IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

IV. Preparation of Blood Group A Substances from Human Sources and a Comparison of Their Chemical and Immunochemical Properties with Those of the Blood Group A Substance from Hog Stomach*

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One of the fundamental problems in the study of the blood group specific substances is the question of whether blood group A (or B or O) substances from different animal species are identical or whether various species elaborate different substances having a general similarity in chemical constitution which is responsible for an immunological cross-reactivity. The solution to this problem requires the isolation in pure form of blood group A substances from two or more species, their characterization by chemical analysis, and a precise assay of their immunochemical activity on a weight basis. Quantitative immunochemical methods have already been used to make precise comparisons of the potency of various samples of the blood group A substance from hog stomach and to obtain a measure of the absolute degree of purity of such preparations (1–3). Thus, it has already been established that, in the region of excess antibody, a given amount of purified blood group A substance from a pool of hog stomachs was only 60 per cent as effective in precipitating anti-A as the same amount of purified A substance from certain individual hog stomach linings. Determination of the proportion of glucosamine added as A substance which was specifically precipitated by excess anti-A provided an estimate of the actual weight of any preparation which combined with its isoantibody. By this method it was shown that, with A preparations from individual hog stomach linings, about 84 per cent of the glucosamine added as A substance was precipitated by excess anti-A, but that only about 60 per cent as much was precipitated by A substance from pools of hog stomachs. These findings were explained by the presence in 30 per cent of the individual hog stomachs of products identical in all properties studied but devoid of blood group A activity. Preparations from random pools of hog stomachs would therefore contain a mixture of these active and inactive substances.

The present study describes the preparation of blood group A substances from human saliva, amniotic fluid, and stomach. These products are charac-

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terized with respect to their potency, purity, and analytical properties. Their non-identity with the blood group A substance from hog stomach is established by activity differences as assayed by quantitative immunochemical methods and by differences in antigenicity and in optical rotation. Differences in the A substances from individuals of subgroups A_1 and A_2 are also reported.

EXPERIMENTAL

Preparation of A Substances.—Saliva from secretors of blood group A was purchased in quantities of from 1 to 7 liters per individual. Two of the individuals, W.G. and A.K., were found to belong to subgroup A_2, the others were of subgroup A_1. Of the latter, G.C., W.H., M.S., and B.K., were shown to be heterozygous A_1O since in each instance one parent belonged to blood group O. Both parents of A.B. were of blood group A, so that this individual could be either homozygous or heterozygous A_1. The samples of saliva were collected over periods of from several days to 3 or 4 weeks in vessels containing several milliliters of chloroform and toluene and were kept in the icebox. Specimens from different individuals were processed separately. The saliva in 1 or 2 liter batches was acidified to about pH 2.3 with concentrated hydrochloric acid and incubated at 37°C. with 5 to 10 mg. crystalline pepsin. After 3 to 4 days the pH was again adjusted to pH 2.3 and after 1 and 2 weeks additional 3 to 6 mg. portions of crystalline pepsin were added and the pH was adjusted when necessary. In most instances, this digestive process resulted in almost complete solution of the insoluble material which usually settles in saliva soon after collection. At the end of 3 weeks, 1 to 2 gm. of sodium acetate was added and the active material precipitated from solution by the addition of 5 volumes of ethanol. The precipitate was centrifuged off, washed, and dried. It was then dissolved in water, any insoluble material removed by centrifugation, and the solution dialyzed in a cellophane sac against several changes of distilled water until no significant amount of nitrogen could be found in the dialysate. This proved to be an important step. Sodium acetate was added to the contents of the dialysis bag and the material reprecipitated by addition of 5 volumes of ethanol, centrifuged, washed as above, and dried. It was then extracted by shaking with 10 ml. of 90 per cent phenol in a shaking machine for 16 to 24 hours. The phenol-insoluble residue was centrifuged off at 2000 R.P.M. for 6 to 12 hours, the clear phenol extract decanted, and the residue again shaken with 5 ml. of 90 per cent phenol. After 3 to 4 phenol extractions, the insoluble residue was triturated with alcohol, washed with alcohol and with ether, and dried. It was then dissolved in 5 to 10 ml. of dilute sodium acetate solution and any small insoluble residue centrifuged off in a refrigerated centrifuge and washed with water or dilute sodium acetate. Five volumes of ethanol were added to the clear solution to precipitate the blood group A substance. The precipitate was washed with ethanol and with ether and dried *in vacuo* over P_2O_5 at room temperature. These products are referred to as the phenol-insoluble fractions.

The clear phenol extracts were combined, a small crystal of sodium acetate was added, and a solution containing equal parts of phenol and absolute alcohol was added dropwise with continuous vigorous stirring until a concentration of 10 per cent alcohol was reached. After standing overnight, the precipitate which formed was centrifuged off. Centrifugation at 2000 R.P.M. for about 8 hours was required. The supernatant was decanted and the precipitate extracted several times with 5 to 10 ml. portions of 90 per cent phenol. A small amount of phenol-insoluble residue was sometimes obtained which was either isolated separately or was combined with the phenol-insoluble fraction. A small crystal of sodium acetate was dissolved in the phenol extract and the phenol-alcohol mixture was again added with vigorous stirring to give a final ethanol concentration of 10 per cent by volume. After standing overnight the precipitate was centrifuged off, triturated with ethanol, washed, and dried. It was

then dissolved in 0.05 per cent sodium acetate, any insoluble residue removed, and 5 volumes of ethanol were added to the supernatant to precipitate the A substance. The precipitate was centrifuged, washed with ethanol and with ether, and dried as above. These fractions are designated as 10 per cent precipitates. Both the phenol-insoluble fractions and the 10 per cent precipitates exhibited blood group A activity.

