IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS*

X. The Preparation of Blood Group A and B Substances and an Inactive Substance from Individual Horse Stomachs and of Blood Group B Substance from Human Saliva

By HAROLD BAER, Ph.D., ELVIN A. KABAT, Ph.D., and VESTA KNAUB

(From the Departments of Neurology and Bacteriology, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, Presbyterian Hospital, New York)

(Received for publication, August 29, 1949)

As part of a study of blood group substances, materials have been isolated from individual horse stomach linings and from the saliva of human beings of blood group B. These products, as prepared by the phenol extraction method (1), have chemical properties similar to the human and hog blood group A and O substances previously studied (2, 3), although the materials of horse origin appear to have a higher nitrogen and lower carbohydrate and fucose content than do the human and hog materials. The horse substances cross-react with type XIV antipneumococcus horse serum (4) and this cross-reaction is increased on heating at 100°C. at pH 1.9 as was found for the hog and human blood group substances (5).

In confirmation of previous findings (6) only the glandular portions of the horse stomach linings yielded active material; some stomachs showed only A or only B activity, others showed both A and B activity. From certain horse stomachs, however, materials were obtained that possessed neither blood group A, B, or O activity as measured by inhibition of isoagglutination although their chemical composition was similar to that of active material obtained from other horse stomachs.

EXPERIMENTAL

Preparation of Substances.—Stomach linings were collected from each of 6 horses through the courtesy of Dr. L. Reddin, Jr., of Lederle Laboratories, and after separation of stomach linings 3, 5, and 6 into glandular and non-glandular portions (6), were placed in alcohol for dehydration. After being filtered and cut into small pieces, the material was dried *in vacuo* in a desiccator and stored in a dried state. The materials were prepared up to this point by Dr. Aaron Bendich. To isolate the blood group substance the stomach linings were first digested with pepsin. This was carried out by adding 300 to 400 ml. of water to each of the dried preparations followed by cautious addition of concentrated HCl until the pH was ap-

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

proximately 2; 10 mg. of crystalline pepsin containing $MgSO_4$ (equivalent to 5 mg. of pepsin) and 10 ml. of toluene were added and the mixture incubated at 37°C. Concentrated HCl was added daily as needed to maintain the pH at about 2, a second portion of pepsin being added after 5 days. By the end of 10 days enzymatic action had apparently become minimal as evidenced by failure of the pH to rise. Stomach linings 2, 4, 3 glandular, and 5 non-glandular were dialyzed against 3 changes of distilled water, the pH adjusted to 2 with HCl, a further portion of pepsin added, and the materials reincubated at 37° C. Analysis of the dialysates by the colorimetric method of Dische and Shettles (7) revealed the presence of methylpentose. If this was fucose then there was twice as much fucose in the dialysates than was present in all the fractions of the substance isolated. Unfortunately, due to the tremendous amount of total materials in the dialysates, it was not possible either to identify the analytical methylpentose as fucose nor to prove that the color produced was specific and related to the methylpentose content.

After digestion the pH was raised to 4 or above with sodium acetate (10 to 20 gm.) and 5 volumes of alcohol was added to the digest to effect precipitation. The precipitate was collected by decantation, centrifuged, and dried over P_2O_5. The dried precipitates were soaked overnight in water, centrifuged, the supernatant removed, and the residue extracted with 6 successive portions of water, the total volume of the extract being 150 to 200 ml. After adding sodium acetate the substances were reprecipitated with 5 volumes of alcohol, dried, and the extraction procedure repeated once more to remove all water-insoluble residues.

