and a 2-cc. aliquot diluted further to 25 cc. Optical density at 260 mμ using a Beckman spectrophotometer (model DU) was found to be 0.720; from this, 18.9 mg. of $Ba_2UTP \cdot 4H_2O$ was calculated[26] to be present in the original 25 cc. of solution, the synthetic sample being thus 95.5% pure. Ion exchange analysis showed only a trace (<2%) of UDP to be present. The synthetic sample was free from "inorganic" phosphates[47] and on paper chromatograms developed in five different solvent systems (Table III) migrated as a single spot.

The procedure for the isolation of UDP was identical with that described above for UTP, except for addition of 6 cc. of ethanol for the precipitation of the barium salt. The concomitantly precipitated sodium chloride was removed during washings with 50% ethanol; wt. of the barium salt, 195 mg. 50 mg. of this sample was dissolved at 0° in ice-cold 0.05 *N* hydrochloric acid and the *mono* barium salt ($C_9H_{12}O_{12}N_2PBa \cdot 3H_2O$)[16] precipitated with equal volume of cold ethanol, collected by centrifugation and washed with ethanol and ether; yield 36 mg. After being exposed to the air for 24 hours, the sample was submitted for analysis.[48]

Anal. Calcd. for $C_9H_{12}O_{12}N_2Ba \cdot 3H_2O$: C, 18.2; H, 3.0; N, 4.7; P, 10.4. Found: C, 18.7; H, 3.3; N, 4.3; P, 10.8. Ratio of labile P to total P, 1:2. The synthetic sample was found to be homogeneous on paper chromatography in five different solvent systems (refs. in Table III) and was free from "inorganic" phosphates.[47] Spectrophotometric estimation carried out as described for the barium salt of UTP showed this sample to be 98% pure with respect to the above formula.

(48) Analysis by Mr. V. Tashinian, University of California, Berkeley.

Paper Chromatography of Uridine Phosphates and "Inorganic Phosphates."—Paper chromatography was used throughout the present work; it was especially useful in following the removal of "inorganic phosphates" from the nucleotides. The solvent systems found most useful were 1 and 2 (Table III), descending technique being used. The solvent systems 3–5 of Table III (ascending technique) were used to confirm the purity and identity of the synthetic nucleotides. Standards were always run side by side since the R_f values varied somewhat with time.

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[CONTRIBUTION FROM THE DEPARTMENTS OF NEUROLOGY AND MICROBIOLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL]

Immunochemical Studies on Blood Groups. XV. The Effect of Mild Acid Hydrolysis on the Glucosamine and Galactosamine in Blood Group Substances[1]

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The glucosamine and galactosamine contents of the dialyzable and non-dialyzable portions of blood group substances which had been subjected to mild acid hydrolysis have been studied. With all of these substances the non-dialyzable fractions showed lower glucosamine–galactosamine ratios than did the original blood group substances. The hexosamines of the dialyzable fractions were strikingly related to blood group activity for the hog and human substances, showing glucosamine–galactosamine ratios of 2 to 4 for the A substances, 13–14 for the O (H) substances; only glucosamine was split off from human B substances. Horse and bovine substances appeared to exhibit species rather than blood group specificity with respect to the splitting off of these hexosamines.

Introduction

It has been known for some time[2] that treatment of blood group substances for two hours at 100° in dilute hydrochloric acid at a *p*H of 1.5–2.0 resulted in almost complete destruction of blood group activity as measured by hemagglutination inhibition tests. At the same time a striking increase in reactivity with type XIV anti-pneumococcal horse serum developed. These changes have been shown to occur in hog,[2] human,[2] horse[3] and cattle[4] substances with A, B or O (H) activity as well as with inactive substances of similar chemical composition. Study of the chemical changes in hog A and O (H) preparations associated with this procedure showed that 60–80% of the fucose together with small amounts of hexosamine, galactose, amino acid nitrogen and oligosaccharide had become dialyzable. The non-dialyzable material, when reisolated by alcohol precipitation, was found to have a substantially lower fucose content but otherwise was very similar to the original material. Mild acid hydrolysis with 1 *N* acetic acid was also shown by Aminoff, Morgan and Watkins[5] to produce similar changes and to result in increased Forssman activity of the A substance. By means of the recently described method[6] for the separation and determination of glucosamine and galactosamine, a re-examination of this reaction was undertaken to see whether more information could be obtained on the chemical changes produced by mild acid hydrolysis and to relate them to immunological specificity.