In one instance, a saliva specimen was not digested with pepsin but was precipitated with alcohol directly and then separated into a water-soluble and water-insoluble fraction. The water-insoluble fraction was digested with pepsin and the alcohol-precipitated digest purified in the usual manner after thorough dialysis. The undigested water-soluble portion after dialysis, yielded only a phenol-insoluble fraction; no fraction corresponding to the 10 per cent precipitates was obtained.

A sample of 440 ml. of human amniotic fluid showing blood group A activity kindly supplied by Dr. L. K. Diamond and Dr. N. Abelson was digested with pepsin and purified as above. This fluid was obtained from a mother of group A, who gave birth to a group O child; accordingly the mother must have been heterozygous, AO.

Four human stomachs from individuals of blood group A were obtained at autopsy. They were kept in 95 per cent alcohol in the refrigerator for several months, and were then dried in a vacuum desiccator over P_2O_5. The blood group A substance was prepared by digestion with pepsin in citrate-HCl buffer at pH 2.3, precipitation of the digest with alcohol, and subsequent purification by the Morgan and King phenol method (4) as described in (3). With all four human stomachs, only negligible amounts of phenol-insoluble fraction were obtained and just as with the hog stomach linings (3) the active substance was concentrated in the 10 per cent precipitates.

The yields and analytical properties of the phenol-insoluble fractions and 10 per cent precipitates of saliva and amniotic fluids and of the 10 per cent precipitates of the human stomachs are listed in Table I.

Analytical Properties.—As in the case of the purified products from hog stomach, the preparations from human saliva, stomach, and amniotic fluid were hygroscopic, absorbing 15 to 20 per cent of their weight of moisture from the air. Accordingly all specimens were dried to constant weight at room temperature in a vacuum desiccator over P_2O_5 before solutions were prepared or analyses performed. Ash, nitrogen, glucosamine, reducing sugar, and relative viscosity determinations were carried out as in (3) and values are given in Table I. Measurements of optical rotation were performed on 0.9 per cent saline solutions containing from 2 to 5 mg. substance per ml. in a 2 decimeter tube using light of wave length 5893Å.

The solubility of specific precipitates of A substance from human saliva and anti-A formed by immunization of human beings of blood groups O and B with purified hog A substance (1–3) was measured as described in (2) by determining the decrease in the amount of antibody nitrogen precipitated from a given volume of serum by a constant quantity of human A substance, as the total volume in which the precipitation was carried out was varied from 1.5 to 6.0 ml. by addition of saline (Table II).

Immunochemical Activity and Purity of the Preparations.—The relative capacity of a given amount of the various samples of A substance purified from human saliva, stomach, and amniotic fluid to precipitate anti-A from the serum of human beings immunized with hog A substance was assayed by the microquantitative precipitin method (5) and interpolation on a quantitative precipitin curve (2, 3). Results are given in Table III. A number of preparations from human salivas, A.B.$_3$ phenol-insoluble; A.B.$_3$, A.B.$_4$, G.C., W.H.$_1$, and W.H.$_2$, 10 per cent precipitates; and W.H.$_2$ digest of H_2O-insoluble, 10 per cent precipitate, proved to be of equal potency in precipitating anti-A and were arbitrarily assigned an activity of 100 per cent. The activity of the other samples is expressed relative to these preparations. Certain of the preparations were tested with several of the sera used so that by reference to these samples it is possible to compare the precipitating capacity of all of the materials tested.

TABLE I

Analytical Properties of Purified Blood Group A Substances Obtained from Human Saliva, Stomach, and Amniotic Fluid