Further purification was effected by extraction with 5 successive portions of 90 per cent phenol (1), the total volume of phenol being 30 to 40 ml. Each extraction was carried out by shaking overnight followed by about 6 to 8 hours of centrifugation. The phenol-insoluble fraction was washed with alcohol and dried *in vacuo*; samples 2, 4, 3 glandular and 5 non-glandular, the samples which had been dialyzed and redigested, possessed negligible amounts of phenol-insoluble material. The clear phenol supernatants were fractionally precipitated by slowly adding with vigorous stirring a solution of equal parts of alcohol and 90 per cent phenol to alcohol concentrations of 15 per cent and 25 per cent. The 15 per cent precipitates were extracted with 4 portions of phenol (total volume of 18 ml.), and after removal of a small quantity of phenol-insoluble material by centrifugation, fractionally precipitated, after adding sodium acetate, by the addition of the 50 per cent ethanol in phenol solution to alcohol concentrations of 15 and 25 per cent, the latter fraction being designated 25 per cent from 15 per cent (*cf.* Table I). The finding that material which was precipitable originally at an alcohol concentration of 15 per cent re-precipitates only partially at this alcohol concentration may be attributable to its solubility being affected by concentration or by the presence of other substances in the phenol. Following the removal of the phenol by repeated washing with alcohol the precipitates were dried *in vacuo*. The dried substances were dissolved in water, centrifuged to remove traces of water-insoluble material, and then precipitated by the addition of sodium acetate and 5 volumes of alcohol. The precipitates were centrifuged, washed with 95 per cent alcohol, and dried. The materials so obtained were white amorphous powders; the analytical properties and yields are listed in Table I.

Two commerical samples of horse substances prepared from mixtures of horse stomachs were available. One of these designated Lederle 35P1A was supplied by Dr. H. D. Piersma of Lederle Laboratories. It was prepared from a pool of horse stomachs each of which was individually tested for the presence of blood group B substance; by this procedure stomachs with only blood group A activity and with no activity were eliminated and only those with B activity or with both A and B activity were used. The second sample, Lilly M336C, was supplied by Dr. J. A. Leighty of Eli Lilly and Co. It was prepared from a random pool of horse stomachs by digestion with pepsin and by shaking with chloroform (*cf.* reference 8). It was refractionated by Dr. Aaron Bendich and a fraction soluble in 90 per cent phenol and

TABLE I

Properties of Blood Group Substances Isolated from Horse Stomach Linings

Preparation	Yield per stomach lining	Ash as Na	N	Reducing sugar as glucose*	Glucosamine*	Glucosamine / Reducing sugar	Glucosamine N / Total N	Fucose	Fucose / Reducing sugar	Acetyl	Activity‡	
											Antiserum to horse A or B substances	Antiserum to human B substance
	mg.	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*		*per cent*	*μg.*	*μg.*
Substances with A activity												
Horse 1 15%	*ca.* 138		6.3	54	23	0.43	0.29	3.2	0.06		5–10	
25% from 15%	*ca.* 314		6.9	48	26	0.55	0.29	4.9	0.10		5–25	
25%	*ca.* 515	1.5	7.4	48	27	0.57	0.28	4.7	0.10		5	
phenol-insol.	*ca.* 62		4.0	42	8	0.18	0.16	1.8	0.04		25	
Substances with B activity												
Horse 2 15%	30		6.4	49, 52	15, 15	0.31	0.19	9.7	0.20	9.1	2§	5
25% from 15%	246		6.1	51	23	0.46	0.30	6.3	0.12		5§	10
25%	157	1.0	6.6	53	29	0.55	0.35	5.2	0.10		5	10
Horse 4 15%	101		6.8	46	22	0.48	0.25	6.9	0.15	9.4	5	5
25% from 15%	144		6.4	48	23	0.48	0.28	4.7	0.10		2	25
25%	376	0.6	7.1	51	28	0.55	0.31	4.7	0.09		5	10
Substances with minimal A, B, or AB activity												
Horse 3 15% gland.	9			52	19	0.36		4.3	0.08		A300 B100	
25% from 15% gland.	336		7.1	42	24	0.56	0.27	3.2	0.08		B300	
Horse 5 15% gland.	151		7.3	49	27	0.54	0.29	3.9	0.08		B100	
25% gland.	293		7.0	44	23	0.54	0.26	4.0	0.09		B100–300	
Substances with neither A, B, or O activity												
Horse 3 25% gland.	285		7.3	52	27	0.53	0.29	4.7	0.09			
Horse 5 25% from 15% gland.	293		7.0	44	23	0.54	0.26	4.0	0.09			
phenol-insol. gland.	101		5.8	40	11	0.28	0.15	3.6	0.09			
Horse 6 15% gland.	*ca.* 313	2.5	7.2	43	23	0.53	0.25	2.6	0.06	8.4		
25% from 15% gland.	*ca.* 564		7.1	42	24	0.56	0.27	3.2	0.08			
25% gland.	*ca.* 306		8.0	41	21	0.52	0.21	3.8	0.09			
phenol-insol. gland.	*ca.* 239		5.5	41	13	0.32	0.18	1.7; 1.8	0.04			
Pooled substances with A and B activity												
Lederle 35P1A		0.96	7.1	51	25	0.49	0.28	6.5	0.13	11.0	A5, B1	5
Lilly			7.0	52	24	0.48	0.27	7.1	0.14		A0.5; B5	
Crude materials (previous to phenol extraction)												
Horse 1			13.6	19	13	0.69	0.07	1.3	0.07			
Horse 2			8.8	47	22	0.47	0.19	5.6	0.12			
Horse 4			11.5	37	16	0.43	0.11	3.7	0.10			
Horse 6 gland.			12.9	21	7	0.32	0.05	1.1	0.05			