Method.—Measured amounts (5–20 mg.) of the materials to be studied were dissolved in a few ml. of water and adjusted to *p*H 1.6 with hydrochloric acid in test-tubes which were then sealed with rubber caps. After immersion in a boiling water-bath for various times (care being taken to release the pressure by puncturing the caps with hypodermic needles), the contents were quantitatively transferred to well washed sausage casings and dialyzed against at least four 25-fold portions of distilled water changed at daily intervals. The dialyzates (dial.) were combined, evaporated down under reduced pressure (water-bath *ca.* 50°), taken up in 2 *N* hydrochloric acid and hydrolyzed at 100° for two hours. The non-dialyzable material (P-1) was transferred from the casing, made up to 2 *N* with concentrated hydrochloric acid and similarly hydrolyzed at 100° for two hours. Both the dialyzable and non-dialyzable fractions were then analyzed for glucosamine and galactosa-

(1) This investigation was carried out under grants from the National Institutes of Health, Public Health Service (RG-34) and the William J. Matheson Commission.

(2) E. A. Kabat, H. Baer, A. E. Bezer and V. Knaub, *J. Exp. Med.*, **88**, 43 (1948).

(3) H. Baer, E. A. Kabat and V. Knaub, *ibid.*, **91**, 105 (1950).

(4) S. M. Beiser and E. A. Kabat, *J. Immunol.*, **68**, 19 (1952).

(5) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *Biochem. J.*, **43**, xxxvi (1948); **46**, 426 (1950).

(6) S. Leskowitz and E. A. Kabat, THIS JOURNAL, **76**, 4878 (1954)

TABLE I

EFFECT OF *p*H AND TIME OF HEATING ON HYDROLYSIS OF BLOOD GROUP SUBSTANCE

				Properties of P-1 Fraction								
Sample no. (Hog 4A)	*p*H	Time of hydrolysis, hr.	P-1 recovery, %	Min. amount inhibiting hemagglutination of A cells by anti-A, μg.	Reducing sugar[a] on hydrolysis, %	Total hexosamine,[a] %	Glucosamine, μg./mg.	Galactosamine, μg./mg.	Ratio glucosamine/galactosamine	Methyl pentose, %	Methyl pentose in dialysate, mg.	Total methyl pentose recovery, mg./100 mg. B.G.S.
I-1	2.3	0	87	0.5	52	30				11.9	0	11.9
I-4	2.3	0.75	83	0.5	52		155	85	1.8	10.6	1.3	11.9
I-7	2.3	2	84	0.5	49	31	145	90	1.6	7.0	2.0	9.0
I-8	2.3	4	64	1.0	49	31	145	90	1.6	6.2	2.4	8.6
I-9	2.3	8	64	20	48	32	160	105	1.5	5.2	3.6	8.8
II-1	1.9	0	92	0.5	53	25	170	85	2.0	11.8	0	11.8
II-4	1.9	0.75	75	0.5	49	29	180	95	1.9	9.3	2.0	11.3
II-7	1.9	2	62	2.0	51	31	170	105	1.6	5.5	3.4	8.9
II-8	1.9	4	63	20	50	31	175	110	1.6	3.3		
II-9	1.9	8	56	>200	45	31	130	100	1.3	2.2	4.4	6.6
III-1	1.5	0	76	0.5	52	27	145	80	1.8	12.6	0	12.6
III-4	1.5	0.75	58	5.0	50	33	165	105	1.6	4.2	3.1	7.3
III-7	1.5	2	54	10.0	49	28	165	115	1.4	2.9	3.9	6.8
III-8	1.5	4	32	>200	47	27	120	120	1.0	2.1	4.0	6.1
III-9	1.5	8	33	>200	40	27	100	120	0.8	1.5	4.4	5.9

[a] After hydrolysis with $2N$ HCl at 100° for 2 hours.