Preparation	Subgroup and genotype	Volume of saliva used	Yield	Ash as Na	N	Reducing sugars as glucose*	Glucosamine*	Ratio $\frac{\text{Glucosamine}}{\text{Reducing sugar}}$	Ratio $\frac{\text{Glucosamine N}}{\text{Total N}}$	Acetyl	Relative viscosity: 0.2 per cent solution in 0.9 per cent NaCl	Optical rotation $[\alpha]_{5893}$	Relative capacity to precipitate anti-A (from Table III)	Proportion of glucosamine of added A substance precipitated by anti-A (from Table IV)
		liters	*mg./liter*	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*			*per cent*	*per cent*
Saliva: Water-soluble portion of alcohol precipitate, not subjected to peptic digestion														
W.H.$_2$ phenol-insol.	A_1O	2.9	12		4.8	55	27	0.49	0.44		1.13	−13°	27;30	25
Saliva: Peptic digest of water-insoluble portion of alcohol precipitate														
W.H.$_2$ phenol-insol.	A_1O	2.9	12		7.7	60	27	0.45	0.27		1.37		50	50
A.K. phenol-insol.	A_2	1.4	13		6.1	57	26	0.46	0.33				33	50
W.H.$_2$ 10% ppt.	A_1O	2.9	31	0.3	5.5	64	30	0.47	0.42	10.1	1.15	−13°	100	61
A.K. 10% ppt.	A_2	1.4	32	1.3		60	29	0.48			1.13	−27.5°	71	82
Peptic digest of whole saliva														
A.B.$_1$ phenol-insol.	A_1	0.22	45			62	34	0.55					100‡	83;95
A.B.$_4$ phenol-insol. 1	A_1	0.95	23		3.6	57	26	0.46	0.57		1.66		74	88
A.B.$_4$ phenol-insol. 2	A_1	0.95	12		4.5	55	32	0.58	0.56		1.15	−8°	90	90
G.C. phenol-insol.	A_1O	0.96	30	2.0	3.9	59	33	0.56	0.65		1.23	−13°	90	90
W.G. phenol-insol.	A_2	2.0	81	1.8	5.5	54	29	0.54	0.41	7.2		−37°	40	59
W.H.$_1$ phenol-insol.	A_1O	2.2	75	0.5	5.5	56	29	0.52	0.41	8.6	1.35	−21°	78	47
W.H.$_2$ phenol-insol.	A_1O	2.5	32	0.6		58	27	0.47			1.37		63	44
M.S. phenol-insol.	A_1O	1.5	10		4.1	62	21	0.34	0.40				58	54
B.K. phenol-insol.	A_1O	1.0	41	1.1	4.9	48	24	0.50	0.38		1.27	−12.5°	48	50
A.B.$_3$ 10% ppt.	A_1	0.22	9			60	35	0.58					100	71
A.B.$_4$ 10% ppt.	A_1	0.95	48	1.3	4.7	56	29	0.52	0.48		1.16	−12°	100	118
G.C. 10% ppt.	A_1O	0.96	77	1.5	5.1	57	28	0.49	0.43	9.2	1.25	−13°	100	77;109;118
W.G. 10% ppt.	A_2	2.0	12		5.6	56	30	0.54	0.42		1.13	−35°	41	50
W.H.$_1$ 10% ppt.	A_1O	2.2	52		5.7	61	32	0.53	0.44	10.3	1.23	−11.5°	100	79
W.H.$_2$ 10% ppt.	A_1O	2.5	21		6.1	61	32	0.53	0.41		1.13	−14°	80;100	84
M.S. 10% ppt.	A_1O	1.5	7	1.1	5.3	50	25	0.50	0.37				86	47
B.K. 10% ppt.	A_1O	1.0	25		6.2	47	21	0.45	0.26		1.13	−20°	46	46
Peptic digest of human stomach														
Human stomach 1			10		6.2	47	25	0.53	0.31				17	13
Human stomach 2			23		6.8	48	25	0.52	0.29		1.11	−2.5°	100	60
Human stomach 3			10		5.3	45	25	0.56	0.37				100	54
Human stomach 4			43		6.7	51	28	0.55	0.33		1.13	−5°	63	41
Peptic digest of human amniotic fluid														
Amniotic fluid I, phenol-insol.		0.44	81		4.2	48	25	0.52	0.47		1.20	−20°	17	38
10 per cent ppt.		0.44	17		5.5	50	22	0.44	0.31				39	23

Values not corrected for ash. * On hydrolysis. ‡ Assumed.

Parallel quantitative precipitin data obtained with samples of A substance from individual hog stomachs (3) are included in Table III, and a comparison of the relative capacities of A substances from hog stomach and human saliva to precipitate hog anti-A formed by human beings is shown in Fig. 1.

As a measure of the actual weight of each preparation of human A substance participating in the reaction with anti-A, washed specific precipitates formed in the region of antibody excess by the addition of known amounts of each specimen to a measured volume of serum were analyzed for their glucosamine content as described in detail in the preceding paper of this series (3). The data are presented in Table IV and the actual amounts of glucosamine in the specific precipitates are given in column 6. The quantity of glucosamine in the precipitate due to the antigen (column 13) was obtained by correcting for the solubility of the specific precipitates in the volumne used (Table II and columns 8 to 11) and for the glucosamine

TABLE II

Solubility of Specific Precipitates of A Substance from Human Saliva and Antibody Formed in Human Beings on Injection of A Substance from Hog Stomach Lining

Serum No.	Volume of serum	A substance added	Region in which precipitate formed	Antibody nitrogen precipitated in volume of ml.					Solubility
				0*	1.5	3.0	4.5	6.0	
	ml.	*μg.*		*μg.*	*μg.*	*μg.*	*μg.*	*μg.*	$\frac{\mu g.}{N/ml.}$
T.R.$_3$	0.5	15‡	Antibody excess	17.6	16.1	14.7	14.7	11.9	1.0
E.K.$_{11}$	0.5	15‡	Antibody excess	20.8	18.0	14.6	12.4	9.6	1.9
T.R.$_3$	0.5	15§	Antibody excess	20.2	18.8	17.2	16.2		0.9
T.R.$_2$	0.5	30§	Antigen excess	26.0		23.9	22.7	21.0	0.8
Average solubility.....									1.2

* By extrapolation.

‡ Phenol-insoluble fraction saliva A.B.$_3$

§ Fraction precipitable from 90 per cent phenol by 10 per cent ethanol, saliva G. C.

content of the antibody (column 12). The proportion of the glucosamine of the A substance found in the precipitate to that added is given in column 14 (*cf.* 3).

Antigenicity Studies.—The antigenicity of several fractions from human saliva was tested in twenty-four human volunteers of the four blood groups. Blood samples were obtained before immunization and each individual was then given two injections subcutaneously into tne arm on successive days of A substance from human saliva. A second blood sample was obtained 10 days after the second injection. The anti-A content of the sera before and after immunization was assayed by the quantitative precipitin method with both hog and human A preparations and by determining the anti-A titer (Table V).

Three of the individuals of group O and one of group B who showed a negligible or weak response to the purified A substance were then given two injections on successive days of a 1:5 dilution of autoclaved saliva from a secretor of blood group A as described by Wiener, Soble, and Polivka (6). The injection was made within 2 weeks after the previous blood sample was taken. Another bleeding taken 10 days later showed no significant antibody response. The three subjects of group O were then given two injections of A substance from hog stomach and serum from blood drawn 10 days after this third course of injections showed a significant rise in anti-A content in all three instances.