* After hydrolysis with 2 N HCl for 2 hours in boiling water bath.

‡ Minimum quantity of substances completely inhibiting hemagglutination of 0.1 ml. of a 4 per cent suspension of A or B erythrocytes by 10 to 20 hemagglutinating units of anti-A or anti-B respectively. The antiserum to horse A and B substances was prepared by injection of human volunteers. The antiserum to human B substance was obtained from a woman of blood group A in whom anti-B had been produced as a consequence of heterospecific pregnancies. The serum was supplied by Dr. John Scudder.

§ These two preparations contained a trace of A activity; *i.e.*, they inhibited isoagglutination of A cells in amounts greater than 100 μg.

TABLE II

The R_F Values of Pure Fucose and Fucose in Concentrated Dialysates of Hydrolyzed Horse Blood Group Substance

Solvent	R_F fucose	R_F horse substance	Reagent
Water-phenol	0.56	0.56; 0.33*	MPD‡
Collidine-water	0.46	0.46	AS§
Butanol-ethanol-water	0.24	0.23	MPD

* It is not known to what this spot corresponds.

‡ MPD, *m*-phenylenediamine reagent.

§ AS, ammoniacal silver reagent.

TABLE III

Cross-Reaction of Horse Blood Group Substances with Type XIV Antipneumococcal Horse Serum and the Effect of Heating at 100°C. for 2 Hours at pH 1.9

Substance	Amount used	Total nitrogen precipitated from 0.5 ml. serum	
		Unheated	Heated 2 hrs. at 100°C.
	μg.	μg.	μg.
Horse 6* glandular 25%	50	2.8	10
	100	3.4	18
	150	3.8	24
	250	9.6	34
	500	14	60
Horse 1* 25%	50	9	18
	100	14	26
	150	18	29
	250	24	44
	500	34	65
Horse 2‡ 25%	50	7	13
	100	9	19
	150	8	23
	250	7	27
	500	5	34

* Serum H635 (1939 bleeding).

‡ Serum H618 (1939 bleeding).

precipitable by 10 per cent ethanol was used for these studies. Analytical data on both of these samples are included in Table I. The methylpentose of the horse blood group substance was identified as fucose by paper chromatography (*cf.* reference 5 and Table II).

Cross-reactions with type XIV antipneumococcal horse serum both before and after heating the blood group substances at 100°C. for 2 hours at pH 1.9 were carried out as previously described (2), and the results are given in Table III.