mine by the previous described method consisting of reduction with sodium borohydride, formation of the N-2,4-dinitrophenylaminohexitols and chromatographic resolution. Total hexosamine values were determined by the Elson–Morgan[7] procedure after evaporating the samples to dryness[8] in a vacuum desiccator, reducing sugar by the Hagedorn–Jensen method,[9] and methylpentose by the Dische–Shettles method.[9a]

Results and Discussion

A study was first made of the effect of various acidities and times of hydrolysis on blood group substances. For this purpose a pooled hog mucin preparation (4 A) with both A and O (H) activity was available.[10] Three 1-g. portions were dissolved in water and made up to *p*H 2.3 (I), 1.9 (II) and 1.5 (III) in 200 ml. Twenty-ml. portions were pipetted into glass tubes, sealed and immersed in a boiling water-bath. Samples were removed at varying intervals and dialyzed against seven changes of distilled water. Both the dialyzate and non-dialyzable portion were analyzed for various constituents; the data are recorded in Table I.

With increasing time of hydrolysis and acidity the hog 4 A blood group substance underwent continuous degradation, as shown by the decreasing recoveries of P-1 fraction and progressive loss in ability to inhibit the hemagglutination of A cells by anti-A. At a *p*H of 2.3 this degradation was fairly slow so that after 8 hours of heating (I-9), 64% of polymerized material which still retained some blood group activity was recoverable. At a *p*H of 1.5, however, only 33% recovery was obtained after 8 hours heating (III-9) and the blood group activity was lost after only 2–4 hours of heating (III-8). While the extent of degradation differed markedly with time and *p*H of hydrolysis, it is noteworthy that the reducing sugar and hexosamine values of the non-dialyzable P-1 fractions are generally similar. The reducing sugar values ranged around 50% and the hexosamine values around 30%, corresponding closely to the values found for the starting material. Methylpentose, as had been previously noted,[2] decreased in the P-1 fractions and increased correspondingly in the dialysate with increasing extent of hydrolysis. The total recovery of methylpentose decreased considerably with increasing acidity and time of hydrolysis, amounting to only 50% at *p*H 1.5 for 8 hours (III-9), indicating appreciable destruction of this component under these conditions.

The simultaneous and progressive removal of methylpentose and loss of blood group activity is accompanied by one other important change which paralleled these two. This is the striking alteration in the ratios of glucosamine to galactosamine in the P-1 fractions. Starting with an initial glucosamine to galactosamine ratio of about 2.0 a continuous drop (more rapid with the more acid solution) is apparent with increasing times of hydrolysis. The ratio appears to approach a value slightly above 1.0; any effort to exceed this by increased time of hydrolysis (III-8, III-9) or increased acidity resulted in such extensive destruction of material as to make the re-isolation of a non-dialyzable fraction difficult.

In an effort to determine the general nature of this phenomenon, a study was next made of a number of blood group substances from various sources which had been hydrolyzed at a known *p*H for 2 hours in connection with another study.[11] About 2–3 mg. portions of the re-isolated P-1 fractions were hydrolyzed with 2 N hydrochloric acid for 2 hours, analyzed for glucosamine and galactosamine (Table II) by the chromatographic technique,[6] and

(7) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).
(8) J. P. Johnston, A. G. Ogston and J. E. Stanier, *The Analyst*, **76**, 88 (1951).
(9) (a) H. C. Hagedorn and B. N. Jensen, *Biochem. Z.*, **135**, 46 (1923); (b) Z. Dische and L. B. Shettles, *J. Biol. Chem.*, **175**, 595 (1948).
(10) *Cf.* A. Bendich, E. A. Kabat and A. E. Bezer, *J. Exp. Med.*, **83**, 485 (1946).
(11) H. Van Vunakis and E. A. Kabat, THIS JOURNAL, **73**, 2977 (1951).