TABLE III

Effectiveness of Purified Blood Group A Substances from Human Saliva, Stomach, and Amniotic Fluid and Hog Stomach in Precipitating Anti-A Formed on Immunization of Human Beings with Hog A Substance

Preparation	Subgroup and genotype	Volume serum used	A substance added, μg.									Relative activity of preparation
			5	10	15	20	30	40	50	60	80	
			Antibody N precipitated									
		ml.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	per cent
A.D.₂ serum used												
A.B.₂ phenol-insol.	A_1	0.5	2.0	9.3	15.6	18.8	23.7	29.5		34.1	38.7	100*
A.B.₂ 10 % ppt.	A_1	0.5	4.5	12.5	17.2	18.4	21.6					100*
Hog 10		0.5	10.8	19.1	23.5	27.1	32.3					196
T.R.₂ serum used												
A.B.₂ saliva phenol-insol.	A_1	0.5	6.7	12.2	18.8	22.7	25.6					100*
A.B.₄ saliva phenol-insol.₁	A_1	0.5			14.0							74
A.B.₄ saliva phenol-insol.₂	A_1	0.5			16.7							90
G.C. saliva phenol-insol.	A_1O	0.5			16.6							90
A.B.₄ saliva 10% ppt.	A_1	0.5			18.7							100*
G.C. saliva 10% ppt.	A_1O	0.5	8.3	14.4	18.8;18.1	21.6	24.1					100*
Hog 4		0.5	12.8	19.3	23.3	25.8	26.2					184
T.R.₁ serum used												
W.H.₂ saliva, digest of H_2O-insol., phenol-insol.	A_1O	0.5			10.5							50
A.K. saliva, digest of H_2O-insol., phenol-insol.	A_2	0.5			8.0							33
W.H.₂ saliva, digest of H_2O-insol., 10% ppt.	A_1O	0.5	8.0	12.8	16.1	19.7		20.5				100*
A.B.₄ saliva, 10% ppt.	A_1	0.5			18.0							100*
W.H.₁ saliva, 10% ppt.	A_1O	0.5			16.1;18.7							100*
W.H.₂ saliva, 10% ppt.	A_1O	0.5			14.4;15.8							80–100
M.S. saliva, 10% ppt.	A_1O	0.5			15.4							86
B.K. saliva, 10% ppt.	A_1O	0.5			10.0							46
W.H.₂ saliva undigested phenol-insoluble	A_1O	0.5					13.1					30
E.K.₁₂ serum used												
W.H.₂ saliva undigested, phenol-insol.	A_1O	0.5	1.4	5.2	8.3	10.1		13.7				27
A.K. saliva digest of H_2O-insol., 10% ppt.	A_2	0.5			14.3							71
W.G.₁ saliva phenol-insol.	A_2	0.5			10.8							40
W.H.₁ saliva phenol-insol.	A_1O	0.5			14.9							78
W.H.₂ saliva phenol-insol.	A_1O	0.5			13.5							63
M.S. saliva phenol-insol.	A_1O	0.5			12.9							58
B.K. saliva phenol-insol.	A_1O	0.5			11.9							48
A.B.₄ saliva 10% ppt.	A_1	0.5			17.1							100*
G.C. saliva 10% ppt.	A_1O	0.5			16.0							100*
W.G. saliva 10% ppt.	A_2	0.5			10.9							41
W.H.₂ saliva 10% ppt.	A_1O	0.5			16.7							100*
Human stomach 1		0.5			5.5							17
Human stomach 2		0.5			16.9							100
Human stomach 3		0.5			16.0							100
Human stomach 4		0.5	6.9	11.0	13.1	15.7		20.7				63
Amniotic fluid I phenol-insol.		0.5			5.8							17
Amniotic fluid I 10 per cent ppt.		0.5			10.7							39

* Assumed.

RESULTS

The blood group A preparations from human saliva, stomach and amniotic fluid appear to be fairly similar in analytical properties to the products obtained from hog stomach linings (3). However, the variations in analytical values from one preparation to another were much greater than those obtained with the hog substances. This is particularly evident from the ratios of glucosamine to reducing sugar and of glucosamine N to total N. Nitrogen ranged from 3.6 to 7.7 per cent, reducing sugar from 47 to 64 per cent, and glucosamine from 21 to 34 per cent. Samples showed a very low ash content. When saliva or amniotic fluid was subjected to peptic digestion the blood group activity was found to be distributed between two fractions, one insoluble in 90 per cent phenol and

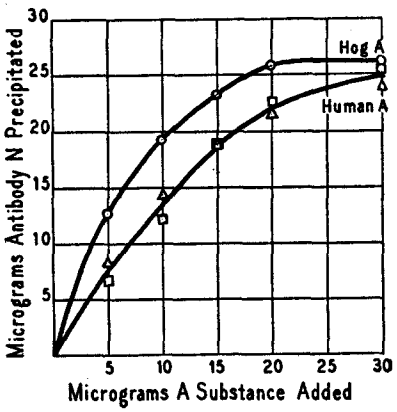

FIG. 1. Quantitative precipitin curves of blood group A substances from hog stomach and human saliva with anti-hog A formed in man. Serum, 0.5 ml. T.R.$_2$; O = hog 4; △ = G.C. 10 per cent precipitate; □ = A.B.$_3$ phenol insoluble.

the other soluble in 90 per cent phenol but precipitable from solution by addition of 10 per cent alcohol by volume (10 per cent precipitate). Blood group A activity was concentrated in the 10 per cent precipitates from human stomachs, no active phenol-insoluble portion being obtained. However, no 10 per cent precipitate was obtained from the water-soluble portion of a saliva which was purified without peptic digestion. Yields of purified products varied from 17 to 127 mg. per liter of saliva or amniotic fluid and from 10 to 43 mg. per human stomach. The relative viscosities of the phenol-insoluble fractions were generally higher than those for the 10 per cent precipitates; A.B.$_4$ phenol-insoluble$_2$ was a small fraction which was originally soluble in phenol and was precipitated by 10 per cent ethanol but did not redissolve in phenol and is probably more closely related to the 10 per cent fraction than to the phenol-insoluble fraction. All preparations were levorotatory, the specific optical rotations of the substances possessing subgroup A_1 specificity ranging from $-2.5°$ to $-21°$