Human B substances, obtained from the saliva of individuals of blood group B, were prepared as previously described (3). The human B substances are also white powders; their properties are given in Table IV.

All analytical procedures were the same as those previously used (2). Activity measurements were carried out by measuring the minimum quantity of substance capable of inhibiting completely hemagglutination of 0.1 ml. of a 4 per cent suspension of A cells by 10 to 20 hemagglutinating units of anti-A or of a similar quantity of B cells by the same amount of anti-

TABLE IV

Properties of Blood Group Substances Obtained from the Saliva of Human Beings of Blood Group B

Preparation		Yield*	Ash as Na	Total nitrogen	Reducing sugar as glucose‡	Glucosamine‡	Glucosamine / Reducing sugar	Glucosamine N / Total N	Fucose	Fucose / Reducing sugar	Acetyl	Activity§		$[\alpha]_D$
												Antiserum to horse B substance	Antiserum to human B substance	
		mg.	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*		*per cent*	*μg.*	*μg.*	*degrees*
S.A.J.‖	phenol-insol.	118		4.5	66	29	0.44	0.49	17	0.26		5	2	−20
	10%	58	0.3	4.9	60	30	0.50	0.47	12	0.20		10	5	−13
E.J.	phenol-insol.	37		3.3	58	23	0.40	0.54	14	0.24		10	5	−16
	10%	59	0.0	4.6	59	30	0.51	0.50	11	0.19		25	10	−20
J.R.M.	phenol-insol.	109		4.1	60	25	0.42	0.49	16	0.27		2	2	
	10%	19		4.2	61	26	0.43	0.48	15	0.25		10	5	−22
J.E.C.‖	phenol-insol.	60		4.2	50	21	0.42	0.38	16	0.32		5	5	−16
	10%	44		4.7	59	28	0.47	0.47	14	0.24		25	5	−21
P.M.	phenol-insol.	384	0.4	3.3	68	20	0.29	0.49	14	0.21	7.7	2	2	+25
	10%	71		5.3	59	29	0.49	0.43	14	0.24	13.0	5	5-10	−27
S.E.	phenol-insol.	62		3.8	53	19	0.36	0.39	13	0.25		25	10	
	10%	22		5.3	45	23	0.51	0.34	12	0.27		25	10	−42

* Yield per 2 liters of saliva except for S.E. where 1 liter of saliva was used.

‡ After hydrolysis with 2 N HCl for 2 hours in boiling water bath.

§ See footnote to Table I.

‖ Heterozygous BO as determined by blood grouping of parents.

B (*cf.* footnote to Table I). Results in Tables I and IV are directly comparable since they were set up simultaneously or by the use of Lilly and Lederle samples as reference standards.

RESULTS

The data in Table I show that the substances isolated as described above from the glandular portion of individual horse stomach linings may be divided into three serological groups, materials with A activity, materials with B activity, and materials with neither A, B, nor O activity. Some preparations were found with very slight A, B, or AB activity but the titer, 100 to 300 μg., was so low as to make the results doubtful. The absence of O activity was indicated by the absence of inhibition of hemagglutination using goat anti-Shiga serum (9, 10, 2) absorbed with A_1B cells as a source of O antibody. The

goat anti-Shiga serum was supplied by Dr. Arthur F. Coca of Lederle Laboratories.

Chemical analysis of the various samples gave nitrogen values ranging from 4.0 to 8.0 per cent, most of the values being near 7 per cent, reducing sugar from 40 to 54 per cent, glucosamine values from 15 to 29 per cent with most samples showing 23–26 per cent, the fucose from 1.7 to 9.7 per cent, and the acetyl from 8.4 to 11.0 per cent. It will be noted that the phenol-insoluble fractions possess the lowest nitrogen and glucosamine values. The sole active phenol-insoluble sample from horse 1 showed the least A activity of any of the horse 1 fractions in its ability to inhibit isoagglutination. Furthermore, the phenol-insoluble fractions can apparently be eliminated if the materials are dialyzed before completing the peptic digestion, since the four preparations that were dialyzed and redigested, 2, 3 glandular, 4, and 5 non-glandular gave negligible quantities of this fraction.

