IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

III. Properties of Purified Blood Group A Substances from Individual Hog Stomach Linings*‡

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(Received for publication, February 23, 1946)

Some of the properties of blood group A specific substances from enzymatic digests of pools of hog stomachs have been presented in the preceding report (1). However, since it was known that not all hog stomachs show A activity (2), it was considered of interest to determine whether better products could be isolated from individual hog stomachs. When stomach linings from a number of individual hogs were each subjected to peptic digestion, and the digests purified by the phenol method of Morgan and King (3), it was found that preparations of high activity could be obtained from some but that others gave immunologically inactive products in the same yield and chemically indistinguishable from the active ones in all properties thus far studied. The active substances were found with one possible exception, within experimental error, to be of equal potency in precipitating anti-A and in inhibiting the hemagglutination of human A cells by anti-A. The other products were not active when tested by these technics and did not give rise to anti-A on injection into humans. Preparations from pools of hog stomachs which probably consist of mixtures of active and inactive substances, are of intermediate activity. A measure of the absolute purity of various active products can be obtained immunochemically by determining the proportion of the glucosamine of the added A substance precipitated by anti-A in the region of antibody excess.

EXPERIMENTAL

Preparation of Substances.—Sample 1A was obtained from hog gastric mucin (4). The other substances were prepared by two methods from individual stomach linings of freshly killed hogs. The stomach linings from hogs 1 to 6 were cut into small pieces and preserved in 95 per cent alcohol in the ice box for several weeks. They were then finely subdivided in a meat grinder, washed with alcohol and ether, and dried *in vacuo*. Attempts to obtain active blood group A substances directly from such dry stomach linings by extraction with 90 per

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University and under a grant-in-aid from the United States Public Health Service.

‡ Aided in part by grants from the William J. Matheson Commission.

cent phenol according to the Morgan and King method (3) without prior enzymatic digestion were unsuccessful. Digestion of the dry linings with crystalline trypsin at pH 7.0 and 8.5 liberated active material from the stomach linings of hogs 1, 3, 4, and 5 only. This method of digestion was discarded, however, because of the difficulty of obtaining pure water-soluble substances in sufficient yield. These products showed low values for glucosamine and reducing sugar.

The best results were obtained with pepsin. In a typical preparation, 10 gm. of the finely divided dried stomach lining was suspended in 75 ml. of citrate-HCl buffer of pH 2.3 containing 1.0 mg. of crystalline pepsin,[1] and the mixture, preserved with toluene, was incubated with occasional mixing at 37°C. for 3 days. The pH was adjusted back to about 2.3 by the careful addition of concentrated HCl, an additional 1.0 mg. of pepsin was added, and after further incubation for 2 days the aqueous digest was separated by centrifugation. The sediment was taken up in 40 ml. of buffer containing 1.0 mg. of pepsin, toluene added, and, after incubation for 2 days, the suspension was centrifuged and the precipitate washed twice with a total of 40 ml. of buffer. To the combined supernatants, clarified by filtration through absorbent cotton, 4 volumes of 95 per cent ethanol were added. A white, stringy precipitate separated which was removed by filtration, washed with alcohol and ether, and dried *in vacuo* over P_2O_5. This product was further purified as described below. Alternatively, freshly obtained hog stomach linings (hogs 7 to 10), cut into small pieces, were allowed to autolyze at 37°C. in 400 ml. of citrate-HCl buffer and toluene at about pH 2.3 for 2 weeks, at which time the pH had risen to 6–7. The residues were filtered off, 5 volumes of ethanol were added to the filtrates, and the stringy white precipitates were filtered, washed, and dried as above. The alcohol precipitates, as well as the original aqueous digests, were assayed for blood group A activity by the hemagglutination inhibition technic (4) and it was found that only the stomach linings from hogs 1, 3, 4, 5, 8, 9, and 10 were active. The inactive preparations also showed no B activity as determined by hemagglutination inhibition tests.