TABLE IV

Glucosamine Analyses on Specific Precipitates of Purified Blood Group A Substances from Human Saliva, Stomach, and Amniotic Fluid and Antibody Formed on Immunization of Human Beings with Purified A Substance Obtained from Hog Stomach

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	Glucosamine Found / 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
Water-soluble portion of alcohol precipitate, not subjected to peptic digestion													
W.H.$_2$ phenol-insol.	60	16	2.0 E.K.$_{12}$	6.0	6	38	33	40	1.21	7	3	4	25
W.H.$_2$ phenol-insol.	120	32	2.0 T.R.$_2$	6.0	11	35	52	59	1.13	12	4	8	25
Peptic digest of water-insoluble portion of alcohol precipitate													
W.H.$_2$ phenol-insol.	60	16	2.0 T.R.$_2$	6.0	9	56	42	49	1.17	11	3	8	50
A.K. phenol-insol.	60	16	2.0 T.R.$_2$	6.0	9	56	32	39	1.22	11	3	8	50
W.H.$_2$ 10% ppt.	60	18	2.0 T.R.$_2$	6.0	15	83	64	71	1.11	16	5	11	61
A.K. 10% ppt.	60	17	2.0 E.K.$_{12}$	6.0	16	94	57	64	1.12	18	4	14	82
Peptic digest of whole saliva													
A.B.$_2$ phenol-insol.	60	20	3.0 T.R.$_2$	6.0	24	118	73	80	1.10	25	6	19	95
A.B.$_3$ phenol-insol.	90	30	3.0 T.R.$_2$	6.0	31	103	113	120	1.06	33	8	25	83
A.B.$_4$ phenol-insol. 1	60	16	2.0 T.R.$_2$	6.0	16	100	56	63	1.13	18	4	14	88
A.B.$_4$ phenol-insol. 2	60	19	2.0 T.R.$_2$	6.0	20	105	67	74	1.10	22	5	17	90
G.C. phenol-insol.	60	20	2.0 T.R.$_2$	6.0	21	105	66	73	1.11	23	5	18	90
W.G. phenol-insol.	60	17	2.0 E.K.$_{12}$	6.0	12	71	43	50	1.16	14	4	10	59
W.H.$_1$ phenol-insol.	60	17	2.0 E.K.$_{12}$	6.0	12	71	60	67	1.12	13	5	8	47
W.H.$_2$ phenol-insol.	60	16	2.0 E.K.$_{12}$	6.0	10	63	54	61	1.13	11	4	7	44
M.S. phenol-insol.	60	13	2.0 E.K.$_{12}$	6.0	10	77	52	59	1.14	11	4	7	54
B.K. phenol-insol.	60	14	2.0 E.K.$_{12}$	6.0	9	64	48	55	1.16	11	4	7	50
A.B.$_2$ 10% ppt.	90	31	3.0 T.R.$_2$	6.0	28	87	113	120	1.06	30	8	22	71
A.B.$_4$ 10% ppt.	60	17	2.0 T.R.$_2$	6.0	23	125	75	82	1.09	25	5	20	118
G.C. 10% ppt.	60	17	2.0 T.R.$_2$	6.0	23	135	75	82	1.09	25	5	20	118
G.C. 10% ppt.	80	22	2.0 T.R.$_2$	6.0	28	128	86	93	1.08	30	6	24	109
G.C. 10% ppt.	60	17	2.0 T.R.$_2$	6.0	17	100	72	79	1.10	19	6	13	77
W.G. 10% ppt.	60	18	2.0 E.K.$_{12}$	6.0	11	61	44	51	1.16	13	4	9	50
W.H.$_1$ 10% ppt.	60	19	2.0 E.K.$_{12}$	6.0	20	105	87	94	1.08	22	7	15	79
W.H.$_1$ 10% ppt.	60	19	2.0 T.R.$_2$	6.0	19	100	75	82	1.09	21	6	15	79
W.H.$_2$ 10% ppt.	60	19	2.0 T.R.$_2$	6.0	19	100	63	70	1.11	21	5	16	84
M.S. 10% ppt.	60	15	2.0 T.R.$_2$	6.0	11	73	62	69	1.11	12	5	7	47
B.K. 10% ppt.	60	13	2.0 T.R.$_2$	6.0	8	62	40	47	1.17	9	3	6	46
Human stomach 1	60	15	2.0 E.K.$_{12}$	6.0	3	17	22	29	1.32	4	2	2	13
Human stomach 2	60	15	2.0 E.K.$_{12}$	6.0	13	87	68	75	1.10	14	5	9	60
Human stomach 3	60	15	2.0 E.K.$_{12}$	6.0	12	80	64	71	1.11	13	5	8	54
Human stomach 4	60	17	2.0 E.K.$_{12}$	6.0	10	59	52	59	1.13	11	4	7	41
Amniotic fluid I phenol-insol.	60	13	2.0 E.K.$_{12}$	6.0	5	38	23	30	1.30	7	2	5	38
Amniotic fluid I 10% ppt.	60	13	2.0 E.K.$_{12}$	6.0	6	46	43	50	1.16	7	4	3	23

* 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.