The non-glandular portions of the horse stomach linings when processed in a manner identical with the glandular portions, yielded relatively small amounts of material and, as would be expected, failure to separate horse stomachs 1, 2, and 4 into glandular and non-glandular portions introduced little, if any, contamination.

Table III shows that the horse blood group substances, as do the human and hog substances (4, 5), precipitate with type XIV antipneumococcus horse serum. When these substances are heated for 2 hours in a boiling water bath at pH 1.9 the cross-reactivity increases, again paralleling the reaction of human and hog blood group substances (5).

By inhibition of isoagglutination, the A substances showed end-points varying from 0.5 μg. for the Lilly preparation to 5 to 25 μg. for certain of the horse 1 fractions using the sera of human beings of blood groups O or B immunized with horse A substance (*cf.* reference 11). While it would appear from these data that the Lilly preparation was more active than the horse 1 substance by inhibition of isoagglutination, nevertheless the horse 1 15 per cent fraction was found to be more active than the Lilly sample in precipitating horse anti-A.[1] This problem is now being studied and the quantitative precipitin data will be recorded in detail shortly.

Preparations from two horses, 2 and 4, possessed B activity and these samples showed end-points of 2 to 5 μg. while the Lederle sample seemed a little more active with an end-point at 1 μg.

The methylpentose of the horse substance has been identified as fucose by the use of paper chromatography in 3 different solvent mixtures, collidine-water, phenol-water, and butanol-ethanol-water (45:5:50). Due to the relatively low fucose content and a substance, not as yet identified, that interferes with the ammoniacal silver reagent, especially in the butanol-ethanol-water

[1] Unpublished data.

solvent, the following procedure was used. Approximately 170 mg. of horse 6 glandular that had accidentally been contaminated with a small amount of horse 1 was dissolved in 17 ml. of water, 17 ml. of 0.1 N HCl added, and the mixture (pH 1.8) heated for 2 hours in a boiling water bath. After cooling, the solution was dialyzed against 6 changes of distilled water, the dialysis bag having first been soaked in 5 changes of distilled water to remove glycerol (5). The dialysate was evaporated to dryness twice (under CO_2 *in vacuo*) and finally made up to a concentration of about 10 mg./ml. of fucose based on a colorimetric analysis by the method of Dische and Shettles (7). Approximately 0.01 ml. of this solution was chromatographed on Whatman No. 1 paper in the 3 solvents, the spots being made visible by means of ammoniacal silver (12) or *m*-phenylenediamine reagent (13). The R_F values are given in Table II.

Table IV shows the properties of the substances prepared from the saliva of individuals of blood group B. The nitrogen varies from 3.3 to 5.3 per cent, the reducing sugar from 45 to 68 per cent, the glucosamine from 19 to 30 per cent, the fucose from 11 to 17 per cent, and the acetyl from 7.7 to 13 per cent. All the human B substances have a negative rotation except one, P. M. phenol-insoluble. The reason for this is unknown. As in the case of the previous human saliva preparations, the materials are divided into phenol-soluble and phenol-insoluble fractions. The factors which determine phenol solubility do not seem to affect the analytical properties determined (Table IV). The quantity of human B substance necessary to inhibit isoagglutination varies from 2 to 25 μg. when tested against anti-horse B serum and from 2 to 10 μg. when titered against anti-human B serum. The inhibition of isoagglutination test also seems to reveal a difference between the 10 per cent precipitates and the phenol-insoluble fractions. In all cases except one the 10 per cent precipitate is the less active fraction while in the one case of S. E. they are equal when using the anti-horse serum; with anti-human serum two samples, S. E. and J. E. C., are equal and for the remainder the phenol-insoluble fractions are more active.