TABLE II

GLUCOSAMINE AND GALACTOSAMINE CONTENT OF NON-DIALYZABLE P-1 FROM VARIOUS SOURCES

Substance	Source	Blood group activity	pH	Glucosamine, μg./mg. P-1	Galactosamine, μg/mg. P-1	Ratio, glucosamine/galactosamine In P-1	In original blood group substance[5]
Hog 18 P-1	Hog gastric mucosa[a]	A	1.65	155	115	1.3	1.9
Hog 30 P-1	Hog gastric mucosa[a]	A	1.65	150	155	1.0	1.4
Hog 27 P-1	Hog gastric mucosa[a]	O (H)	1.62	150	125	1.2	2.3
Hog 29 P-1	Hog gastric mucosa[a]	O (H)	1.8	195	115	1.7	2.3
W.H.$_1$ C_6H_5OH ins.	Human saliva[b]	A	1.7	135	125	1.1	1.7
A.B.$_5$ C_6H_5OH ins. P-1	Human saliva[b]	A	1.7	125	120	1.0	1.6
P.M. C_6H_5OH ins. P-1	Human saliva[c]	B	1.7	110	75	1.5	2.9
Horse 3, 25% P-1	Horse stomach[c]	Inactive	1.7	135	105	1.3	1.6
Horse 4, 25% P-1	Horse stomach[c]	B	1.7	150	110	1.4	1.7
Cow 22, 10% P-1	Cow abomasus[4]	Inactive	1.7	180	120	1.5	2.3
Cow 26, 10% P-1	Cow abomasus[4]	B	1.7	145	105	1.4	1.8
Cow 31, 10% P-1	Cow abomasus[4]	A	1.7	170	110	1.5	1.7

[a] *Cf.* reference 10. [b] E. A. Kabat, A. Bendich, A. E. Bezer and S. M. Beiser, *J. Exp. Med.*, **85**, 685 (1947). [c] See reference 3.

the results calculated per mg. of P-1 material for ready comparison. From the first two columns of Table II, it can be seen that the materials chosen for this survey represent a fairly complete sampling of all the blood group substances available to us. Moreover, as seen in the last column, these substances had initial glucosamine–galactosamine ratios ranging from 1.4 to 2.9 which was also just about the limits of variation observed amongst all the blood group substances previously studied.[6] Yet despite the wide range of source, specificity and glucosamine–galactosamine ratio of the materials studied, all behaved similarly toward the mild acid hydrolysis in that they gave strikingly lower glucosamine–galactosamine ratios in the resulting P-1 fractions. The ratios in all cases seemed to approach 1.0 with the amount of decrease in ratio varying from 0.2 in the case of cow 31–10% to 1.4 for P. M. C_6H_5OH ins. Thus in all cases it appeared that glucosamine was being preferentially split from the blood group substance leaving a residue relatively enriched in galactosamine.

To confirm these findings, a study was made of the glucosamine–galactosamine content of the dialysate following mild acid hydrolysis. A large number of hog and human materials were subjected to hydrolysis at *p*H 1.6 for 2 hours at 100° and dialyzed against at least 4 changes of water. The dialyzable portion was concentrated by vacuum distillation and the non-dialyzable portion (when necessary) by lyophilization. Both were then hydrolyzed with 2 *N* HCl and analyzed for glucosamine and galactosamine[6] and the results listed in Table IIIA.

Since under the conditions selected for the mild acid hydrolysis (*p*H 1.6, 2 hours at 100°) approximately 30% of the starting blood group substance became dialyzable, the size of the starting sample was set at around 10 mg. in order to give sufficient hexosamine in the dialysate for analysis by the chromatographic technique. All results in Table IIIA and B are given on the basis of 10 mg. of sample for comparability. The over-all recovery of hexosamine in a complete experiment involving hydrolysis, dialysis and analysis of dialysate and non-dialyzable P-1 fraction averaged 72% of the amount calculated from the Elson–Morgan values on the original blood group substance. In view of the large number of manipulations involved, this cannot be considered unsatisfactory.

The amount of hexosamine rendered dialyzable by mild acid hydrolysis of the specimens studied ranged from 6–38%.[12] There was no correlation apparent between this amount and the blood group activity of the preparation. There was, however, a distinct relation between the source of the blood group specimen and the amount of hexosamine released. Thus, in all the hog and human preparations studied, the amount of hexosamine rendered dialyzable by hydrolysis at *p*H 1.6 ranged from 15–29% with an average value of 23%. On the other hand, all the horse preparations studied, regardless of blood group activity, gave only from 6–10% (average 8%) dialyzable hexosamine following this same treatment. The bovine materials, with one exception, gave dialyzable hexosamines ranging from 23–38% with an average of 30% of the total initial hexosamine being released. This may be compared with the results obtained in studies with small enzyme[13] in which it was found that in hog A preparations up to 14% of the total hexosamine was rendered dialyzable with only a 10-fold loss in activity as measured by hemagglutination inhibition. It is evident that mild acid hydrolysis effects considerably more drastic changes in blood group substances than the snail enzymes previously studied.

