity with Type XIV antiserum probably due to its content of polymerized material. A small amount of the polysaccharide (1.5 mg.) in the dialysate could be precipitated by alcohol and was shown to precipitate with Type XIV antibody.

Further study of the hog 15 dialysate by paper chromatography after concentration *in vacuo* under CO_2, showed the presence of four components. From the R_F values in both butanol-ethanol-water and collidine-water, fucose and very small amounts of galactose and glucosamine were identified. There was also a component which was essentially immobile (slow) and a component (glycerol from the dialysis bag) which moved more rapidly than the fucose and was seen only when chromatograms were developed with ammoniacal silver (Table V).

Quantitative paper chromatography showed essentially all of the methylpentose to be present in the fucose spot both in the butanol and collidine chromatograms. In butanol this spot contained a small amount of glucosamine which appeared to be in a polymerized form since the amount found was increased on hydrolysis and it is probable that these small quantities of material were included in the fucose spot by uncertainties in cutting the papers.

Both the slow moving and the galactose-glucosamine spots consisted of polysaccharide since marked increases in the glucosamine and reducing sugar occurred on hydrolysis. The non-glucosamine nitrogen (amino acids) also occurred largely in these two fractions. Whether this is bound to the polysaccharides in the dialysate or has been liberated by the heating and merely moves at the same rate as the polysaccharides is uncertain. The unhydrolyzed reducing sugar values for the galactose-glucosamine and the slow spots were 5.7 and

12.4 mg. in butanol and 11.0 and 6.9 in collidine, while the sum of the values for the two spots in each solvent is the same. It is evident therefore, that butanol and collidine effect a different resolution of the polysaccharides present.

The main non-dialyzable fractions from hog 15 (P1, P2) isolated by alcohol precipitation, and showing the enhanced reactivity with the Type XIV antiserum, had essentially similar glucosamine and reducing sugar contents, both hydrolyzed and unhydrolyzed, and an acetyl content and nitrogen similar to that of untreated blood group substances. The fucose content had decreased from 9.2 to 1.5 and 1.7 per cent and the optical rotation had increased from +10° to +25° and +30° for the P2 and P1 fractions respectively.

With the hog 29 substance, the heating was carried out only for 1 hour at pH 1.8 and these milder conditions increased the reducing sugar and glucosamine from 10 to 17 and from 1.8 to 2.4 per cent respectively, corresponding to the liberation of 18.5 mg. of reducing sugar and 1.6 mg. of glucosamine from the 265 mg. of material used. The optical rotation increased from −5° to +7° a change in the same direction and of the same magnitude as that on heating the hog 15 material. Loss of blood group activity and increased reactivity with Type XIV antiserum occurred.

The dialysate contained only 15.3 mg. of reducing sugar and 1.2 mg. of glucosamine, about one-half that found in the case of the hog 15 sample; on hydrolysis these values were increased to 30.6 and 7.4 mg. indicating that some polysaccharide was present. The dialysate contained 17.9 of the 25 mg. of fucose in the starting material.

Results of paper chromatography of the hog 29 dialysate were similar to those with hog 15. Most of the reducing sugar and almost all the methylpentose of the unhydrolyzed concentrated dialysate were found in the fucose spot. The bulk of the polymerized material appeared in the slow component. The galactose-glucosamine component constituted but a minor fraction of the total dialysate and from the R_F value and the finding that the reducing sugar was not increased on hydrolysis, it would appear that it consists chiefly of free galactose. As in the case of the hog 15 dialysate, glycerol from the dialysis sac appeared as a very fast component when chromatograms were developed with ammoniacal silver, but only insignificant quantities of the constituents analyzed for in Table V were present.

The non-dialyzable fractions from the hog 29 substance were similar to those with hog 15 (Table IV), but the yield of non-dialyzable material was much greater due to the milder conditions of hydrolysis and their fucose contents were somewhat higher indicating that only about 60 per cent of the fucose had been split off. The optical rotation had increased from −5° to +10°, the latter value being considerably lower than that of the corresponding hog 15 substance. The cross-reactivity of the P1 and P2 substances was much greater than the original material.

Since galactose is the only sugar known to be present in the blood group substances beside glucosamine and fucose, the quantity of galactose liberated can be estimated for the hog 15 substance by subtracting the total glucosamine (23 mg.) plus the fucose calculated as reducing sugar ($17.1 \times 2/3 = 11.4$ mg.) from the total reducing sugar; thus 57 mg. − 23 mg. − 11.4 mg. = 22.6 mg. of galactose as reducing sugar. Multiplying by 4/3 the factor for converting the reducing power of galactose to weight gives 30 mg. of galactose. If it is assumed that for each molecule of glucosamine there is one galactose, subtraction (30 − 23) shows 7 mg. of galactose over and above that necessary for combination with glucosamine. A similar calculation for hog 29 again using the data in Table V reveals the presence of 14.8 mg. of galactose and 7.4 mg. beyond the amount necessary for combination with glucosamine.

Studies on a hydrolysate of A substance from human saliva $W.H._2$ 10 per cent precipitate (10) by paper chromatography in butanol-ethanol-water and in collidine-water served to identify the methylpentose as fucose.