Data on the precipitation of human B substance with type XIV antipneumococcus horse serum before and after heating have already been recorded (5).

The purification that phenol extraction achieves is demonstrated by the data at the bottom of Table I for the crude materials after peptic digestion and alcohol precipitation as compared with the purified preparations. Horse 2 was in a fairly pure condition even before phenol treatment while the other substances were grossly impure, nitrogen values being very high, about 9 to 14 per cent as compared to 4 to 8 per cent for the purified substances, glucosamine ranged from 7 to 22 per cent in comparison with 23 to 26 per cent for purified substances, etc. It would appear therefore, that phenol extraction followed by fractional alcohol precipitation removed material high in nitrogen and low in glucosamine and reducing sugar, probably protein.

DISCUSSION

The blood group B substance isolated from human saliva possesses the same analytical composition as the human A and O substances from saliva (3, 14) and the substance isolated from the saliva of a non-secretor of blood group A (15). It may be pointed out that, since no data were given, the non-secretor could have been heterozygous AO and possibly was a non-secretor of A substance but a secretor of O or of Lewis (20) substance. Were this the case, the substance isolated would have been O or Lewis (20) substance and would be expected to have the same composition as the other saliva materials.

The glandular portion of horse stomachs, however, yields materials of somewhat different composition from those isolated from other sources. The horse substances tend to have lower hexosamine and reducing sugar and higher total nitrogen and higher non-hexosamine nitrogen than human, hog, or cow materials. The substance prepared from horse saliva by Landsteiner (16) has analytical values similar to those from horse stomach. This may indicate either a higher amino acid content or the presence of some other nitrogen-containing compound. The presence of a relatively low fucose content points toward a similarity to the cow substances (17). However, as with the blood group substances from other species (5), the fucose exists as end groups (*cf.* reference 18) as evidenced by the ease with which it is split off by heating at pH 1.9 (*cf.* reference 19). The consequent increase in the cross-reactivity with type XIV antipneumococcus horse serum after heating shows that the horse substance is similar in at least some structural details to the hog and human substances; *i.e.*, they all have a straight or branched chain of galactose-N-acetyl glucosamine with fucose end groups at various points. There is, nevertheless, one striking difference between hog, human, and horse substances. Certain horse stomachs possess a substance whose analytical composition is similar to that of other horse stomach materials but which shows neither blood group A, B, or O activity by hemagglutination inhibition tests. Some cow stomachs also yielded such materials (17).

All the substances isolated do not have an identical composition but instead show a range of analytical values well beyond the errors in the analytical procedures. This is not unexpected due to the complexity of the starting materials. The materials of hog origin exhibit a relatively small range of values except for fucose, while the human B as well as the A substances (3) display an unexpectedly large range of values for reasons that have not as yet been completely determined. The horse substances also show a relatively wide range of values, nitrogen varying from 4.0 to 8.0 per cent, hexosamine from 15 to 29 per cent, etc. In the case of the horse substances careful fractionation by alcohol precipitation from phenol can, apparently, lead to more homogeneous material. For instance, the lowest and highest values for hexosamine, the lowest and highest values for reducing sugar after hydrolysis,

and the highest value for total nitrogen are found in the 15 per cent and 25 per cent precipitates (Table I). On the other hand the material obtained by refractionation of the 15 per cent precipitate at alcohol concentrations of 15 per cent and 25 per cent, gives 25 per cent precipitates showing a relatively smaller range of analytical values, total nitrogen varying from 6.1 to 7.1 per cent, glucosamine from 23 to 26 per cent, reducing sugar from 42 to 51 per cent. By the use of quantitative precipitin data, which will be reported in a future publication, it may be possible to determine the relationship between blood group activity and the degree of fractionation.