The results of this study of the hog and human blood group substances revealed an even more striking correlation between blood group activity and behavior toward mild acid hydrolysis. As had been previously demonstrated,[6] blood group substances showed characteristic glucosamine–galactosamine ratios for each blood group, *i.e.*, an average of 1.6 for hog and human A, 2.2 for hog O and 2.8 for human B. From an examination of the glucosamine–galactosamine ratios of the P-1 residues in column 5, Table IIIA, it is again apparent that all the hog and human substances studied showed a

(12) Glucosamine + galactosamine in dialysate divided by total hexosamine (Table III).

(13) C. Howe and E. A. Kabat, THIS JOURNAL, **75**, 5542 (1953).

TABLE IIIA

EFFECT OF MILD ACID HYDROLYSIS ON HOG AND HUMAN BLOOD GROUP SUBSTANCES

Material	Blood group activity		Glucosamine, μg./10 mg. B.G.S.	Galactosamine, μg./10 mg. B.G.S.	Ratio, glucosamine/galactosamine	Original ratio,[a] glucosamine/galactosamine	Total hexosamine recovered, μg./10 mg. B.G.S.	Total hexosamine by Elson–Morgan, μg./10 mg. B.G.S.	Hexosamine recovery, % of Elson–Morgan	Ratio, glucosamine/galactosamine recovered
Hog 18[a] (7.7 mg.)	A	Dial.	710	190	3.7	1.9	2130	3100	69	1.8
		P-1	660	570	1.2					
Hog 30[a] (8.0 mg.)	A	Dial.	600	245	2.5	1.4	2615	3200	82	1.3
		P-1	900	870	1.0					
Hog 27[a] (9.9 mg.)	O(H)	Dial.	775	55	14.0	2.2	1785	3000	60	2.6
		P-1	520	435	1.2					
Hog 42 (4%)[e] (10.6 mg.)	O(H)	Dial.	595	45	13.0	2.2	2105	2900	73	2.1
		P-1	830	635	1.3					
W.H.$_2$ 10%[b] (10 mg.)	A	Dial.	600	215	2.8	1.6	2210	3000	74	1.4
		P-1	690	705	1.0					
A.B.$_5$ 10%[b] (8.5 mg.)	A	Dial.	330	165	2.0	1.6	2150	2900	74	1.2
		P-1	840	815	1.0					
W.G. C_6H_5OH ins.[b] (10 mg.)	A_2	Dial.	405	95	4.3	2.2	1920	2900	66	2.0
		P-1	865	555	1.6					
A.K. 10%[b] (9.6 mg.)	A_2	Dial.	395	115	3.4	2.1	1830	2900	63	1.7
		P-1	755	565	1.3					
E.J. 10%[e] (6.0 mg.)	B	Dial.	450	Trace		2.5	2030	3000	71	2.3
		P-1	970	610	1.6					
E.J. C_6H_5OH ins.[c] (4.8 mg.)	B	Dial.	640	Trace		3.1	1995	2300	87	2.5
		P-1	785	570	1.4					
J.R.M. C_6H_5OH ins.[c] (9.1 mg.)	B	Dial.	550	Trace		2.8	1890	2500	76	2.4
		P-1	775	565	1.4					
J.C. 10%[c] (8.4 mg.)	B	Dial.	640	Trace		2.8	2105	2800	75	2.3
		P-1	830	635	1.3					

