

## SEROLOGIC METHOD OF PURIFYING BLOOD GROUP A SUBSTANCE

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THE USE OF blood group A and B substance for the "conditioning"<sup>12</sup> of group O blood for transfusion (i.e., partially neutralizing the anti-A and anti-B agglutinins in the group O plasma) has today acquired considerable importance, and would probably continue to be used in time of war.<sup>18</sup> The A substance used for this purpose has been prepared from hog stomach linings, and the B substance from horse stomachs (this B substance having also some "A" activity). Representative methods of preparation are described by Kabat.<sup>10</sup>

Regarding any material to be added to blood before it is used for transfusion, the question of purity is, of course, important. When the use of these A and B substances was first introduced, little was known about their chemical composition, although information suggesting their safety was available. And, in fact, the work of Kabat<sup>1, 9</sup> has since shown that the early materials were certainly mixtures, containing some active and some inactive material. It was felt at the time that the preparation of a material known to be 100 per cent pure, even in small amounts, would be worth while, as it would provide a standard of potency with which materials offered for large scale use could be compared.

It is, of course, possible that chemical methods alone could yield material which would be completely pure and 100 per cent reactive, and some of Kabat's later work suggests that he has come very close to achieving this aim. Nevertheless, it seemed desirable to test an entirely different method of preparation, one in which the method of purification was primarily serologic. The present communication deals with the results obtained by application of this method.

It has long been known<sup>15</sup> that it is often possible, by injecting rabbits with human erythrocytes of blood group A, to obtain precipitating antibodies for A substance. It was shown<sup>3, 10</sup> that such antibodies would precipitate the A substance prepared from hog stomachs. It is obvious that this affords a delicate and specific method of separating serologically active A substance from inactive carbohydrates which are present and which are similar in their chemical properties. If the precipitate which results when a crude preparation of group A substance and a precipitating antibody produced by the injection of human group A erythrocytes is washed thoroughly with saline, it will contain only serologically active A substance, anti-A precipitin (a modified rabbit globulin), and possibly traces of lipids and various components of complement.<sup>8</sup> Removal of the antibody should leave a group A substance of a high degree of purity.

## METHODS

1. *Production of antisera.* Rabbits were injected three times weekly with one cc. of a 30 per cent suspension in saline of washed human erythrocytes of group A. To avoid possible variations due to indi-

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vidual differences in blood group antigens, the cells of a single individual (WCB) were always employed. Bleedings showed that 15 out of 20 of the animals had produced group-specific precipitins within five weeks after the injections were started. The preliminary tests were done by the interfacial technic, using crude A substance. The pooled active sera were assayed for antibody content by the method of optimal proportions.<sup>5</sup> The results indicated a content of antibody nitrogen varying from 0.2 to 1.0 mg. per cc.

2. *Source of A substance.* The A substance used was a preparation of the Eli Lilly Company, designated as intended for laboratory work only (Lot numbers R 15 and 330). Analytical data for the latter preparation are given by Kabat.<sup>9</sup>

3. *Preparation of specific precipitates.* After determination of the optimal proportions ratio of a given pool of serum (previously filtered), a solution of A substance containing the calculated optimal amount, plus a 10 per cent excess, was added. The mixture was allowed to stand in the icebox overnight, and the precipitate centrifuged off and washed until the washings were free from protein, as judged by the absence of opalescence when saturated with picric acid. From three to five washings were usually required.

4. *Treatment to remove the antibody.* Various treatments designed to remove the antibody from the antibody-antigen complex were tried, using a total of 18 samples of crude blood group A substance totalling 362.5 mg. They included (a) digestion with trypsin, (b) digestion with chymotrypsin, (c)

TABLE 1.—*Typical Inhibition Test. Comparison of activity of Lilly 1 per cent A Substance and Papain plus phenol treated A-Anti-A Precipitate*

Results of tests with A<sub>1</sub> cells

Anti-A iso immune serum plus	Saline control	Dilution of solution of A substance						
		Undil.	1:10	1:10 <sup>2</sup>	1:10 <sup>3</sup>	1:10 <sup>4</sup>	1:10 <sup>5</sup>	1:10 <sup>6</sup>
1% A substance . . . . .	+4	—	—	—	—	—	+1	+2
0.1% treated A-Anti-A precipitate . . . . .	+4	—	—	—	+1	+2	+2	+3

The symbol — indicates a negative reaction, +1, +2, etc., indicates positive reactions of different strength, +4 being complete (solid) agglutination.

digestion with papain activated with cysteine hydrochloride,<sup>11</sup> (d) treatment in the Waring Blendor with chloroform and amyl alcohol,<sup>17</sup> (e) treatment at room temperature with 90 per cent phenol,<sup>13</sup> (f) treatment at 100 C with 90 per cent phenol, (g) treatment with 0.25 N trichloroacetic acid,<sup>2</sup> (h) subjection to pressures of 9000 atmospheres<sup>1</sup> for twenty-four hours, (i) digestion with papain followed by treatment with half-saturated ammonium sulfate or by treatment with 22.2 per cent sodium sulfate, or by treatment with trichloroacetic acid, or by treatment with 90 per cent phenol, (j) denaturation by heating to 100 C. for one hour, followed by papain digestion, (k) heating to 55 C. in ethylene glycol.

After treatment by the above methods, the material was precipitated by the addition of an excess of alcohol, centrifuged, the soluble material taken up in saline or water, reprecipitated, and redissolved in saline. The solution was then tested for A activity by the inhibition technic,<sup>16</sup> using the original material for a control (see table 1).

