# ISOELECTRIC FOCUSING AND IMMUNOCHEMICAL ANALYSIS OF PROTEINS OF THE "DIAFERM-3" ANTITETANUS SERUM

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The isoelectric points (PIs) of proteins present in the "Diaferm-3" antitetanus serum, a mixture of partially purified $[F'ab]_2$-fragments of native molecules of antitetanus immunoglobulins, their antitoxic activity, and their immunochemical properties were investigated by isoelectric focusing in a pH gradient of ampholines. The PI values and electrophoretic mobility of the preparation were compared. Proteins with antitoxic activity were shown to have PI values within the range 9.1-5.15, and from their electrophoretic mobility they can be classed as $\gamma$G- and $\beta$-globulins. No antitoxic activity was found in macroglobulins with the mobility of $\alpha$-globulins.

KEY WORDS: antitetanus serum; isoelectric focusing of proteins; immunoglobulins.

Proteins of "Diaferm-3" antitoxic sera are the $[F'ab]_2$-fragments of native globulin molecules obtained by peptic hydrolysis. Since the difference between the specificities of the original globulins and their fragments are due to differences in the composition of the protein molecule, the investigation of the charge on the protein molecule is particularly important. Investigation of the charge on proteins of commercial preparations may also be useful from the practical point of view in order to establish the theoretical basis for methods of purifying preparations to remove inactive fractions. The object of the present investigation was to study the isoelectric focusing in a pH gradient of ampholines [19]. The LKB (Sweden) apparatus was used to determine the isoelectric focusing of proteins of the "Diaferm-3" antitetanus serum. The following solutions were used to create a linear gradient in the column: 1.5 ml 40% ampholine + 49 ml $H_2O$ + serum (50 or 100 mg protein) and 1.5 ml 40% of ampholine + 34 ml $H_2O$ + 23 g sucrose. The space around the cathode was filled with 1.95 M sucrose solution in 1% NaOH, and that near the anode with 1% $H_2SO_4$ solution. Absorption was recorded by the Uvicord-11 instrument at 280 nm. The pH values in the samples (2 ml) were measured on the LPU-01 pH-meter.

The immunological analysis of the fractions was carried out by double diffusion and immunoelectrophoresis in agar [1, 4], using rabbit serum against horse serum proteins and tetanus toxin purified on Sephadex G-100; the latter was also used as a standard for the mobility of $\beta$-globulin [13, 21]. The relative electrophoretic mobility was determined by electrophoresis in agar. The mobility of the components was calculated relative to the mobility of bovine albumin and high polymer dextran. Antitoxic activity was determined by titration in mice.

## EXPERIMENTAL RESULTS

Investigation of PI of the "Diaferm-3" serum proteins began with isoelectric focusing in a wide range of ampholine pH values. As Fig. 1 shows, the serum is a mixture of proteins with PI values from 3 to 10.

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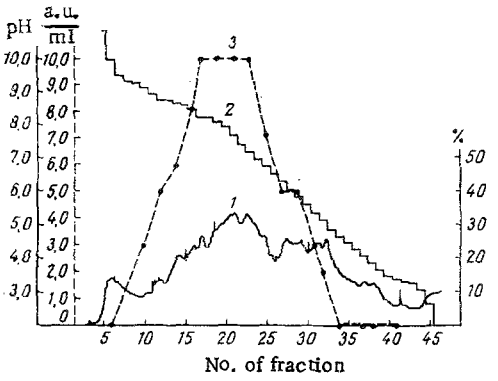


Fig. 1

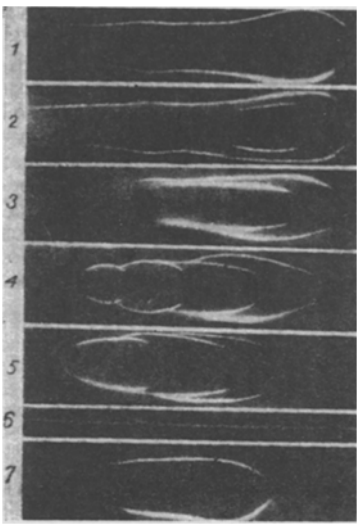

Fig. 2

Fig. 1. Fractionation of "Diaferm-3" antitetanus serum by isoelectric focusing at pH 3-10: 1) absorption of eluate at 280 nm (in %); 2) pH values; 3) results of determination of antitoxic activity (in a.u./ml).

Fig. 2. Immunoelectrophoresis of fractions obtained by isoelectric focusing at pH 3-10. Wells contain fractions with the following PI values: 1) 8.45 ("slow" γG-globulins); 2) 7.65 ("slow" and "medium" γG-globulins); 3) 6.75 ("medium" and "fast" γG-globulins); 4) 5.85 ("fast" γG-globulins, β-, $\alpha_1$-, and $\alpha_2$- globulins); 5) 5.35 (β-, $\alpha_1$-, and $\alpha_2$-globulins); 6) pyronine (30 mm); 7) tetanus toxin (mobility of β-globulins). Gutters contain rabbit antihorse serum to reveal fractions and "Diaferm-3" antitetanus serum to reveal tetanus toxin.