TABLE IIIB

EFFECT OF MILD ACID HYDROLYSIS ON HORSE AND BOVINE BLOOD GROUP SUBSTANCES

Material	Blood group activity		Glucosamine, μg./10 mg. B.G.S.	Galactosamine, μg./10 mg. B.G.S.	Ratio, glucosamine/galactosamine	Original ratio,[a] glucosamine/galactosamine	Total hexosamine recovered, μg./10 mg. B.G.S.	Total hexosamine by Elson–Morgan, μg./10 mg. B.G.S.	Hexosamine recovery, % of Elson–Morgan	Ratio, glucosamine/galactosamine recovered
Horse 1–25%[c] (6.8 mg.)	A	Dial.	265	Trace		2.0	2315	2700	83	1.9
		P-1	1250	800	1.6					
Horse 2–25%[c] (11.1 mg.)	B	Dial.	160	Trace		1.5	1670	2900	58	1.3
		P-1	775	710	1.1					
Horse 4–25%[c] (13.3 mg.)	B	Dial.	175	Trace		1.7	2165	2800	77	1.6
		P-1	1150	810	1.4					
Horse 5–15%[c] (10 mg.)	Trace B	Dial.	195	Trace		1.9	1715	2700	64	1.4
		P-1	795	725	1.1					
Horse 3–25%[c] (17.3 mg.)	Inact.	Dial.	215	40	5.4	1.5	1280	2700	48	1.3
		P-1	505	520	1.0					
Horse 6–25%[c] (8 mg.)	Inact.	Dial.	150	Trace		1.5	1830	2100	87	1.4
		P-1	920	760	1.2					
Cow 18–10%[d] (10 mg.)	A	Dial.	295	230	1.3	1.4	950	1400	68	1.1
		P-1	205	220	0.9					
Cow 40–10%[d] (12 mg.)	A	Dial.	665	265	2.5		2170	2800	78	1.8
		P-1	720	520	1.4					
Cow 26–10%[d] (10 mg.)	B	Dial.	480	240	2.0	1.8	2185	3100	71	1.6
		P-1	855	610	1.4					
Cow 24–10%[d] (13.1 mg.)	B	Dial.	710	220	3.2		2330	2900	80	2.1
		P-1	870	530	1.6					
Cow 22–10%[d] (10 mg.)	Inact.	Dial.	405	220	1.8	2.3	2140	3400	63	1.8
		P-1	960	555	1.7					
Cow 28–10%[d] (10.3 mg.)	Inact.	Dial.	405	300	1.4	1.4	1680	2300	73	1.2
		P-1	510	465	1.1					
								Av.	72	

[a] *Cf.* reference 10. [b] See footnote (b) Table II. [c] See reference 3. [d] See reference 4. [e] C. Howe and E. A. Kabat, unpublished data.

marked decrease in ratio regardless of blood group and tended to approach a ratio slightly above 1.0. The most remarkable result of this study lies in the hexosamine ratios obtained for the dialyzable portion. Both the hog and human A substances which have been shown to be serologically very similar had

approximately 2–4 times as much glucosamine as galactosamine split off by mild acid hydrolysis. The hog O (H) substances had considerably more (13 and 14 times as much) glucosamine than galactosamine split off, and the human B substances had practically only glucosamine split off. It is apparent from the results listed in the last column that, within experimental error, the starting glucosamine-galactosamine ratio may be reconstructed from the dialysate and P-1 data.

The implications of these findings for the eventual elucidation of the structure of the polysaccharide moiety of the blood group substances are several. First, there would appear to be a portion of the blood group substance of somewhat greater lability than the major part. Much of this labile fraction is fucose and hexosamine. Thus while 2 *N* hydrochloric acid is required for the complete hydrolysis of the sugar moiety of the blood group substance in 2 hours at 100°, *p*H 1.6 is sufficient for splitting off this labile fraction. Whether this portion exists in furanose form or is attached through some other acid-labile grouping is at present unknown. It has been shown previously, however, that the fucose split off during mild acid hydrolysis appears chiefly as free fucose which from methylation studies[14] appears to be attached as an end group. A study of the relationship between fucose content and cross-reactivity with type XIV antibody had also been made previously[15] and it was shown that at least in hog materials the fucose content was inversely related to the extent of cross-reactivity with type XIV anti-pneumococcus sera. Since, however, blood group A substances which have been shown to be identical in blood group A activity may vary considerably in their fucose content, it was postulated that a large portion of the fucose present was unrelated to blood group activity, but served only to shield to some extent the cross-reacting sites on the main polysaccharide chain. That the fucose may not be as important as the other acid labile portion in shielding the main polysaccharide chain is indicated by the behavior previously observed with the horse[3] and cattle[4] preparations. These materials have low fucose contents to begin with and therefore it appears likely that the increase in cross-reactivity with type XIV anti-pneumococcal polysaccharide following mild acid hydrolysis is due to the splitting off of the hexosamine-containing residues rather than to the small amounts of fucose removed.