## RESULTS

None of these methods, unfortunately, yielded a product which was any more active than the starting material and at the same time completely soluble in saline. Methods (i) (papain followed by phenol) and (h) (high pressures) were, on the whole, the most successful in denaturing the antibody and releasing an active antigen. Nevertheless, the resulting products were never more active than the starting material, and were often less soluble in saline. The phenol method (e) uniformly gave products insoluble in saline, which were, moreover, only 1/100 as active as the starting material. Method (i) (papain followed by phenol) gave a

product which was soluble with some difficulty, but only 1/10 as active as the starting material. The other methods were even less successful, for the resulting products were either inactive or insoluble or both.

#### DISCUSSION AND CONCLUSIONS

At the time this work was begun, no estimate was possible of the percentage of the crude A substance which was specifically active. It was provisionally (and, as it proved, incorrectly) estimated that the active material did not amount to over 5 per cent at the most. If this had been correct, it is likely that one or more of the above methods would have resulted in a significant degree of purification. From Kabat's<sup>9</sup> later results, however, it is now apparent that over half of the material was serologically active, and consequently that no great degree of purification, from the serologic point of view, remained to be accomplished. Kabat's work also suggests that chemical methods have been equal to the task of producing material nearly or perhaps quite serologically pure.

The decreased solubility of the A substance after it had been precipitated with antibody and subjected to the above treatments could possibly be explained by the assumption that some of the polar groups of the A substance remained in combination with fragments, of undetermined size, of the antibody, since none of the treatments rendered it less soluble, when applied to solutions of A substance directly.

It would seem that none of the above methods offer an ideal solution to the problem of completely eliminating the antibody from a compound of antibody and antigen, even though the blood group A substance, used in these experiments, is chemically much more stable than most antigens. The converse problem, of removing *some* relatively pure antibody from an antibody-antigen compound, leaving some insoluble antibody-antigen compound to be discarded, is obviously much easier, and has been solved for several systems by various workers.<sup>6, 7, 11, 14</sup>

#### REFERENCES

- <sup>1</sup> BENDICH, A., KABAT, E. A., AND BEZER, A. E.: Immunochemical studies on blood groups. III. *J. Exp. Med.* 83: 485-497, 1946.
- <sup>2</sup> BOIVIN, A., MESROBEANU, I., AND MESROBEANU, L.: Technique pour la préparation des polysaccharides microbiens spécifiques. *Compt. rend. soc. de Biol.* 113: 490-492, 1933.
- <sup>3</sup> BOYD, W. C., AND WARSHAVER, E. R.: Serological relationship of blood group A and B antigens; production of anti-B precipitins. *J. Immunol.* 50: 101-106, 1945.
- <sup>4</sup> BRIDGMAN, P. W.: *The Physics of High Pressure*. London, G. Bell & Sons, Ltd., 1931.
- <sup>5</sup> DEAN, H. R., AND WEBB, R. A.: The influence of optimal proportions of antigen and antibody in the serum precipitation. *J. Path. & Bact.* 29: 473-492, 1926.
- <sup>6</sup> FELTON, L. D.: Dissociation of the specific protein precipitate of antipneumococcus horse serum, and a comparison with a protein isolated by chemical means from this immune serum. *J. Immunol.* 22: 453-467, 1932.
- <sup>7</sup> HEIDELBERGER, M., AND KENDALL, F. E.: Quantitative studies on antibody purification. I. *J. Exp. Med.* 64: 161-172, 1936.
- <sup>8</sup> —, AND MAYER, M.: Quantitative studies on complement or alexin. IV. *J. Exp. Med.* 75: 285-295, 1942.
- <sup>9</sup> KABAT, E. A., BENDICH, A., AND BEZER, A. E.: Immunochemical studies on blood groups. II. *J. Exp. Med.* 83: 477-484, 1946.

- <sup>10</sup> KABAT, E. A., AND BEZER, A. E.: Immunochemical studies on blood groups. I. *J. Exp. Med.* 82: 207-215, 1945.
- <sup>11</sup> KALMANSON, G. M., AND BRONFENBRENNER, J.: Restoration of activity of neutralized biologic agents by removal of the antibody with papain. *J. Immunol.* 47: 387-407, 1943.
- <sup>12</sup> KLENDSHOJ, N. C., AND WITEBSKY, E.: Transfusion of O blood conditioned by the addition of blood group specific substances. *J. A. M. A.* 128: 1091-1093, 1945.
- <sup>13</sup> MORGAN, W. T. J., AND KING, H. K.: Studies in immunochemistry. 7. *Biochem. J.* 37: 640-651, 1943.
- <sup>14</sup> NORTHROP, J. H.: Purification and crystallization of diphtheria antitoxin. *J. Gen. Physiol.* 25: 465-485, 1942.
- <sup>15</sup> SCHIFF, F.: Über gruppenspezifische serumpräcipitine. *Klin. Wchnschr.* 3: 679-680, 1924.
- <sup>16</sup> —, AND BOYD, W. C.: *Blood Grouping Technic*. New York, Interscience Publishers, Inc., 1942.
- <sup>17</sup> SEVAG, M. G., LACKMAN, D. B., AND SMOLENS, J.: The isolation of the components of streptococcal nucleoproteins in serologically active form. *J. Biol. Chem.* 124: 425-436, 1938.
- <sup>18</sup> TISDALL, L. H., GARLAND, D. M., AND WIENER, A. S.: A critical analysis of the value of the addition of A and B group-specific substances to group O blood for use as universal donor blood. *J. Lab. & Clin. Med.* 31: 437-443, 1946.