IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

II. Properties of the Blood Group A Substance from Pools of Hog Stomachs and of Specific Precipitates Composed of "A" Substance and Homologous Human Antibody*‡

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The finding that purified blood group A and B substances specifically precipitate the corresponding isoantibodies from the serum of normal and immunized individuals and that the amounts of these antibodies could be estimated by the microquantitative precipitin method (1) introduced by Heidelberger and MacPherson (2) has made it possible to apply the technics of quantitative immunochemistry to the characterization and study of these substances. For example, the relative purity of different preparations can be ascertained by comparing the amounts of each preparation required to precipitate a given amount of antibody. In addition, these procedures can be applied to study the stability of the blood group substances (for a review of the scope of quantitative immunochemical methods *cf.* references 3–5). The present study describes the use of these methods in comparing the relative purity of preparations of blood group A substance obtained by several methods from pools of hog stomachs together with a consideration of the pH stability range of the A substance. Data are also included on the chemical properties of the A substance, on the solubility of A anti-A specific precipitates, and on the magnitude of losses involved in washing specific precipitates.

EXPERIMENTAL

Blood Group A Substances.—Preparations 1A and 2A were obtained as described in (1) by dissolving hog gastric mucin in 90 per cent phenol and precipitating with alcohol at a concentration of 10 per cent by volume according to the Morgan and King phenol method (6). 11A was obtained in a manner similar to 1A. 306C and 330C were prepared and supplied by Dr. J. A. Leighty of Eli Lilly and Co.; both were obtained from autolyzed hog stomach linings, the former by alcohol precipitation and shaking with chloroform (7) and the latter by removal of protein by heating to 70°C. at pH 4.3. 960-P-S-2 was obtained from Dr. L. A. Kazal of Sharp and Dohme; it was prepared from hog stomachs by a procedure involving peptic autolysis, tryptic digestion, alcohol precipitation, and removal of protein by heating to 85°C.

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TABLE I

Stability of Purified Blood Group A Substance to Various pH Levels at 37°C. and at 100°C.

pH of solution*	Hemagglutination inhibition activity: μg. of substance used‡ 100	50	25	10	5	2	1	0.5	Activity by quantitative precipitin tests: Amount added to 0.5 ml. antiserum	Antibody N precipitated	Equivalent amount of unchanged 1A§	Proportion of original activity
									μg.	μg.	μg.	*per cent*
Solutions kept at 37°C. for 48 hrs.												
1.02	−	−	−	−	−	−	+++	++++	40	25.9	39	98
2.97	−	−	−	−	−	−	+++	++++	40	26.3	41	103
4.56	−	−	−	−	−	−	++	++++	40	27.0	43	108
5.12‖	−	−	−	−	−	−	++	+++	40	28.6	47	118
6.89	−	−	−	−	−	−	+	+++				
7.58	−	−	−	−	−	−	+	++++				
9.03	−	−	−	−	−	−	+	++++				
9.57	−	−	−	−	−	++	+++	++++	40	27.7	44	110
10.74	−	−	−	−	−	+++	+++	++++	40	28.4	46	115
Solutions kept at 100°C. for 2 hrs.												
1.02	++++	++++	++++	++++	++++	++++	++++	++++	100	1.0	3	3
2.97	−	−	−	−	−	−	+++	+++	40	25.1	38	95
4.56	−	−	−	−	−	−	−	+++	40	25.6	39	98
5.12‖	−	−	−	−	−	−	+	+++	40	27.5	43	108
6.89	−	−	−	−	−	−	+	++++	40	26.5	41	103
7.58	−	−	−	−	−	++	+++	++++	40	25.6	39	98
9.03	−	−	−	++	+++	++++	++++	++++	100	23.5	34	34
9.57	−	−	++	+++	++++	++++	++++	++++	100	18.1	25	25
10.74	−	−	++	++++	++++	++++	++++	++++	100	18.2	25	25

−, +, ++, +++, and ++++, denote complete, strong, moderate, slight, and no inhibition of hemagglutination, respectively.

Complete loss of activity of 1A was found after 3 hours at 37°C. in N NaOH as measured by inhibition of hemagglutination.

* Solutions made up to contain 1.0 mg. of 1A per ml.

‡ Added to 0.1 ml. of a 1:25 dilution of serum E.K.a (titer 256).