Serologically, the A and B substances isolated from individual horse stomachs did not show as high activity by inhibition of isoagglutination as did the commercial samples from pools of horse stomachs. This, in addition to the finding of an inactive substance in other stomachs would suggest that some horse stomachs probably have considerably greater A or B activities. Further quantitative immunochemical studies are required to establish whether the substances isolated from individual horse stomachs are mixtures with inactive substance as might be expected from genetic considerations (*cf.* reference 2).

It is interesting to note in carrying out the inhibition of isoagglutination with horse and human substances using homologous and heterologous antibody that with both horse anti-B and human anti-B complete inhibition is obtained with somewhat less homologous antigen than with the heterologous antigen. Relative assays of agglutination inhibition in Tables I and IV are comparable since they were determined for both human and horse B substances by reference to the Lederle and Lilly samples as standards. As shown in Tables I and IV, column 12, the difference between homologous and heterologous antigens is small but consistent. This indicates that the B substances of different species are similar but not identical, a conclusion that had previously been reached with the hog and human A substances (3) and with horse and cow B substances (17).

SUMMARY

Blood group substances have been isolated from the saliva of human beings of blood group B and from the linings of individual horse stomachs. The properties of the human B substances are similar to those of hog and human blood group substances previously isolated. The horse substances showed lower hexosamine and reducing sugar and higher total and non-hexosamine nitrogen than do the materials from the other species. Materials isolated from individual horse stomachs possess either A or B activity or both. Certain stomachs yielded products of identical analytical composition but with neither blood group A, B, or O activity as measured by their ability to inhibit isoagglutination. Fucose has been identified as a constituent of the horse blood group substances.

BIBLIOGRAPHY

1. Morgan, W. T. J., and King, H. K., *Biochem. J.*, 1943, **37**, 640.
2. Bendich, A., Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83**, 485; *J. Am. Chem. Soc.*, 1947, **69**, 2163.
3. Kabat, E. A., Bendich, A., Bezer, A. E., and Beiser, S. M., *J. Exp. Med.*, 1947, **85**, 685.
4. Kabat, E. A., Bendich, A., Bezer, A. E., and Knaub, V., *J. Exp. Med.*, 1948, **87**, 295.
5. Kabat, E. A., Baer, H., Bezer, A. E., and Knaub, V., *J. Exp. Med.*, 1948, **88**, 43.
6. Kazal, L. A., Higashi, A., Brahinsky, R., DeYoung, M., and Snow, L. E., *Arch. Biochem.*, 1947, **13**, 329.
7. Dische, Z., and Shettles, L. B., *J. Biol. Chem.*, 1948, **175**, 595.
8. Witebsky, E., and Klendshoj, N. C., *J. Exp. Med.*, 1940, **72**, 663.
9. Eisler, M., *Z. Immunitätsforsch.*, 1930, **67**, 38.
10. Landsteiner, K., and Levine, P., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 309.
11. Witebsky, E., Klendshoj, N. C., and McNeil, C., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 167.
12. Partridge, S. M., *Biochem. J.*, 1948, **48**, 251.
13. Chargaff, E., Levine, C., and Green, C., *J. Biol. Chem.*, 1948, **175**, 67.
14. Landsteiner, K., and Harte, R. A., *J. Biol. Chem.*, 1941, **140**, 673.
15. Harte, R. A., *J. Biol. Chem.*, 1947, **167**, 873.
16. Landsteiner, K., *J. Exp. Med.*, 1936, **63**, 185.
17. Beiser, S. M., and Kabat, E. A., *J. Am. Chem. Soc.*, 1949, **71**, 2274.
18. Bray, H. G., Henry, H., and Stacey, M., *Biochem. J.*, 1946, **40**, 124.
19. Aminoff, D., Morgan, W. T. J., and Watkins, W. M., *Biochem. J.*, 1948, **43**, xxxvi (abstract).
20. Grubb, R., and Morgan, W. T. J., *Brit. J. Exp. Path.*, 1949, **30**, 198.