The absorption curve divides into three sectors: the first in the pH range 9.25-10.0, the second with many small peaks in the range 6.5-9.1, and the third between 3.0 and 6.3. Proteins of the second and third sectors present antitoxic activity, although by far the greater part of the activity (80%) belonged to proteins of the second segment.

The immunochemical characteristics of proteins with different PI values were studied by immunoelectrophoresis (Fig. 2, Table 1). The most acid proteins possessed the highest electrophoretic mobility. Since proteins with the mobility of γG-globulins gave three arcs on immunoelectrophoresis at different distances from the well (Fig. 2), it was concluded that this fraction consists of three groups of proteins differing in mobility. These proteins were conventionally called "slow," "medium," and "fast" γG-globulins (Table 1). The precipitation test with tetanus toxin gave only proteins with the mobility of γG- and β-globulins.

The antitoxic activity of the electrophoretic fractions differed appreciably. The activity of the "slow" γG-globulins was 11-17 a.u./mg protein, while that of the "medium" and "fast" γG-globulins was 8-9 a.u./mg protein, and that of the β-globulins 3-8 a.u./mg protein. The antitoxic activity of the $\alpha_1$- and $\alpha_2$-globulins was particularly low (0-5 a.u./mg protein). This small activity itself might have been due to the presence of β-globulins as impurities, for globulin fractions uncontaminated with β-globulins showed no antitoxic activity.

To separate the "Diaferm-3" serum proteins more accurately, narrow ampholine pH zones were used (8-10, 7-9, 6-8, 5-7, 4-6, 3-5). In this way it was possible to achieve better fractionation of the components. For example, on fractionation within the pH range 8-10 (Fig. 3A) three clearly distinct peaks were obtained. Correlation between the absorption and antitoxic activity curves will be apparent from Fig. 3A, but in their electrophoretic mobility the proteins of these peaks belonged to the same group – to the "slow," γG-globulins. Fractions with PI values from 3 to 6 possessed the greatest heterogeneity. The specific antitoxic activity of the γG- and β-globulins was increased, whereas the α-globulins (without β-globulins as impurities) possessed no antitoxic activity. It will be clear from Fig. 3B that proteins with PI values

TABLE 1. PI and Relative Electrophoretic Mobility of Some Serum Proteins

| "Diaferm-3"* | | | Human plasma | | | Horse serum |
|---|---|---|---|---|---|---|
| proteins with mobility of globulins | PI | relative mobility | globulins | PI [17]† | relative mobility [9] | PI |
| γG: | | | | | | |
| slow | 9,96—7,65 | 0,064±0,009 | $\gamma_2$ | 8,0—6,5 | | 7,6 [10], 6,8‡ [22] |
| medium | 7,65—6,5 | 0,185±0,026 | | | 0,170 | 6,1‡ [22], 5,6 [10] |
| fast | 7,20—5,85 | 0,310±0,017 | $\gamma_1$ | 6,0; 5,0 | | 5,57† [12] |
| β | 5,85—5,15 | 0,390—0,022 | β | 5,8; 5,4; 4,2 | 0,474 | 5,1 [16] |
| $\alpha_2$ | 5,85—4,6 | 0,563±0,033 | $\alpha_2$ | 4,4; 5,2; 3,0 | 0,697 | 4,8 [14] |
| $\alpha_1$ | 5,85—4,6 | 0,772±0,014 | $\alpha_1$ | | 0,865 | |

*Results of the present investigation.
†Figures in parentheses represent literature citation.
‡Antitetanus serum; values calculated from results of determination of absolute electrophoretic mobility.

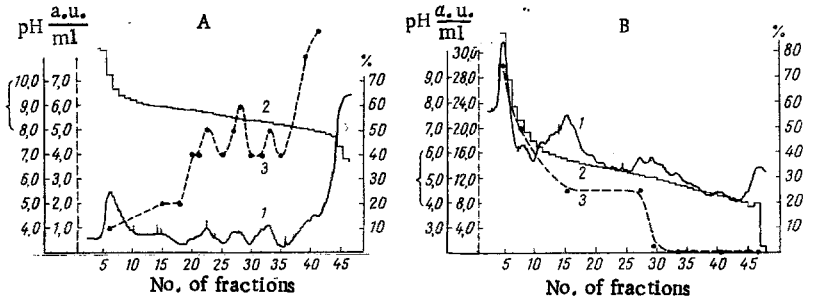


Fig. 3. Fractionation of "Diaferm-3" antitetanus serum by isoelectric focusing at pH 8-10 (A) and pH 4-6 (B). Legend as in Fig. 1.

below 5.0 (they accounted for 30% of the absorption in the pH range 4-6) possessed no antitoxic activity. These proteins had the mobility of α-globulins.