The alcohol precipitates from both active and inactive stomachs were purified by the phenol method of Morgan and King (3) as follows: 1.0 gm. of the dried alcohol precipitate was shaken for 1 day with 10 ml. of 90 per cent phenol; the insoluble portion was removed by centrifugation, suspended in 10 ml. of phenol, and again shaken for 1 day. After centrifugation, the residue was reextracted with 5 ml. of 90 per cent phenol. The combined viscous supernatants were centrifuged until perfectly clear. Occasionally, in some of the preparations, a white lipid material would accumulate at the surface. Most of this lipid could be removed by skimming with a spatula, and the remainder by filtration with gentle suction through a Schleicher and Schüll filter paper No. 589–1H. It is essential that the phenol solutions be perfectly clear and free of this lipid or the final products will be contaminated and will not be completely soluble in H_2O or saline. To the clear phenol solution, stirred mechanically, a mixture of equal volumes of ethanol and 90 per cent phenol was slowly added dropwise until the final alcohol concentration reached 10 per cent by volume, and after standing overnight at room temperature, the transluscent jelly-like precipitate was separated by centrifugation. This was redissolved in 90 per cent phenol by shaking. The solution was clarified by prolonged centrifugation and then reprecipitated with alcohol-phenol as above. It may be necessary to add a small crystal of sodium acetate to ensure flocculation. The precipitate was finely suspended by rubbing in ethanol and allowed to soak overnight to remove phenol. The white product was washed 3 times with alcohol and with ether and finally dried to constant weight *in vacuo* over P_2O_5. The substances obtained from all the hog stomach linings studied were completely soluble in H_2O or saline; the yields and analytical properties are listed in Table I.

[1] Purchased from the Plaut Research Laboratories Co., Lehn and Fink Co., Bloomfield, New Jersey.

Analytical Methods.—Ash, nitrogen, glucosamine, and reducing sugar were determined as in (1). Acetyl was determined after hydrolysis for 2 hours with 25 per cent *p*-toluenesulfonic acid as described by Heidelberger, Kendall, and Scherp (5).

Viscosity determinations were carried out at 25°C. in a 2 ml. Ostwald viscometer using 0.2 per cent solutions in 0.9 per cent saline; the values recorded in Table I were obtained by dividing the time of flow for the solutions by that of 0.9 per cent saline.

The relative activity of the purified active preparations from hogs 1 to 10 and 1A was measured qualitatively and quantitatively as described in (references 1 and 4); the quantitative data for the active substances are listed in Table II.

TABLE I

Properties of Purified Blood Group A and Inactive Substances Obtained from Individual and Pooled Hog Stomach Linings

Preparation	Method	Activity of original digest	Yield per lining	Ash as Na	N	Reducing sugar as glucose*	Glucosamine‡	Ratio $\frac{\text{Glucosamine}}{\text{Reducing sugar}}$	$\frac{\text{Glucosamine N}}{\text{Total N}}$	Acetyl	Relative viscosity: 0.2 per cent solution in 0.9 per cent NaCl
			mg.	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*	
Hog 1	Peptic digestion	Active	230		6.4	55	32	0.58	0.39		1.52
" 2	" "	Inactive	600	0.7	5.9	56	32	0.57	0.42	9.4	1.63
" 3	" "	Active	410	0.4	6.4	58	34	0.58	0.42	10.2	1.65
" 4	" "	"	320	0.9	6.5	55	33	0.60	0.40	9.4	1.50
" 5	" "	"	980	0.4	6.6	58	34	0.59	0.41	10.0	1.56
" 6	" "	Inactive	490	0.7	6.1	59	34	0.58	0.44	9.9	1.47
" 7	Autolysis	"	310	1.1	5.7	58	32	0.55	0.44	9.3	1.56
" 8	"	Active	380	0.9	5.9	61	34	0.55	0.45	11.3	1.39
" 9	"	"	400	0.8	6.0	57	33	0.54	0.45	10.2	1.49
" 10	"	"	660	0.9	6.1	59	34	0.58	0.43	10.7	1.71
1A§				1.0	6.1	56	30	0.54	0.39	9.1	1.38

Values not corrected for ash.

* Reducing values given were obtained by the Hagedorn-Jensen method after hydrolysis for 2 hours with 2N HCl.

