FRACTIONATION OF HUMAN IMMUNE γ-GLOBULIN

By JOHN R. CANN, RAYMOND A. BROWN,* AND JOHN G. KIRKWOOD

(From the Gates and Crellin Laboratories of Chemistry,† California Institute of Technology, Pasadena)

AND JOHN H. HINK, JR.

(From the Cutter Laboratories, Berkeley, California)

(Received for publication, February 18, 1950)

Equine and bovine serum proteins have recently been fractionated by means of a physical method utilizing an electrophoretic adaptation of the principles of the Clusius column (1–4), first described and tested by Kirkwood (5) and Nielsen (6). The method of electrophoresis-convection has now been applied to the fractionation of human γ-globulin. The γ-globulin was prepared by ethanol fractionation (7) from the plasma of individuals hyperimmunized to *Hemophilus pertussis* organisms. The resulting fractions of γ-globulin have been characterized electrophoretically, and the protective antibody activity and agglutinin titer have been measured.

The material used in this investigation was Fraction II of pooled immune human plasma prepared by the Cutter Laboratories from hyperimmunized donors. The apparatus and experimental procedures used in the fractionations reported here were essentially the same as those described for the fractionation of bovine γ-globulin (3, 4). Fractionations were carried out in phosphate buffer, ionic strength 0.1, at a field strength of 1.7 to 2.0 volt per cm. for 48 to 71 hours. The initial protein concentration was 2.0 to 2.8 gm. of protein per 100 ml. Fractions possessing different mean mobilities and isoelectric points were obtained by proper choice of the operating pH.

The agglutinin titers of the fractions were obtained by the Flosdorf method (8) with two different antigen strains. Protective activities of the fractions were determined by mouse protection tests. In these tests, the determinations of the virulence of the challenge and the protective activity of the fractions were carried out simultaneously in order to rule out any change in virulence of the culture. Each of the fractions was injected subcutaneously at doses of 10, 2, and 0.4 mg., with fifteen mice at each level. 6 hours following immunization, a challenge dose of virulent *H. pertussis* organisms was administered to each mouse by intracerebral injection. The virulence of the challenge was determined on forty-eight

* Postdoctorate Fellow of the United States Public Health Service, National Institutes of Health.

† Contribution No. 1394.

mice, twelve mice being tested for each of four dilutions. Death and survivals were recorded after 10 days, those mice substantially paralyzed on the 10th day being considered as dead. The results were analyzed by the method of Reed and Muench (9).

A sample of γ-globulin obtained from the plasma of donors hyperimmunized to *H. pertussis* organisms was carried through three stages of fractionation. The scheme used in this fractionation may be briefly described as follows. In the first stage the γ-globulin was fractionated at pH 8.10. The fractions withdrawn from the top and bottom reservoirs of

TABLE I

Fractionation of Fraction II, Sample 1

Fraction	Per cent of original γ-globulin	Mean mobility,* $-10^5 \times \bar{u}$	Mean isoelectric point†	$-10^5 \times \frac{\Delta\bar{u}}{\Delta pH}$	Protective activity,‡ ID_{25} per mg. N	Agglutinin titer§ against	
						Strain P26A	Strain P39A
		cm.² sec.⁻¹ volt⁻¹	*pH*	*cm.² sec.⁻¹ volt⁻¹ (pH unit)⁻¹*			
Unfractionated γ-globulin		1.27	7.55	0.78	1.4 (1.8)	1:1280	1:1280
Top 1	24	0.88	7.86	1.17	5.0 (2.7)	1:1280	1:1280
" 2	27	1.23	7.47	1.00	1.3	1:640	1:1280
" 3	17	1.60	6.96	0.55	2.1	1:640	1:640
Bottom 3	32	1.44	7.28	1.32	1.9	1:640	1:640

* The mean mobilities were determined in barbital buffer, pH 8.6, ionic strength 0.1, and protein concentration 1 per cent.

† The mean isoelectric points were determined in cacodylate-chloride buffer, ionic strength 0.1, and protein concentration 0.5 per cent.

‡ The challenge dose was 100,000 organisms or 250 LD_{50}. The values in parentheses are results of check assays with 100,000 organisms or 910 LD_{50}.