Finally, mention must be made of the considerable loss (60%) of the total antitoxic activity of the preparation tested during isoelectric focusing.

The isoelectric focusing method revealed considerable heterogeneity of the preparation studied. For comparison, 1-2 fractions were found on gel-filtration of the "Diaferm-3" preparation on Sephadex G-100 and G-200 [6-8], and 3-4 fractions on electrophoresis [2, 5, 15]. The heterogeneity can be explained by the heterogeneity of the material used for proteolysis, and also by the presence of a variety of peptic hydrolysis products in "Diaferm-3." Even γG-antibodies purified on immunosorbents showed greater microheterogeneity [3]. Products of digestion of horse T-globulin by pepsin, moreover, have the mobility of γG-globulin; consequently, in the course of proteolysis the heterogeneity of the γG-globulin fraction is increased [18, 20, 23]. The results showing the connection between antitoxic activity and the fractions of γG- and β-globulins agree with the results of investigations by other workers [7, 15].

The results given in Table 1 for the PI values and electrophoretic mobility of the "Diaferm-3" serum proteins, which are basically $[F'ab]_2$-fragments of horse antitoxic globulin, were similar to the corresponding values for globulins of native human plasma and horse serum. This agreement accords with the view that the electrophoretic mobility of the whole globulin molecule is determined chiefly by the $F_{AB}$-fragment [11].

It is an interesting fact that gel-filtration of Sephadex G-200 showed that the proteins with mobility of α-globulins studied were high-molecular-weight macroglobulins. Previously only low-molecular-weight

products of peptic proteolysis were found in the α-globulin fraction [8, 15]. Comparison of the results obtained by isoelectric focusing and electrophoresis showed that mobility increases with a decrease in PI.

In the present experiments highest activity was found in the most acid and least alkaline proteins of the "Diaferm-3" serum (Figs. 1 and 3A). These observations are in agreement with observations that after purification of the "Diaferm-3" preparation on anionic and cationic exchange resins the activity of the preparation increases appreciably [8, 15].

## LITERATURE CITED


1. G. I. Avelev and V. S. Tsvetkov, Vopr. Onkol., No. 6, 62 (1960).
2. V. V. Budylina and Z. I. Sosina, Zh. Mikrobiol., No. 1, 141 (1963).
3. A. E. Gurvich, R. B. Kapner, and R. S. Nezlin, Biokhimiya, No. 1, 144 (1959).
4. A. I. Gusev and V. S. Tsvetkov, Lab. Delo, No. 2, 43 (1961).
5. T. I. Davydova and I. Ya. Sil'd, in: Stimulation and Mechanisms of Antibody Biosynthesis [in Russian], Vol. 5, No. 1, Leningrad (1966), p. 113.
6. G. N. Kryzhanovskii and R. M. Kulygina, Byull. Éksperim. Biol. i Med., No. 10, 67 (1972).
7. V. B. Freiman, A. I. Mikhailov, R. L. Shurer, et al., in: Toxins, Toxoids, and Antitoxic Sera [in Russian], Moscow (1969), p. 214.
8. Yu. A. Khavkin, T. M. Chizhova, T. A. Batalova, et al., in: Toxins, Toxoids, and Antitoxic Sera [in Russian], Moscow (1969), p. 241.
9. P. Dole and E. Braun, J. Clin. Invest., 27, 708 (1944).
10. H. F. Deutsch and J. C. Nichol, J. Biol. Chem., 176, 797 (1948).
11. J. L. Fahey, Immunochemistry, 1, 121 (1964).
12. J. F. Larquer, Arch. Biochem., 77, 350 (1958).
13. J. F. Larquer, J. Immunol., 76, 393 (1956).
14. M. Lavergne and M. Raynaud, Ann. Inst. Pasteur., 119, 27 (1970).
15. G. Hristova, Z. Immun.-Forsch., 138, 439 (1968).
16. B. Polis, H. W. Schmukler, J. Custer et al., J. Am. Chem. Soc., 72, 4965 (1950).
17. G. E. Perlmann and P. Kaufman, Ibid., 67, 638 (1945).
18. Relyveld and M. Raynaud, Ann. Inst. Pasteur, 92, 246 (1957).
19. H. Haglund, Sci. Tool, 14, 17 (1967).
20. H. E. Schultze, H. Haupt, et al., Immunochemistry, 2, 273 (1965).
21. M. F. Sheff, M. B. Perry, and S. J. Zacks, Biochim. Biophys. Acta, 100, 215 (1965).
22. E. L. Smith and T. D. Gerlough, J. Biol. Chem., 167, 679 (1947).
23. J. Van der Scheer et al., J. Immunol., 41, 349 (1941).