‡ Glucosamine determined according to (6) after hydrolysis for 2 hours with 2N HCl.

§ 1A was obtained from Wilson's gastric mucin (4).

Glucosamine Analyses on Specific Precipitates.—The following experiments were carried out to determine what proportion of the glucosamine of the added A substance was carried down in A anti-A specific precipitates formed in the region of antibody excess (4). Saline solutions of the A preparations were accurately pipetted into 10 ml. pyrex conical centrifuge tubes and 1 to 4 ml. portions of serum, previously clarified by centrifugation in the cold at 2000 R.P.M. for several hours, were added. The contents of the tubes were thoroughly mixed, the tubes covered with sterile caps, incubated at 37° for 1 hour, and kept in the refrigerator for 7 to 8 days. Blanks containing no A substance were included. The tubes were centrifuged in the cold and the precipitates washed twice in the cold with 3.0 ml. portions of chilled saline. This is essentially the procedure described in (4). The washed precipitates were

dissolved in 0.2 ml. of concentrated HCl in the pyrex centrifuge tubes. One ml. of H_2O was added to adjust the normality to 2.0 and the tube, sealed in an oxygen flame, was kept in a boiling water bath for 2 hours. After cooling, the contents were carefully neutralized in the cold with 3N NaOH to pH 6–7 and the final volume was adjusted to 5.00 ml. with H_2O. Samples of 4.00 ml. were removed and analyzed for glucosamine (6). The determinations were carried out in duplicate; the average values are listed in Table III.

Parallel duplicate determinations of antibody nitrogen precipitated were carried out and the results listed in Table III. These values were corrected for solubility by adding 1.6 μg. antibody nitrogen (*cf.* 1) dissolved per ml. for the total volume in each determination. The values of the glucosamine contents of the specific precipitates (column 6, Table III) were corrected for solubility by multiplying by the solubility correction factor obtained by dividing the corrected antibody N value by the uncorrected (values in column 9 divided by those of

TABLE II

Effectiveness of Various Purified Blood Group A Substances from Hog Stomachs in Precipitating Anti-A from 1.0 Ml. of E. K.$_{4-10}$ Serum

A substance added	Antibody N precipitated by preparation from hog No.							
	1	3	4	5	8	9	10	1A*
μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
5				12.5			14.5	
10				23.2			25.6	
15				32.8				
20				38.8			36.3	
30	48.1	46.3	48.6	46.3	43.8	40.7	43.1	
40				51.3			49.7	38.3‡; 42.1§
60							52.5‖	

All determinations carried out in a total volume of 2.0 ml; values not corrected for solubility.

* From gastric mucin.

‡ 56 per cent as active in precipitating anti-A as the preparation from hog 10 (1).

§ 60 per cent as active in precipitating anti-A as the preparation from hog 5 (1).

‖ Slight excess of A substance in supernatant.

column 8, Table III) and are based upon the assumption that the composition of the saturated solution of the A anti-A complex is the same as that of the specific precipitate. The glucosamine content of the antibody was estimated by determining the glucosamine to nitrogen ratio of preparations of normal γ globulin and by multiplying the corrected antibody N values by this ratio. A sample of human γ globulin was prepared according to the procedure described by Kendall (7) by repeated ammonium sulfate fractionation of normal serum and the water-soluble γ globulin obtained after thorough dialysis against several changes of distilled water. The glucosamine to nitrogen ratio was found to be 0.0688. Electrophoretically separated human γ globulin gave a ratio of 0.0716. The average of 0.07 was multiplied by the antibody N values to determine the glucosamine content of the anti-A (column 12). Subtraction of these values from those of column 11 gave the amount of glucosamine due to A substance in the precipitate.