§ The titers are expressed as the titers of 2 per cent solutions of the fractions.

the electrophoresis-convection apparatus are designated as Top 1 and Bottom 1, respectively. Bottom 1 was then refractionated in the second stage at pH 7.78, the resulting fractions being designated as Top 2 and Bottom 2. In the last stage of fractionation, carried out at pH 6.50, Bottom 2 was separated into two fractions, Top 3 and Bottom 3. The electrophoretic and immunological data obtained on the resulting fractions are presented in Table I, where $\bar{u}$ is the mean mobility at pH 8.6 and $\Delta\bar{u}/\Delta pH$ the slope of the mobility-pH curve in the neighborhood of the mean isoelectric point. This fractionation yielded four electrophoretically unique fractions of γ-globulin. These fractions possess a mean mobility spectrum ranging from -0.88×10^{-5} to -1.60×10^{-5} cm.² sec.$^{-1}$ volt^{-1} at pH 8.6 in barbital buffer and a mean isoelectric point spectrum from 7.86 to 6.96 in cacodylate-chloride buffer.

The results of the assays for protective antibody show that, although all of the fractions possess protective antibody, more protective activity is associated with the fraction of lowest mobility, Top 1, than with the other fractions and the unfractionated γ-globulin. The conclusion that Top 1 possesses more protective activity than the unfractionated material

TABLE II

Fractionation of Fraction II, Sample 2; Electrophoretic and Immunological Properties of Fractions

Fraction	Per cent of Top 1	Mean mobility,* $-10^5 \times \bar{u}$	Mean isoelectric point†	$10^5 \times \beta$‡	Protective activity,§ ID_{50} per mg. N
		cm.² sec.⁻¹ volt⁻¹	*pH*	*cm.² sec.⁻¹ volt⁻¹*	
Unfractionated γ-globulin...		1.26	6.90	0.56	1.3
Top 1.....		0.96	7.15		2.1
Bottom 1.....		1.37			
Top 4.....	10	1.28			<0.9
" 5.....	21	1.02	7.28		1.6
" 6.....	23	0.96	7.36	0.53	2.0
" 7.....	17	0.87			2.8
Bottom 7.....	29	0.91	7.46		2.1

* Mean mobilities were determined in barbital buffer, pH 8.7, ionic strength 0.1, and protein concentration 1 per cent.

† The mean isoelectric points were determined in tris(hydroxymethyl)aminomethane buffers, ionic strength 0.1 and protein concentration 0.5 per cent. These buffers were 0.02 N in the hydrochloride of the amine and 0.08 N in sodium chloride. Discrepancies of the order of 0.6 pH unit between isoelectric points determined in cacodylate and tris(hydroxymethyl)aminomethane buffers have been found. These discrepancies are at least in part due to the different temperature coefficients of the pH of these buffers. pH values were measured at 25°, while the isoelectric points were determined at 2°.

‡ The standard deviations of the mobility distributions were determined from reversible boundary spreading experiments. These experiments were carried out in tris(hydroxymethyl)aminomethane buffer at the mean isoelectric points of the proteins.

§ The challenge dose was 100,000 organisms or 690 LD_{50}.

is supported by the results of two independent assays for protective antibody. All four fractions gave nearly the same agglutinin titer against two different antigen strains.

In order to accomplish still further enrichment of protective antibody, a five stage fractionation of a different sample of γ-globulin from hyperimmunized donors was carried out. The results of the electrophoretic and immunological characterization of the various fractions are presented in Table II, where $\bar{u}$ is the mean mobility at pH 8.7 and β the standard

deviation of the mobility distribution in the neighborhood of the mean isoelectric point.

The first stage of fractionation was carried out under the same experimental conditions as were employed in the initial stage of the first fractionation. A composite of the top fractions obtained in ten separate experiments was made. Electrophoretically, this composite resembles Top 1 obtained in the first fractionation as closely as can be expected, considering possible differences in the two samples of unfractionated γ-globulin.[1] Accordingly, this composite has also been designated as Top 1. The immunological assay of the composite Top 1 confirms the previous finding that more protective activity is associated with the low mobility fraction of γ-globulin than with the unfractionated material.