It is apparent from the results in Table III that the hexosamine content of the acid labile portion in contradistinction to the fucose is strikingly related to blood group specificity in the hog and human substances.

The residues (P-1) after removal of these labile portions have very similar chemical compositions (reducing sugar, N, hexosamine) differing quite markedly only in fucose and glucosamine–galactosamine ratios from the original substance. Since mild acid hydrolysis has been shown to increase greatly the ability of blood group substances to cross-react with type XIV anti-pneumococcal polysaccharide, the inference was drawn that the blood group substances were stripped down by this treatment to polysaccharide chains resembling more closely that of the type XIV pneumococcal polysaccharide which consists of N-acetylglucosamine and galactose (1:3).[16]

Thus the attractive hypothesis presents itself that the blood group substance has a skeleton (P-1) which can cross-react with antiserum to the type XIV pneumococcal polysaccharide and which is made up of galactose, N-acetylglucosamine[10] and N-acetylgalactosamine in ratios of about 2:1:1. To this would be attached in some acid-labile arrangement, fucose and various oligosaccharide side chains consisting in part of N-acetylglucosamine and N-acetylgalactosamine in different ratios depending upon the various blood group specificities. In the native blood group substances, these side chains would confer blood group specificities on and prevent access of type XIV anti-pneumococcal polysaccharide antibodies to the skeleton, thus rendering the substances less readily precipitable by the cross-reacting antibody. Following removal of these side chains by mild acid hydrolysis, the resulting skeleton (P-1) would lose blood group specificity and gain in ability to precipitate with the antibody to the type XIV pneumococcal polysaccharide by virtue of the exposure of more sites reactive with this antibody. Work is currently being undertaken to clarify structure and specificities of the oligosaccharides split off by mild acid hydrolysis.

In previous work[6] on blood group substances isolated from horse and bovine sources, no correlation could be found between glucosamine–galactosamine ratios and A, B, O specificity. This, together with certain serologic evidence,[4,17,18] pointed to the existence of other specificities on these molecules with their ABO specificities being a cross-reaction due perhaps to only a small portion of the total structure. An investigation of the effect of mild acid hydrolysis on these substances has provided further evidence consistent with this hypothesis.

Six horse preparations having blood group A, B or no activity were studied by mild acid hydrolysis. The results in Table IIIB show that in common with hog and human materials, all preparations following mild acid hydrolysis exhibit a decrease in glucosamine–galactosamine ratios in the non-dialyzable P-1 residue. These decreases range from a value of 0.8 with horse 5-15% to 0.3 for horse 4-25%. However, in contrast to the hog and human preparations the horse blood group substances showed a great preponderance of glucosamine in the dialysates regardless of blood group activity. Thus while 150–265 μg. of glucosamine per 10 mg. of blood group substance appeared in the dialysate, only slight amounts of galactosamine were found. These results could be explained in terms of a specificity common to all the horse preparations, the chemical basis for which lay in the acid-labile glucosamine residues. It is interesting furthermore that whereas horse blood group A preparations re-

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acted rather poorly with anti-hog A and anti-human A sera,[19] horse blood group B preparations have been shown[20] to be almost as effective as human B preparations in the precipitin reaction with human isoimmune serum. This might in some way be a reflection of the finding that human B preparations give practically only glucosamine on mild acid hydrolysis.

The six bovine materials studied (Table IIIB) also showed a behavior characteristic of the species rather than of ABO specificity. While all six preparations showed some decrease in glucosamine–galactosamine ratios following mild acid hydrolysis, the composition of the dialyzable hexosamine showed no apparent relation to ABO activity. Rather, all six cattle preparations showed hexosamine ratios in the dialysate ranging only from 1.3 to 3.2, and in most cases not significantly different from those of the original unhydrolyzed material. Since bovine materials have been shown to react relatively poorly in the precipitin reaction with anti-hog A and anti-human A[4] and B,[20] it is not altogether surprising that these materials exhibit a behavior toward mild acid hydrolysis unlike that of the hog and human materials. Moreover, since bovine substances possess a common bovine specificity[4] and contain the so-called J factor of cattle,[17,18] it appears likely that a correlation between the hexosamine ratio of the dialysate and some specificity other than A, B or O might be a possibility. No explanation is apparent from the data presented for the capacity of cow B substances to precipitate human antibody to horse B substance.[4]