TABLE V

Antibody Response of Human Beings to Injections of Blood Group A Preparations from Human Saliva

Subject	Blood group	Anti-A level before immunization as determined with			First course		Anti-A level 10 days after 1st course as determined with			Second course		Anti-A level 10 days after 2nd course as determined with			Third course		Anti-A level 10 days after 3rd course as determined with		
		Hog A	Human A	Titer	Material linjected	Amount*	Hog A	Human A	Titer	Material injected	Amount	Hog A	Human A	Titer	Material injected	Amount*	Hog A	Human A	Titer
		μg. N/ml.	μg. N/ml.			mg.	μg. N/ml.	μg. N/ml.			ml.	μg. N/ml.	μg. N/ml.			mg.	μg. N/ml.	μg. N/ml.	
J.S.	O	0.2	1.3	2–4	A.B.$_4$ phenol-insoluble‡	1.0	4.1	2.2	4	Autoclaved saliva	0.4§	2.7	1.1	4	Hog 2A	1.0	9.1	7.7	8
H.S.	O	2.9	2.2	4	A.B.$_4$ phenol-insoluble‡	1.0	3.4	1.4	8	" "	0.4	4.4[¤]	2.5[¤]	4	Hog 2A	1.0	12.2(9.8)¶	8.0(8.9)¶	32
E.S.	O	0.2	0.1	0	A.B.$_4$ phenol-insoluble‡	1.0	0.9	0.9	0–2	" "	0.4	1.6	1.3	0	Hog 2A	1.0	6.2	5.6	16
Sc.	O	0.7	0.2	2	A.B.$_4$ phenol-insoluble‡	1.0	2.3	1.0	4										
Sh.	O	2.9	2.0	2–4	A.B.$_4$—10%**	1.0	10.8	10.1	16										
L.C.	O	1.0	0.9	0	W.H.$_2$—10%**	1.0	2.0	1.7	0										
E.D.	O	1.3	0.3	0	W.H.$_2$—10%**	1.0	0.9	0.4	0										
J.G.	O	10.6	9.7	4	W.H.$_2$—10%**	1.0	34.2	35.0	16										
W.N.	O	1.9	0.8	0	W.H.$_2$—10%**	1.0	2.7	2.0	8–16										
C.W.	O	1.4	0.7	1	W.H.$_2$—10%**	1.0	5.5	5.3	8–16										
El	O	2.4	2.3	4	W.H.$_2$ undigested‡‡	1.0	3.1	3.1	8–16										
M.M.	O	1.2	0.5	0	W.H.$_2$ undigested‡‡	1.0	5.8		8										
Me	O	1.6	0.4	0	W.H.$_2$ undigested‡‡	1.0	2.4		2										
O.B.	B	0.2	0.0	0	A.B.$_4$—10%**	1.0	4.3	1.4	8										
L.P.	B	0.0	0.0	0	A.B.$_4$—10%**	1.0	1.7	0.9	4–8	" "	0.4	1.5	1.3	2–4					
Ca	B	2.3	1.9	0–1	W.H.$_2$—10%**	1.0	2.0	1.8	1–2										
E.J.	B	1.5	1.1	1	W.H.$_2$—10%**	1.0	2.6	0.9	4–8										
P.M.	B	1.0	0.0	0	W.H.$_2$—10%**	1.0	20.2	18.8	8										
J.M.	B	1.6	0.0	0	W.H.$_2$—10%**	1.0	4.8	1.4	8										
Jo	B	1.3	0.7	0	W.H.$_2$ undigested ‡	1.0	1.0	0.5	2										
L.K.	A	0.0	0.0	0	A.B.$_4$—10%**	1.0	0.0	0.0	0										
A.O.	A	0.0	0.0	0	A.B.$_4$—10%**	1.0	0.0	0.0	0										
Bu	A_2	0.5	0.3	0	W.H.$_2$ undigested‡‡	1.0	0.4	0.2											
V.K.	A_2B	1.3	0.3	0	A.B.$_4$—10%**	1.0	0.9	0.1	0										

* 2 injections of 0.5 mg. each. ‡ Phenol-insoluble fraction. § 2 injections of 1.0 ml. 1:5 diluted saliva.

[¤] Another sample of serum taken 2 months later without further injections gave 4.4 and 3.6 μg. antibody N per ml. with hog A and human A respectively.

¶ Values in parentheses obtained on a sample of blood drawn 16 days later. ** 10 per cent precipitate. ‡‡ Phenol-insoluble fraction of saliva not subjected to peptic digestion.

and those from individuals of subgroup A_2 varying from $-27.5°$ to $-37°$. Several of the A_1 samples were obtained in such small yield that accurate optical rotations were difficult to obtain; with those samples obtained in good yield specific rotations ranged from $-13°$ to $-21°$. The difference in specific rotation between the A_1 and A_2 substances is considered to be significant.

The solubility of specific precipitates of A substance from human saliva and anti-A formed by immunization of human beings with hog A substance varied from 0.8 to 1.9 μg. N per ml. saline with an average of 1.2 μg. N per ml. (Table II) in good agreement with the average solubility of 1.6 μg. N per ml. previously reported for hog A-anti-A specific precipitates (2).