Subsequent stages of fractionation were designed to separate Top 1 into subfractions. The composite Top 1 was fractionated at pH 6.38, the resulting fractions being designated as Top 4 and Bottom 4. Bottom 4 was then fractionated at pH 7.10 to yield Top 5 and Bottom 5. Bottom 5 in turn was fractionated at pH 7.63 to yield Top 6 and Bottom 6. Finally, Bottom 6 was separated into two fractions, Top 7 and Bottom 7, by fractionation at pH 8.00. The mobilities of the resulting subfractions range from -0.87×10^{-5} to 1.28×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.7 in barbital buffer.[2] In agreement with the results of the first fractionation, the immunological characterization of these subfractions revealed that greater protective activity is associated with low mobility fractions than with high mobility fractions.

The rather broad distribution of protective antibody among the fractions may in part be due to the fact that the γ-globulin was prepared from pooled plasma. Of course, there is some question as to whether the protective antibody, if derived from a single donor, might not also exhibit a broad distribution. Several factors might give rise to such a heterogeneity. The first of these is the fact that *H. pertussis* immunity is probably given by several different strains of bacteria. Also, it is conceivable that both bacterial- and toxin-produced antibodies are responsible for immunity.

[1] The fact that the two samples of γ-globulin possessed the same mean mobility at pH 8.6 does not of course imply that they possessed the same distribution of mobilities.

[2] It is of interest that little or no apparent separation was accomplished in the last two stages of fractionation, despite the fact that the operating pH of successive stages differed by at least 0.4 pH unit. This is somewhat difficult to understand, especially in view of the large amount of transport obtained in these runs and the fact that the standard deviation of the mobility distribution of Top 6 was found to be the same as that of the unfractionated γ-globulin.

SUMMARY

A sample of human antipertussis γ-globulin has been separated into four electrophoretically unique fractions by electrophoresis-convection. These fractions possess a mean mobility spectrum ranging from -0.88×10^{-5} to -1.60×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.6 in barbital buffer and a mean isoelectric point spectrum from 7.86 to 6.96 in cacodylate-chloride buffer. All of the fractions gave nearly the same agglutinin titer against two different antigen strains. Although all fractions showed protective activity, the greatest activity was associated with the fraction of lowest mobility. This was confirmed by the fractionation of a second sample of immune γ-globulin and subsequent subfractionation of the low mobility material.

The authors are indebted to Dr. Dan Campbell for helpful discussion of the immunological significance of the results.

Addendum—Since preparation of this manuscript, Hink and Johnson (10) have reported their studies on the distribution of antibodies among plasma fractions prepared by ethanol fractionation (7) from the plasma of individuals hyperimmunized to *H. pertussis* organisms. Their results indicate that the protective activity in antipertussis hyperimmunized human plasma occurs almost entirely in the purified γ_2-globulin, Fraction II. Less than half of the *H. pertussis* agglutinins was recovered in Fraction II, which contained 90 per cent of the γ_2-globulin of the plasma. The remaining agglutinin activity was found in Fraction III-1. Their Fraction II was the material used in this investigation.

BIBLIOGRAPHY

1. Cann, J. R., Kirkwood, J. G., Brown, R. A., and Plescia, O. J., *J. Am. Chem Soc.*, **71**, 1603 (1949).
2. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **71**, 1609 (1949).
3. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **71**, 2687 (1949).
4. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Biol. Chem.*, **181**, 161 (1949).
5. Kirkwood, J. G., *J. Chem. Phys.*, **9**, 878 (1941).
6. Nielsen, L. E., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **68**, 181 (1946).
7. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., *J. Am. Chem. Soc.*, **71**, 541 (1949).
8. Flosdorf, E. W., McGuinness, A. C., Kimball, A. C., and Armstrong, J. G., *J. Pediat.*, **19**, 638 (1941).
9. Reed, L. J., and Muench, H., *Am. J. Hyg.*, **27**, 493 (1938).
10. Hink, J. H., Jr., and Johnson, F. F., *J. Immunol.*, **64**, 39 (1950).