Antigenicity of the Preparations.—Before immunization, samples of blood were obtained from normal individuals of blood groups A, B, and O. The individuals then received two subcutaneous injections of saline solutions of the preparations on successive days and blood

samples were withdrawn 10 days after the last injection. The hemagglutination titers were measured as in (4). The amount of antibody removed by the various preparations before and after immunization was determined by the microquantitative precipitin method (8). The data are listed in Table IV. Two individuals, F. Be. and H. H. of groups O and B respectively, who had been injected with the inactive preparation from hog 7, were given a second course of

TABLE III

Glucosamine Analyses on Specific Precipitates of Purified Blood Group A Substances from Hog Stomach and Homologous Human Antibody in the Region of Antibody Excess

1	2	3	4	5	6	7	8	9	10	11	12	13	14
A substance	Amount used	Glucosamine in added A substance	Volume serum used	Total volume	Glucosamine found in specific precipitate	Found / Glucosamine / Added	Antibody N precipitated	Antibody N precipitated corrected for solubility	Solubility correction factor*	Glucosamine found in specific precipitate corrected for solubility‡	Glucosamine due to antibody§	Glucosamine in precipitate due to antigen‖	Proportion of glucosamine of added A substance precipitated¶
	μg.	μg.	ml.	ml.	μg.	per cent	μg.	μg.		μg.	μg.	μg.	per cent
A.D.$_{1+2}$ serum used													
1A	60	18	1.0	6.0	11	61	43	53	1.23	14	4	10	56
1A	100	30	1.0	6.0	19	63	57	67	1.18	22	5	17	57
1A	140	42	1.0	6.0	24	57	68	78	1.15	28	5	23	55
E.K.$_{4-10}$ serum used													
1A	120	36	3.0	6.0	24	67	115	125	1.09	26	9	17	47
1A	120	36	3.0	6.0	28	78	126	136	1.08	30	10	20	56
Hog 1	90	29	3.0	6.0	33	114	144	154	1.07	35	11	24	83
" 3	90	31	3.0	6.0	35	113	139	149	1.08	38	10	28	90
" 4	90	30	3.0	6.0	35	117	146	156	1.08	38	11	27	90
" 5	90	31	3.0	6.0	33	106	139	149	1.08	36	10	26	84
" 8	90	31	3.0	6.0	32	103	131	141	1.08	35	10	25	81
" 9	90	30	3.0	6.0	28	93	122	132	1.08	30	9	21	70
" 10	60	20	4.0	8.0	23	115	124	137	1.11	25	10	15	75
" 10	80	27	4.0	8.0	32	119	145	158	1.09	35	11	24	89
" 10	90	31	3.0	6.0	32	103	129	139	1.08	35	10	25	81

* Column 9 divided by column 8.

‡ Column 6 multiplied by column 10.

§ Antibody N precipitated multiplied by 0.07 (ratio of glucosamine to N in γ globulin).

‖ Column 11 minus column 12.

¶ Column 13 divided by column 3.

injections with 1A and the active product from hog 8 respectively, and another portion of serum was obtained and analyzed as above. The sera obtained from the first bleeding before immunization are designated by the subscript x, the sera obtained from the first bleeding after immunization are designated by subscript 1; later samples are numbered in order. To obtain relatively large quantities, sera from several bleedings of an individual were pooled and the pool designated, for example, as K_{4-10}, which refers to a pool of the fourth to the tenth bleedings.

TABLE IV

Antibody Response of Humans to Two Injections of Purified Blood Group A or Inactive Preparations from Individual Hog Stomachs and from Gastric Mucin