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Chromanones, Thiochromanones and 2,3-Dihydro-4(1H)-quinolones

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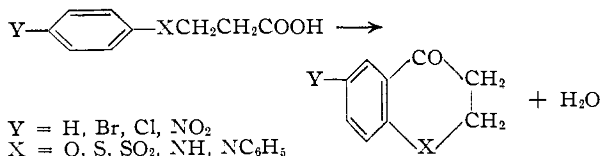
Polyphosphoric acid is an effective reagent for bringing about the ring closure of 3-aryloxypropionic acids into 4-chromanones, 3-arylmercaptopropionic acids into 4-thiochromanones and N,N-diaryl-β-alanines into 2,3-dihydro-1-aryl-4(1H)-quinolones. 8-Methyl-4-thiochromanones were included in the study. Phosphoryl chloride causes cyclization of N-*p*-chloro- and N-*p*-bromophenyl-N-*p*-tolylsulfonyl-β-alanine into 6-halo-2,3-dihydro-1-*p*-tolylsulfonyl-4(1H)-quinolone but the yields are low. Also, 3-(*p*-nitrophenoxy)-propionic acid produces 6-nitro-4-chloro-2H-benzopyran in good yield on heating with phosphoryl chloride, a process involving not only ring closure but also halogenation at the carbonyl. With 3-phenylmercaptopropionic acid, however, phosphoryl chloride gives rise to 3-chloro-4-thiochromanone. Aspects of this unusual C–H chlorination are discussed. Both physical and chemical evidence was used in establishing structures. Reactions carried out on the thiochromanones were oxidation to the 1-dioxides by hydrogen peroxide, halogenation at position 3 by bromine or sulfuryl chloride and hydrazone formation. 6-Nitro-4-chromanone reacts with sulfuryl chloride to yield 3-chloro-6-nitro-4-chromanone. This α-chloro ketone reacts with thiourea to produce a yellow crystalline solid, presumably a thiazolobenzopyran derivative. The 3-substituted propionic acids from which these heterocyclics are synthesized are prepared conveniently from propiolactone.

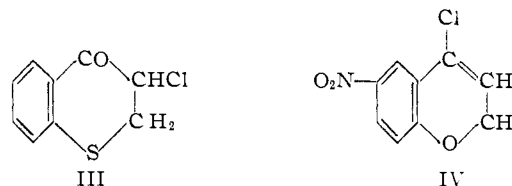
These cyclizations are known: 3-aryloxypropionic acids[1,2] into chromanones by phosphorus pentoxide,[1] 3-arylmercaptopropionic acids into thiochromanones by sulfuric acid[3] and N,N-diphenyl-β-alanine into 2,3-dihydro-1-phenyl-4(1H)-quinolone by phosphorus pentoxide in boiling xylene.[4] This general equation covers these ring closures



Recently it has been shown that the precursors of these heterocyclics may be made from propiolactone, since the latter reacts with sodium phenoxide to yield 3-phenoxypropionic acid,[2] sodium phenyl sulfide to yield 3-phenylmercaptopropionic acid[2] and arylamines to yield N-aryl-β-alanines.[5] In view of this, we became interested in studying the reaction further, using polyphosphoric acid and phosphoryl chloride as reagents for effecting these ring closures.

Polyphosphoric acid proved to be very effective. Examples tested with it were 3-phenylmercaptopropionic acid (I) into 4-thiochromanone, 3-(*p*-nitrophenoxy)-propionic acid (II) into 6-nitro-4-chromanone and N,N-diphenyl-β-alanine into 2,3-dihydro-1-phenyl-4(1H)-quinolone. Polyphosphoric acid has been used previously[6] for intramolecular acylations to give isocyclic rings, but it has not been used in the synthesis of heterocyclic compounds.

Phosphoryl chloride also promoted cyclizations of I and II, but the compounds formed contained chlorine, that from I being 3-chloro-4-thiochromanone (III) although in poor yield, and that from



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