The relative capacity of the various human A and several hog A substances to precipitate hog anti-A is given in Table III. In general, the phenol-insoluble fractions from saliva showed a significantly lower capacity to precipitate anti-A than did the 10 per cent precipitates, with the exception of A.B.$_3$ and G.C. phenol-insoluble which were among the best in precipitating power. In this connection it is noteworthy that the sample of saliva which was not subjected to peptic digestion and from which only phenol-insoluble active material was obtained was even poorer in precipitating capacity. Human stomachs 2 and 3, and saliva preparations A.B.$_3$ phenol-insoluble, and A.B.$_4$, G.C., W.H.$_1$, and W.H.$_2$ 10 per cent precipitates all showed the same potency in precipitating anti-A (± 1 μg. antibody N for samples of 15 μg. A substance), and by this criterion were the most active materials obtained. These products, however, were only 51 to 54 per cent as effective as hog A substance in precipitating anti-A. From the data in Table III and Fig. 1, the hog A substance was calculated to have a precipitating activity of 196 and 184 per cent referred to that of the best human saliva preparations. Of considerable interest is the finding that the products from A.K. and W.G., the two individuals of subgroup A_2 were much less effective in precipitating anti-A than those from persons in subgroup A_1. W.G. yielded phenol-insoluble and 10 per cent fractions with 40 and 41 per cent of the precipitating power of the best saliva and stomach samples and the corresponding products from A.K. were 33 and 71 per cent as active. The amniotic fluid fractions and human stomach 1 were very low in capacity to precipitate anti-A and human stomach 4 fell into the same range of precipitating activity as did the materials from A_2 individuals and some of the phenol-insoluble fractions from persons of subgroup A_1.

The ratios obtained (Table IV) for the quantity of glucosamine in the various samples precipitable by anti-A to that added provide an additional independent criterion of the purity of the preparations (3). The results parallel those in Table III in many respects. The purest preparations by this criterion are seen to be those from A.B. and G.C. and W.H.$_1$ and W.H.$_2$ 10 per cent precipitates in which close to 100 per cent of the glucosamine added as antigen was precipitated by excess anti-A. Two of these individuals (G.C. and W.H.) were heter-

ozygous, the other is indeterminable from blood grouping of the parents. In another group of salivas, those from M.S., B.K., and some samples from W.H., all three of whom were shown to be heterozygous A_1O as well as those from the two individuals of subgroup A_2, W.G. and A.K., and the samples from human stomachs 2, 3, and 4, only about one-half of the glucosamine could be precipitated by excess anti-A. A third group consists of human stomach 1, the sample from undigested saliva of W.H., and the amniotic fluid from which an even smaller proportion of the glucosamine could be precipitated by anti-A.

Several additional correlations become evident by comparing the analytical properties of various preparations with the two estimates of immunochemical activity obtained from Tables III and IV. This may be seen from the last two columns of Table I in which the data on relative capacity to precipitate anti-A and the proportion of the glucosamine of each preparation precipitated by anti-A are tabulated. The products which showed the greatest capacity to precipitate anti-A fall into two groups with respect to their proportion of specifically precipitable glucosamine: One group including products from A.B. and G.C. and $W.H._1$ and $W.H._2$ 10 per cent precipitates shows maximal precipitating activity, and essentially all the glucosamine of these products is specifically precipitable by anti-A; the second group, comprising the saliva samples from M.S. 10 per cent precipitate and $W.H._2$ digest of water-insoluble 10 per cent precipitate, and human stomachs 2 and 3 was about equal to A.B. and G.C. in capacity to precipitate anti-A, but only about one-half of their glucosamine was specifically precipitable. The saliva samples from subgroup A_2 as well as B.K. and M.S. phenol-insoluble and human stomach 4 showed about one-half the capacity to precipitate anti-A and a corresponding proportion of their glucosamine was specifically precipitable. Finally, the lowest capacity to precipitate anti-A and a correspondingly low content of specifically precipitable glucosamine was found in the amniotic fluid preparations, human stomach 1, and the sample of saliva from W.H. which was not subjected to peptic digestion. Several of these samples showed significantly lower ratios of glucosamine N to total N.

Table V shows the results obtained on immunizing twenty-four individuals with several human A preparations from saliva. No antibody response was noted in the three individuals of group A, one of whom was of subgroup A_2, or in the A_2B subject. In the twenty volunteers belonging to groups O or B, antibody levels before immunization, with one exception, varied from 0 to 3 μg. N per ml.; after immunization, the antibody level rose in one instance to 18 to 20 μg. N per ml., in another to 10 μg. N per ml., and in two others to 5 μg. N per ml.; the remaining individuals showed an even poorer antibody response. In one instance, J.G., an initial antibody level of about 10 μg. N/ml. was found, the highest level of presumably naturally occurring anti-A thus far encountered. On immunization an increase to about 35 μg. antibody N per ml. was obtained.

The two individuals showing the best antibody response, Sh. and P.M., as well as J.G., were injected with the 10 per cent precipitates of A.B.$_4$ and W.H.$_2$, indicating that these materials were antigenic despite the peptic digestion and subsequent purification. In general, especially in the sera obtained before immunization and in those sera in which little or no increase in antibody was found, the hog A substance usually gave slightly higher values than did the human A substance. However, in those instances in which the best antibody responses were obtained, and the amount of A substance to be added could be judged with better precision, hog and human A substances removed the same amount of antibody. It is significant that the three individuals who gave a slight or negligible response to purified human A substance and also failed to respond to injections of autoclaved human A saliva (6) developed a definite antibody response on subsequent injection of hog A substance. In general, increases in titer paralleled rises in antibody nitrogen within experimental error.

DISCUSSION

The data presented above indicate the relatively complex nature of the problem and the difficulties of obtaining purified blood group substances from human sources, in contrast to the previous studies on the blood group substances prepared from the stomach linings of individual hogs (1–3). Thus, it is apparent that chemical differences must exist between the A substance as secreted into saliva or pseudomucinous ovarian cyst fluid (7) and that obtained by digestion of stomach linings with pepsin since the former are insoluble in 90 per cent phenol while the latter are completely soluble in phenol. Indeed, while digestion of saliva with pepsin renders a portion of the A substance soluble in phenol, this conversion is not complete and a phenol-insoluble fraction is invariably obtained (Table I). This difference in phenol solubility appears to be correlated in general with a lower relative viscosity, but does not appear to be related to the analytical properties or optical rotation. All of the preparations from human sources were levorotatory while the hog stomach A substances were dextrorotatory. The finding that the product from undigested saliva showed a lower degree of activity by the two immunochemical criteria employed indicates the desirability of peptic digestion in purification. It would also appear that the materials isolated from peptic digests are simpler products than are those secreted directly into saliva.