Subject	Blood group	Amount of antibody removed by inactive fraction before immunization	Anti-A level before immunization		1st course: Material injected			Amount of antibody removed by inactive fraction 10 days after immunization	Anti-A level 10 days after immunization		2nd course: Material injected			Amount of antibody removed by inactive fraction after 2nd course of immunization	Anti-A level after 2nd course of immunization	
					Preparation No.	Amount injected	Source				Preparation No.	Amount injected	Source			
		μg.N/ml.	μg.N/ml.	titer		mg.		μg.N/ml.	μg.N/ml.	titer		mg.		μg.N/ml.	μg.N/ml.	titer
Bd.	O		0.0	1	2A	2.0	Gastric mucin	0.0	14.6	64						
C.A.	A		0.0	0	2A	2.0	" "		0.2	0						
C.Sh.	A		0.0	0	2A	2.0	" "		0.2	0						
F.Be.	O	0.8	1.8	4	Hog 7	2.0	Inactive hog stomach	0.8	2.2	4	2A	2.0	Gastric mucin	1.8	26.3	128
H.H.	B	0.6	0.7	2	" 7	2.0	" " "	1.0	1.1	2	8	2.0	Active hog stomach	0.0	3.3	32
W.G.	A	0.3	0.2	0	" 7	2.0	" " "	0.5	0.4	0						
W.H.	A	0.2	0.0	0	" 7	2.0	" " "	2.0	2.0	0						
Lu.	O		2.1	2	Hog 8	2.0	Active hog stomach	2.6	8.5	32						
Du.	A		0.0	0	" 8	2.0	" " "		0.4	0						
L.S.	A		0.0	0	" 8	2.0	" " "		1.8	0						
Gl.	O		1.6	2	Hog 10	2.0	Active hog stomach		3.6	8						
P.St.	O		0.8	2	" 10	2.0	" " "		2.5	8						
T.R.	B		4.0	1	" 10	2.0	" " "	0.8–1.5	52	128						

Values not corrected for solubility.

Table V provides a comparison of the amounts of antibody nitrogen precipitated by active and inactive fractions from the sera of Group O and B individuals who had been immunized with A substance.

RESULTS

It can be seen from Table I that active and inactive products of uniform analytical properties can be obtained by two methods from hog stomach in yields of from 230 to 980 mg. per hog stomach lining. The substances contained 5.7 to 6.6 per cent of N, 55 to 61 per cent of reducing sugars (as glucose), 30 to 34 per cent of glucosamine, and 9.1 to 11.3 per cent of acetyl. Relative viscosity values varied from 1.38 to 1.65. No significant analytical differences between the active and inactive substances were found. Preparation 1A,

TABLE V

Antibody Nitrogen Precipitated by Purified Blood Group A and Inactive Substances from Sera of O and B Individuals after Immunization with Active Preparations

Blood sample	Blood group of subject	Preparation used for immunization	Antibody N precipitated from serum by	
			A substance	Inactive substance from hogs 2, 6, or 7
			μg./ml.	μg./ml.
E. K_{4-10}	O	Lilly 330	53	4.8
Bd_{1-5}	O	2A	16.6	0.0–1.2
T. R_1	B	10	52	0.8–1.5
H. H_2	B	8	3.3	0.0
F. Be_2	O	2A	26.3	1.8
Lu	O	8	8.5	2.6

Values not corrected for solubility.

obtained from a random pool of hog stomachs, had a similar chemical composition.

Table II lists the results of a comparison of the effectiveness of 1A with the active products in precipitating the antibody from 1.0 ml. of E. K_{4-10} serum. This serum contained about 52.5 μg. of precipitable antibody N per ml. The preparations from hogs 1, 3, 4, 5, 8, and 10 appeared equally active within experimental error, whereas that from hog 9 seemed to be slightly less active and 1A only 56 to 60 per cent as active in precipitating anti-A as the preparations from hogs 10 and 5 respectively as estimated from the calibration data in the table.

Table III summarizes the data obtained by determining the glucosamine contents of specific precipitates formed in the region of antibody excess from the active substances and homologous antibody. With 1A, the specific precipitates were found to contain glucosamine equivalent to 57 to 78 per cent of that added as 1A, whereas with the preparations from hogs 1, 3, 4, 5, 8, and 10 values of

103 to 119 per cent were obtained. A slightly lower value for the hog 9 precipitate (93 per cent) was obtained. These values, listed in column 6, are subject to two main corrections, one due to the solubility of the specific precipitates and the other due to the glucosamine content of the antibody. The solubility correction factors are listed in column 10 and vary from 1.15 to 1.23 for precipitates containing 53 to 78 μg. of antibody nitrogen and from 1.07 to 1.11 for the larger precipitates. The glucosamine contents of the antibody portion of the specific precipitates, estimated by determining the glucosamine to nitrogen ratio of normal human γ globulin and multiplying the corrected antibody nitrogen values in column 9 by this ratio, are listed in column 12. When these corrections are applied, it can be seen, from the last column, that 47 to 57 per cent of the glucosamine, average 54, of 1A added was precipitated, whereas 75 to 90 per cent, average 84, of the glucosamine of the preparations from hogs 1, 3, 4, 5, 8, and 10 came down in the specific precipitates. As in the comparisons of precipitating power (Table II), a smaller value for the product from hog 9 was obtained. From the ratio 54/84, 1A appeared to be about 64 per cent as pure as the active preparations from the individual hog stomachs on the basis of its glucosamine content.