§ From calibration curve of serum A.D. 1+2: 10, 20, 30, 40, 50, 70, 100, and 150 μg. of 1A precipitated 4.3, 12.0, 21.4, 26.1, 28.6, 33.8, 35.8, and 35.4 μg. antibody N respectively from 0.5 ml. portions of serum in a total volume of 3.0 ml.

‖ In acid citrate dextrose (ACD) solution; a medium used in preserving whole blood for transfusion (10).

Sera.—Human sera containing anti-A in high titer were prepared as described by Witebsky, Klendshoj, and McNeil (8) by immunization of volunteers of groups O or B with blood group A substance (1). The potency of these sera was determined both by their hemagglutination titer and their anti-A content (1).

Methods.—The relative activity of different samples containing blood group A substance was measured either qualitatively by determining the minimum amount of each preparation

which completely inhibited the hemagglutination of 0.1 ml. of 4 per cent suspension of washed A erythrocytes by 0.1 ml. of a given dilution of a serum containing anti-A (1), or quantitatively by comparing, in the region of antibody excess, the relative amounts of A substance required to precipitate a given amount of antibody nitrogen from a measured volume of serum containing anti-A. The latter method involves determination of the amounts of antibody nitrogen precipitated by the addition of varying amounts of a standard preparation of *A* substance to measured volumes of serum up to the point of complete removal of antibody (*cf.* references 3 and 4); the total volume is kept constant. After 1 hour at 37°C. and 1 week in the refrigerator, the precipitates are centrifuged off, washed twice in the cold with saline as described in (references 1 and 9), and analyzed for nitrogen by the modified Folin-Ciocalteau

TABLE II

Properties of Purified Blood Group Substances Obtained from Pools of Hog Stomachs

Preparation	Source	Relative potency compared with 1A	Ash as Na	N	P	S	Reducing sugar (as glucose)*	Glucosamine*	Ratio $\frac{\text{Glucosamine}}{\text{Reducing sugar}}$	$\frac{\text{Glucosamine N}}{\text{Total N}}$
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
1A	Gastric mucin	100	1.0	6.1	0.98	0.36	56	30	0.54	0.39
2A	" "	100		5.9			56	31	0.55	0.41
11A	" "			5.6			56	32	0.56	0.45
306 (Lilly)	Hog stomach linings	100	3.0	5.8	1.00	0.35	51	26	0.51	0.35
330 (Lilly)	" "	62	0.9	6.8‡	0.80	0.52	59‡	28‡	0.48	0.32
960-P-S-2 (Sharp & Dohme)	Hog stomachs	58		5.3§			46	25	0.56	0.38

* On hydrolysis with 2N HCl for 2 hours at 100°C.

‡ Of soluble portion; the preparation was 98.5 per cent soluble in H_2O.

§ Analysis kindly supplied by Dr. E. Brand.

tyrosine method (2). The supernatant serum is assayed for anti-A by hemagglutination tests or for excess of A substance by hemagglutination inhibition tests (1). The precipitation data obtained with this preparation are plotted to serve as a calibration curve. The relative A activity of other samples may be determined by addition of an amount of the sample insufficient to precipitate all of the antibody from the volume of serum used. The amount of antibody nitrogen precipitated by this amount of sample, under the same conditions of total volume, time, and temperature is interpolated on the calibration curve and the amount of the standard preparation to which it corresponds is found. The ratio of the value found to the weight of substance added represents the relative potency of the sample in precipitating anti-A as compared to the standard.

pH Stability Range of the Blood Group A Substance.—A series of solutions of preparation 1A at different pH was prepared by the addition of 2.0 ml. of a solution of 1A in water containing 2.0 mg./ml. to 2.0 ml. of a series of buffer solutions varying in pH from 1 to 11, including one set in acid-citrate dextrose (ACD) buffer (10) at pH 5.12. After mixing, the pH of each solu-

tion was determined and each solution divided into two equal portions. One set was kept at 37°C. for 48 hours with a drop of chloroform in each tube as a preservative, the other set in sealed tubes was heated in a water bath at 100°C. for 2 hours. No changes in pH were noted after this treatment. Each solution was then assayed for activity by hemagglutination inhibition and by quantitative precipitin tests (Table I).