DISCUSSION

The data presented clearly establish (Tables I to III) that blood group A and O substances from hog stomach and the A and B substances from human saliva show an increased capacity to precipitate Type XIV antipneumococcal antibody after heating at 100° at pH 1.5–2.0 although their blood group activity is lost by this treatment. These observations suggest a similarity of structural pattern between these various substances which provides the basis for this behavior; similarities in chemical composition have already been noted (3, 8, 10) and a sample of A substance from human saliva was shown to contain fucose. Study of the chemical changes associated with this mild hydrolysis, in the case of hog 15 (chiefly A substance) and hog 29 (O substance), showed that the predominant change was the liberation of about four-fifths and two-thirds of the fucose respectively in a dialyzable form. In addition, partial depolymerization of the blood group substances occurred as evidenced by the finding of small amounts of free galactose and, in the case of hog 15, of glucosamine as well as considerably larger quantities of polymerized glucosamine-galactose units in the dialysate. In the case of the hog 29 preparation, which had been heated at a somewhat higher pH and for a shorter period than the hog 15 substance, much less partially depolymerized material was found in the dialysate. Appreciable quantities of amino acid nitrogen (non-glucosamine nitrogen) were found in the dialysate and this nitrogen was present in the galactose-glucosamine and slow spots obtained by paper chromatography. Whether this amino acid nitrogen is attached to the polysaccharide in the dialysate has not yet been determined.

The non-dialyzable residues, comprising the bulk of the original blood group substance, showed enhanced reactivity with Type XIV antiserum and were

devoid of blood group activity. They had essentially the same analytical composition as the original blood group substance except for the marked reduction in methylpentose content and the increase in optical rotation (Table IV). This is not surprising, however, since it is doubtful whether the elimination of the fucose in these two instances, would significantly affect the glucosamine, reducing sugar, nitrogen, or acetyl values.

The correlation between the immunochemical and the chemical data establishes a portion of the structure of the blood group substances. The Type XIV specific carbohydrate (SXIV) and the hog blood group A and O substances have been shown to consist of *N*-acetylglucosamine and galactose residues. By virtue of the cross-reaction of the blood group substances with Type XIV antiserum, it would seem reasonable that both the SXIV and the blood group substances possess in the basic chain a series of *N*-acetylglucosamine-galactose residues; the sequence in the various substances not necessarily being identical, but giving rise to patterns with certain similarities of structure at various points on the chain, much as in the case of the structures proposed for SIII and SVIII to explain their cross-reactivity with their heterologous antipneumococcal horse sera (20, 21). The blood group substances, unlike SXIV, contain fucose residues (8, 9, 15) and by methylation studies, Bray, Henry, and Stacey (9) have shown these to be present as end groups. If these fucose end groups were attached to the main *N*-acetylglucosamine-galactose chain at various points, they would project outward and might very well prevent the cross-reacting groupings from combining with antibody. Removal of these fucose residues might either provide additional cross-reacting sites on the main chain or might make possible a closer approach of the antibody molecule to the reactive groupings on the main chain and thereby increase the extent of the cross-reaction as has been observed. The fact that the fucose may be easily removed without effecting appreciable hydrolysis of the main galactose-glucosamine chain is further evidence that the fucose does, indeed, occur as end groups and is not an integral part of the main polysaccharide chain. Further examination of the data reveals that, for both the hog 29 and hog 15 substances, hydrolysis resulted in the splitting off of almost identical amounts of fucose, of galactose beyond that necessary for combination with glucosamine, and of amino acid nitrogen. Data of this nature indicate that the mild conditions used in these experiments produce an orderly cleavage of blood group A and O substances. Fucose and galactose are split off in a ratio of roughly 3:1, and a definite proportion of the non-glucosamine nitrogen (amino acids) is liberated suggesting that these substances form an easily hydrolyzed unit or units attached to the main polysaccharide chain. It further indicates that there are two types of non-glucosamine nitrogen (amino acids). The labile amino acids may possibly be responsible for conferring blood group activity on the galactose-glucosamine polysaccharide, since as will be demonstrated in the following paper, the fucose

(or at least some of it) is not necessary for blood group activity. Further studies will be needed to clarify these relationships.

The structural relations thus far proposed are in agreement with the data. However, they do not provide any explanation of the previous report (1) that various samples of blood group A and O substances from individual hog stomachs show different capacities to react with Type XIV antiserum. This will be treated in the accompanying paper (22) in which it is demonstrated that the hog blood group substances vary in their fucose content and that the capacity of the substance to cross-react with Type XIV antiserum is inversely related to its fucose content, providing strong additional evidence for the structural relations proposed.

SUMMARY

1. The effect of heating at varying pH on the cross-reactivity of hog blood group A and O substances with Type XIV antibody has been investigated. The hydrolysis of blood group A, B, and O substances from hog and human sources at pH 1.5–1.8 resulted in destruction of blood group activity and a marked increase in cross-reactivity with Type XIV antipneumococcus horse serum.

2. Analysis revealed the liberation of reducing sugar, most of which was dialyzable. It was further shown that the major part of the reducing sugar was fucose with small amounts of free and polymerized galactose and glucosamine. Non-glucosamine nitrogen, probably amino acids, also was found in the dialysate.

3. Paper chromatographic separation of the concentrated dialysate confirmed the presence of fucose and showed that the majority of the galactose and glucosamine occurred in polymeric form.

4. Quantitative analysis of the substances isolated from the paper showed good recovery of the various components, indicating that practically all of the material in the dialysate could be accounted for.

5. The methylpentose of human blood group A substance was identified as fucose.

6. The structure of the blood group substances is interpreted in terms of these results.

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