Table IV summarizes data on the antigenicity in man of several of these preparations. It is evident that the injection of 2A or the active products from hogs 8 and 10 into individuals of groups O and B produced a significant rise in anti-A titer and in antibody N precipitable by active A substance. However, very little or no antibody in the sera of these immunized individuals was precipitable by the inactive preparations. Immunization of individuals of group A with these active substances produced no anti-A agglutinins and only very small amounts of precipitable nitrogen, the highest value being 1.8 μg N per ml. (*cf.* 4).

On immunization of a subject of group O (F. Be.) and one of group B (H. H.) with the inactive substance from hog 7, no change in anti-A titer nor any significant increase in antibody nitrogen precipitable by either active or inactive material was noted. These individuals each received subsequent injections of an active A preparation and their sera then showed a definite rise in anti-A titer and in antibody nitrogen precipitable by A substance. Only a very slight increase in antibody precipitable by the inactive product was noted in the serum of F. Be.; in the case of H. H., no antibody was precipitated by the inactive substance. Of two group A subjects who received injections of inactive substance no response occurred in one (W. G.), the other (W. H.) showed a slight response (2.0 μg. N/ml). In both instances, the same amounts of antibody nitrogen were precipitated by the inactive and active products.

The relative amounts of anti-A and non-anti-A in the sera of immunized individuals of groups O and B may be seen in Table V which lists the amounts of antibody nitrogen precipitable from these sera by active and inactive prepara-

tions. In all but one instance, the proportion of antibody to the inactive fraction constituted less than 10 per cent of the total antibody.

DISCUSSION

The problem of establishing the degree of purity or homogeneity of complex substances such as proteins and polysaccharides has proven extraordinarily difficult. A variety of procedures including constancy of analytical properties and a constant ratio, in several preparations, of activity to dry weight or to nitrogen, occurrence of one component in the ultracentrifuge or in the Tiselius electrophoresis apparatus, constant solubility in the presence of increasing quantities of solid phase, and several immunochemical criteria have been proposed. None of these criteria alone furnishes unequivocal proof of purity and in many instances even the combination of several of these methods may not prove adequate (*cf.* 9). These difficulties are exceptionally well illustrated in the present study.

The data presented in this communication clearly indicate that from certain hog stomachs materials which show blood group A activity can be obtained by the Morgan and King phenol method (3) after peptic digestion, whereas from other stomachs treated in the same manner, products of identical composition with respect to nitrogen, glucosamine, reducing sugar, and acetyl, but devoid of blood group A activity result (Table I). Viscosity measurements on these active and inactive materials suggest that differences in shape or polymerization are not involved. Products from gastric mucin obtained by random pooling of hog stomachs also show the same properties and without quantitative assays of their capacity to precipitate anti-A (Table II) it would be impossible, from the analytical data alone, to determine whether any active preparation consisted only of active material or of a mixture of active and inactive substances. It follows, therefore, that, in the present instance, constancy of analytical properties provides little information about the purity of the A substance.

By the quantitative precipitin method it was found that six of the seven active preparations from individual hog stomachs were equally effective in precipitating anti-A (Table II), and that the seventh sample was only slightly less effective. These six preparations, therefore, within experimental error, satisfy the criterion of constant ratio of activity to dry weight. The value of this evidence is augmented by the finding that the corresponding product obtained from the pool of stomachs (1A), and presumably containing a mixture of active and inactive substances, was only 56 to 60 per cent as effective in precipitating anti-A weight for weight as were the materials from the individual active stomachs. However, these data do not provide any estimate of the absolute degree of purity of the A substance, since it could well be argued, thus far, without fear of contradiction, that the preparations from the active hog stomachs were uniformly composed, for instance, of 99 per cent by weight of the

inactive substance and only 1 per cent by weight of active A substance of unknown properties which, like the inert material, was soluble in phenol and precipitated at a concentration of 10 per cent alcohol by volume.