In contrast to the uniform behavior of the purified blood group A preparations from individual hog stomach linings, the purified preparations from saliva fall into several distinct groups as judged by the two quantitative immunochemical criteria employed for their characterization (2, 3). None of the human A substances showed more than about one-half the capacity of an equal weight of the hog A substance to precipitate homologous anti-hog A formed in human beings of blood groups O or B, although the products from the saliva of three

individuals, A.B., G.C., and several samples from W.H., were of equal purity as judged by almost complete precipitation of their glucosamine in the region of antibody excess (Tables I, III, and IV). The reaction of human saliva and hog stomach A substances with anti-hog A would thus appear to be in the nature of a cross-reaction (8); it is, however, one in which the same total amount of antibody can be precipitated by an excess of either antigen (Fig. 1). A study of the reciprocal reaction of human and hog A substances with anti-human A formed in man is contemplated and this should provide further information about the specificities of the hog and human A substances.

Saliva from the two individuals of subgroup A_2 yielded purified products, identical in analytical properties but having a significantly higher levorotation than those from persons of subgroup A_1. The A_2 preparations showed a lower capacity to precipitate anti-hog A than did the best A_1 preparations and only about one-half of their glucosamine was specifically precipitable by excess anti-A. Such behavior is compatible either with the presence of 50 per cent of material which is not A substance or with a cross-reaction between the A_2 substance and anti-hog A (*cf.* 8). The precipitin reaction of hog and human A_1 and A_2 substances with their respective homologous and heterologous sera will be studied quantitatively to establish which of these two alternatives is correct.

Only about one-half of the glucosamine of the other A_1 saliva products was found to be precipitable by excess anti-A. It is significant that blood grouping of the parents of these individuals showed them to be heterogyzous, A_1O, and it is possible that some of these products contain a considerable amount of O substance which would satisfactorily account for their reduced capacity to precipitate anti-A and for the lower percentage of their glucosamine precipitable by anti-A. On this basis G.C. and W.H., the heterozygous individuals, who yielded products, the glucosamine of which was almost completely precipitable by anti-A, might be non-secretors of O substance; however A.B. might be homozygous or heterozygous but a non-secretor of O substance. It is also possible that the amount of O substance if secreted by these individuals may be insufficient to affect the glucosamine analyses of specific precipitates. Studies by Landsteiner and Harte (9) indicated that O saliva yielded products of the same analytical compositon as did A and B saliva and Morgan and coworkers (10, 11) obtained similar data for A, B, and O substances from pseudomucinous ovarian cyst fluids. In addition human saliva from individuals of blood group A was found to show both A and O specificity (7), the genotype of the donors was not given, however.

In the case of the amniotic fluid, the low activity in precipitating anti-A and the corresponding low proportion of specifically precipitable glucosamine strongly suggest that the preparations consist of a mixture of A and O substances. Inasmuch as the mother was group A and necessarily heterozygous, AO, since the fetus was group O, the assumption that the amniotic fluid prob-

ably contained O substance derived from the fetus and from the heterozygous mother in addition to A substance of maternal origin would satisfactorily account for the immunochemical findings. Indeed the presence of O substance in the amniotic fluid is indicated from studies on the cross-reactions of human saliva and amniotic fluid A substances with Type XIV antipneumococcal horse serum which will be reported subsequently (12), as will further studies on the reactivity of the various preparations with anti-O sera.

The anomalous observation that human stomachs 2 and 3 and the saliva preparations of M.S. and W.H.$_2$ digest of water-insoluble 10 per cent precipitate were more efficient in precipitating anti-A than would be anticipated from their content of specifically precipitable glucosamine requires further study.

Little can be said of the reason for the variations in the preparations from human stomach, except that the entire stomach had to be used, in contrast to the experience with hog stomachs (3) in which only the mucous lining was selected.

From the data in Table V, it can be seen that the human A substance is antigenic in individuals of groups O and B. It appears to be a poorer antigen than the hog A substance in that a smaller proportion of individuals show good antibody levels (*cf.* 1–3). In addition, the finding that three individuals who had shown only a slight or negligible response to human A substance, and to A saliva (6) developed an appreciable response to hog A substance suggests that differences in antigenicity between the hog and the human A substances may exist.

SUMMARY

1. Blood group substances have been prepared from human saliva, stomach, and amniotic fluid from individuals of blood group A_1 and A_2. Several of the saliva samples were obtained from individuals shown to be heterozygous, A_1O.

2. The purified blood group A substances from human sources were similar in nitrogen, glucosamine, reducing sugar, and acetyl content. The A_1 and A_2 substances differed in optical rotation. All of the human A samples were levorotatory while those from hog stomach were dextrorotatory.

3. By two immunochemical criteria the various human preparations could be shown to fall into distinct groups, with respect to purity. The best products showed maximal activity and almost all of their glucosamine was specifically precipitable by anti-A. These samples of human A substance were only about one-half as effective in precipitating antibody to hog A substance formed in man as was homologous hog A substance although the same total amount of antibody was precipitable by excess of either antigen.

4. Human blood group A_1 substance was found to be antigenic in individuals of blood groups B and O but was not as good an antigen as hog A substance.

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