TABLE III

Solubility of A Anti-A Specific Precipitates in Saline

Serum No.	Volume of serum	A substance added	Region in which precipitate formed	Antibody nitrogen precipitated in volume of ml.							Solubility
				0*	1.5	2.5	3.0	4.0	4.5	6.0	
	ml.	μg.		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg. N/ml.
E.K.$_3$	0.5	40‡	Antibody excess	25.8	23.8		20.4		19.1	17.0	1.5
E.K.$_3$	0.5	100‡	Antigen "	42.4	39.4		35.0		32.1	28.3	2.4
Bd$_{1-5}$	1.5	20§	" "	23.9		22.0		20.0		18.8	0.9
Bd$_{1-5}$	1.5	100§	" "	23.1		20.5		17.5		16.4	1.2
F.P.$_{1-4}$	1.5	30‡	Antibody "	20.0		15.7		13.6		10.0	1.6
F.P.$_{1-4}$	1.5	100‡	Antigen "	31.2		26.1		24.1		19.5	1.9
Average solubility											1.6

* By extrapolation.

‡ Preparation 1A.

§ Preparation from hog 8 (*cf.* following paper).

TABLE IV

Effect of Washing on A Anti-A Specific Precipitates

No. of washings*	Antibody nitrogen in precipitate formed in	
	Antibody excess	Antigen excess
	μg.	μg.
2	25.2	32.3
4	23.8	29.2
6	23.5	28.0

* Each washing carried out at 0°C. with 3.0 ml. saline.

Properties of Blood Group A Substances.—Some of the chemical properties of the different preparations are listed in Table II. Ash, sulfur, and phosphorus determinations were performed by Mr. William Saschek. Reducing sugar (as glucose) was determined after hydrolysis with 2 N HCl at 100°C. for 2 hours by the Hagedorn-Jensen method. Glucosamine was determined by a modification (11) of the procedure described by Meyer, Smyth, and Palmer (12) for the Elson-Morgan method (13). It was demonstrated that maximum liberation of glucosamine resulted under these conditions of hydrolysis. Relative activity was determined quantitatively as described above.

Solubility of A Anti-A Specific Precipitates.—The solubility of A anti-A specific precipitates

in saline was estimated by adding a given amount of A substance to a constant amount of antiserum; the total volume in which precipitation occurred was varied between 1.5 and 6.0 ml. by the addition of different volumes of 0.9 per cent saline. After 1 hour at 37°C. and 1 or 2 weeks in the refrigerator the amounts of antibody nitrogen in the twice-washed precipitates were determined (1, 2) and the solubility calculated from a graph of antibody nitrogen precipitated plotted against total volume. Solubility determinations were carried out in the regions of excess antibody and of excess antigen (Table III).

Effect of Washing on Specific Precipitates.—To estimate the losses in washing specific precipitates a series of specific precipitates was analyzed after 2, 4, and 6 washings with cold saline (Table IV).

RESULTS

The data in Table I indicate that solutions of the blood group A substance, when kept for 2 days at 37°C. over a pH range from 1.02 to 10.7 are stable as measured both by hemagglutination inhibition and by quantitative precipitin tests. After 2 hours at 100°C. no significant loss of activity appeared when the pH was between 2.97 and 7.58 *cf.* 14); at pH 1.03, however, practically complete loss of A activity occurred and partial destruction resulted from exposure to a pH of 9.03 or higher. Within experimental error the results of the hemagglutination inhibition and the quantitative precipitin tests ran parallel; the latter method, however, was the more precise.

The chemical data in Table II show that preparations of fairly constant chemical composition can be obtained by the Morgan and King phenol method (6). Preparation 2A which was obtained from 1A by shaking with chloroform (*cf.* 1) showed no increase in activity as measured by quantitative precipitin tests, nor did any significant change in physical and chemical properties result. Sample 306C (Lilly) prepared by shaking with chloroform (7), appeared equal to 1A and to 2A in precipitating activity although it had a somewhat lower glucosamine and reducing sugar content. 330C (Lilly) and 960-P-S-2 (Sharp and Dohme) were only about 60 per cent as active as 1A although 330C contained more glucosamine and reducing sugar than did 306C.

The results of solubility determinations on A anti-A specific precipitates from three different sera are shown in Table III. Values from 0.9 to 2.4 μg. of antibody N per ml. were found with an average solubility of 1.6 μg. antibody N per ml. for dilutions made with saline. No significant differences in solubility between specific precipitates formed in the region of antibody excess and those formed in the presence of excess of A substance were found.

The magnitude of errors introduced in washing the specific precipitates in the cold with saline (1) estimated from Table IV appears to be about 3.0 μg. N or less per two washings each with 3.0 ml. of cold saline.