To assess the validity of this objection and in addition to obtain a measure of the actual purity of the A substance, a method was required which would provide an estimate of the actual weight of material added as A substance which participated in the reaction with anti-A. A method of accomplishing this was derived from a consideration of the quantitative course of the precipitin reaction (9, 10). It has been established for a large number of systems (*cf.* 9, 10) involving the precipitation of an antigen by its homologous antibody that, in the region of antibody excess, the presence of antigen could not be detected in the supernatant and that all of the antigen could be assumed to be in the precipitate. The A anti-A system had also been shown recently to behave in a similar manner (4). The validity of this assumption was established by Stokinger and Heidelberger (11) who found that 96 to 101 per cent of the iodine of thyroglobulin was precipitated by excess of homologous rabbit antibody; Heidelberger and Kendall (12) had earlier shown that practically complete precipitation of an azo protein occurred in the presence of excess antibody.

With this as a basis, therefore, it could be postulated that, if the preparations showing A activity were pure, then in the presence of excess anti-A, the entire amount of antigen should be present in the specific precipitate, and hence all of its glucosamine should be precipitated by excess anti-A. Conversely, if only a small proportion by weight of the active preparations was actually the A substance, little or none of the glucosamine added should be found in the specific precipitate.

The results obtained by analyses of specific precipitates for glucosamine, after correction for solubility and for the assumed glucosamine content of the antibody (Table III, column 14) show that an average of 84 per cent of the glucosamine of active preparations from hogs 1, 3, 4, 5, 8, and 10 added as A substance came down in the specific precipitate. Only 70 per cent of the glucosamine of the preparation from hog 9 and an average of 54 per cent of the glucosamine of preparation 1A was precipitated. The ratio of the averages of the percentage of glucosamine of 1A precipitated to those for the six active preparations of 64 per cent is in excellent agreement with the ratio of 58 per cent for their respective capacities to precipitate anti-A (Table II). The preparation from hog 9 also was slightly less pure than those from the other individual hogs as estimated by both methods. These data definitely provide information about the absolute degree of purity of these preparations.

The average value of 84 per cent for the absolute purity of the preparations from the individual hog stomachs is subject to several uncertainties. The most significant of these arises from the use of normal human γ globulin as the reference standard for the antibody nitrogen determinations and in correcting for

the glucosamine content of the antibody in the specific precipitates. In the absence of purified anti-A, the assumption implied in using normal γ globulin as standard was necessary for calculating the results. However, there is some evidence to indicate that anti-A may be of higher molecular weight than normal human γ globulin (13) so that the present figures might have to be revised somewhat when purified anti-A becomes available, should its glucosamine to nitrogen ratio or color value with the Folin reagent per unit of nitrogen differ from that of normal human γ globulin.

The other source of uncertainty results from the finding that a small proportion of the antibody in the sera used is not anti-A (Tables IV and V). This amounts to 10 per cent of the total antibody nitrogen in serum E. $K._{4-10}$, and does not seem to be antibody to the inactive fraction since variations of from 3 to 500 μg. in the amount of inactive material used did not appreciably change the amount of antibody N precipitated. It would thus appear to be antibody to some other contaminating substance present in small amounts. This conclusion is supported by the findings in Table IV and in reference (4) that small amounts of antibody are formed in some individuals of group A on injection of the various preparations. This antibody has been shown (4) to be unrelated to the A anti-A system. However, since this antibody is present in the specific precipitates, it increases the magnitude of the correction for glucosamine due to the antibody and hence tends to lower the final estimates of absolute purity by a maximum of 5 per cent.

As indicated, the above estimate of absolute purity rests on the assumption that the amount of added glucosamine which comes down in the specific precipitate is proportional to the weight of A substance in the specific precipitate; *i.e.*, that the material which precipitates is a single substance. If this assumption is not made, however, it can be calculated by multiplying the fraction of added glucosamine in the specific precipitate (0.84) by the percentage of glucosamine in the preparation (33, Table I), that at least 28 per cent of the weight of substance added is involved in the precipitation of anti-A. Since both the Type XIV pneumococcal polysaccharide (14) and the anthrax polysaccharide (15) both of which are composed of galactose and *N*-acetyl glucosamine, have been found to be serologically very closely related to the blood group A substance, it is highly probable that both the acetyl and the galactose (14) in the active preparations are essential for blood group A activity in addition to the glucosamine. These three constituents make up 75 to 80 per cent of the total weight of material and if the ratio of glucosamine precipitated to that added as antigen is taken as a measure of all of these constituents, then a minimal estimate of about 65 per cent is obtained for the absolute purity of these preparations considering only the glucosamine, galactose, and acetyl, which thus far can be considered to be involved in activity.

Little can as yet be said about the rôle of the amino acids in these prepara-

tions. Apart from the fact that they appear to be present in all active products thus far obtained (3, 14, 16), there is no evidence to show that they are either important or of no significance. Theoretically it should be possible to determine by a procedure similar to that used for glucosamine, whether or not any one or more of the amino acids in these products is contained in the A anti-A specific precipitate. In practice, however, this presents great difficulties since the amount of any amino acid from the antibody portion of the precipitate would be much greater than that derived from the antigen. Studies by Dr. E. Brand on the amino acid composition of sample 1A are given as an appendix to this paper. Further studies are contemplated by Dr. Brand to determine whether any differences with respect to amino acid composition exist between the active and inactive samples.

The immunochemical principles outlined above may prove applicable to the estimation of the absolute purity of complex biological materials especially when the antigen contains some unique characteristic group which can readily be measured. They may be especially useful to determine whether a given constituent of a biologically active preparation is involved in or is essential for activity.

SUMMARY

1. Studies on a number of individual hog stomachs have shown that substances with blood group A activity cannot be obtained from all hogs. Of ten stomachs studied, only seven yielded products with blood group A activity. All ten purified preparations, however, showed identical properties with respect to nitrogen, reducing sugar, glucosamine, acetyl, and relative viscosity. Six of the seven active samples were of equal potency in precipitating anti-A; the seventh was slightly less active.

2. Preparations from random pools of hog stomachs, although possessing the same analytical properties, were of lower activity than those from individual active stomachs as determined by the microquantitative precipitin method.

3. An immunochemical method for estimating the absolute purity of the blood group A substance by determining the proportion of its glucosamine precipitated by excess anti-A was developed. Values of about 84 per cent for the purity of six of the seven purified active preparations from the individual hog stomachs were obtained.

4. The inactive products, unlike the active ones, did not stimulate the production of anti-A on injection into human beings and did not precipitate anti-A or inhibit hemagglutination of A erythrocytes by anti-A.

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APPENDIX

*Composition of Purified Blood Group "A" Substance**

By ERWIN BRAND, PH.D., AND LEO J. SAIDEL

Constituent	Preparation 1A‡		Proportion of total nitrogen
	Per cent	Nitrogen	
		per cent	*per cent*
Total N		6.1	100
Glucosamine§	30.4	2.38	39.0
Glycine	1.6	0.30	4.9
Valine	0.7	0.08	1.3
Isoleucine	0.3	0.03	0.5
Proline	3.3	0.40	6.6
Phenylalanine	0.1	0.01	0.1
Tryptophane	0.2	0.02	0.3
Histidine	0.6	0.16	2.6
Lysine	1.0	0.19	3.1
Aspartic acid	0.8	0.08	1.3
Glutamic acid	1.3	0.12	2.0
Serine	1.9	0.25	4.1
Tyrosine	0.3	0.02	0.3
Reducing sugar as glucose§	56		
Total N accounted for			66.1

* From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York. Work carried out under a Contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Columbia University.

‡ *Cf.* Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1945, **82**, 207.

§ Kabat, E. A., Bendich, A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83**, 477.

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