DISCUSSION

The data in Table I, in addition to providing information about the pH stability of the A substance, also make it possible to judge the precision of the

microquantitative precipitin method when applied to the estimation of the relative activity of samples of A substance. In the eleven instances in which 40 μg. samples of A substance, after exposure to various conditions of temperature and pH, were added to 0.5 ml. of antiserum, amounts of antibody nitrogen varying from 25.1 to 28.6 μg. with an average of 26.7 μg. were obtained. Interpolating these values on the calibration curve (data in footnote, Table I), gave corresponding quantities of A substance varying from 38 to 47 μg. with an average of 41.8 μg. of A substance, or percentage recoveries ranging from 95 to 118 per cent. The uneven displacement of values about 100 per cent is due to the use of 26.1 μg. N precipitated by 40 μg. of 1A in the calibration curve. From the average of all points the error might be as much as ± 12 per cent in the determination of an amount of A substance by a single analysis. The precision of the antibody nitrogen determinations is in good agreement with the value of ± 2 μg. antibody nitrogen reported by Heidelberger and MacPherson (2). However, somewhat greater variation results when the antibody nitrogen values are substituted in the calibration curve to obtain the amounts of the standard preparation to which they correspond. It is apparent, however, that the use of the quantitative precipitin method to determine amounts of A substance is much more precise than the usual serological methods involving inhibition of hemagglutination, where it is frequently difficult to ascribe significance to results which differ by 50 to 100 per cent.

The data on the relative activity of the blood group substances in Table II, were obtained by this method and further indicate its utility in comparing the potency of different products.

In analyzing for such small amounts of antibody nitrogen, the total volume in which the precipitation of anti-A is carried out may become of considerable significance. In the usual type of quantitative precipitin estimation (*cf.* references 3 and 4) in which amounts of from 100 to 1000 μg. of antibody nitrogen are determined, errors due to the solubility of specific precipitates may be neglected. However, when small amounts of anti-A nitrogen are determined by the micro method, a considerable error may be introduced if the solubility of 1.6 μg. nitrogen per ml. (Table III) is neglected. For example, a value of 20.4 μg. antibody nitrogen found by carrying out the precipitation in a volume of 3.0 ml. would become 25.8 μg. antibody nitrogen when corrected to zero volume to account for solubility—an error of 26 per cent. It is apparent, therefore, that for reliable comparisons antibody nitrogen values should be corrected to zero volume or that the volume in which the tests were carried out should be specified. Even when this is done, however, the final result may well be as much as 3.0 μg. N less than the true value, since the procedure of washing twice in the cold with 3.0 ml. portions of saline involves losses of this order, as may be seen from Table IV. This error would be present in all the determinations since the standard procedure of washing is always used.

The solubility of specific precipitates of A substance and human anti-A in saline (Table III) is only about one-fifth to one-half that reported for specific precipitates of egg albumin, serum albumin, thyroglobulin, and Type III pneumococcal polysaccharide and their homologous rabbit antibodies (15–18).

Within experimental error, the blood group A substance is stable to heating at 100°C. at pH levels between pH 2.97 and 7.58 and also in the ACD medium at pH 5.1 (10). This indicates that it would be possible to incorporate the A substance directly into the bottle containing the ACD solution prior to sterilization when it is desired to neutralize the anti-A agglutinins in a group O blood (14) before transfusion. Earlier studies by Witebsky have shown that no decrease in activity detectable by the hemagglutination inhibition method resulted from autoclaving the A substance in this medium (14).

SUMMARY

1. The microquantitative precipitin method can be used to compare the relative activity of different preparations of the blood group A substance from hog stomachs and to study the effect of chemical treatment upon its stability.

2. With samples of about 25 μg. antibody nitrogen, an error of ±1.7 μg. antibody nitrogen will result in an error of ±12 per cent in the estimation of the amount of A substance.

3. The blood group A substance showed no significant loss of activity at 37°C. after 48 hours at pH 1.07 to 10.7 or after 2 hours at 100°C. over a pH range from 2.97 to 7.58. Exposure at 100°C. at pH 1.03 or at 9.03 or higher resulted in loss of activity. Parallel results were obtained by the hemagglutination inhibition and quantitative precipitin methods.

4. The solubility of specific precipitates of the blood group A substance from hog stomach and its homologous antibody formed in man was found to be about 1.6 μg. antibody N/ml.

5. A comparison is given of the chemical properties and activity of blood group A substances obtained by several procedures from pools of hog stomachs.

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