following explanation of the contradiction observed between data cited [9] and obtained in the present investigation may be suggested. Lipid peroxidase activity is determined by the action of selenium (GP) and nonselenium (GT) peroxidases. Since changes in the peroxidase activity of GP and GT during progressive atherosclerosis are opposite in direction and may compensate one another, differences in total lipid peroxidase activity between the normal and affected regions may prove to be not significant.

The data given in this paper are thus evidence that lipid peroxidase activity in zones of atherosclerotic damage to the human aorta may arise through a decrease in GT activity. This is a very important fact for the pathogenesis of atherosclerosis, having regard to the important role of GT in detoxication of membrane acyl hydroperoxides, the content of which rises sharply in the course of progressive atherosclerosis of the aorta [7].

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BIOCHEMICAL AND IMMUNOCHEMICAL STUDY OF STRUCTURAL HETEROGENEITY OF POLYMERIZED HEMOGLOBIN

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The use of polymerized hemoglobin (Hb), containing pyridoxal-5'-phosphate as regulator of reversible oxygenation (PH-PP) is evidently the most promising approach to the creation of a blood substitute with a gas transporting function [1, 4, 5]. Experiments in animals have shown that PH-PP circulates for a long time in the blood stream, has a half-elimination time of 25-48 h, transports virtually sufficient oxygen to the erythrocytes, and maintains the survival of animals after replacement of 95% of the circulating blood volume [2, 7, 8]. During

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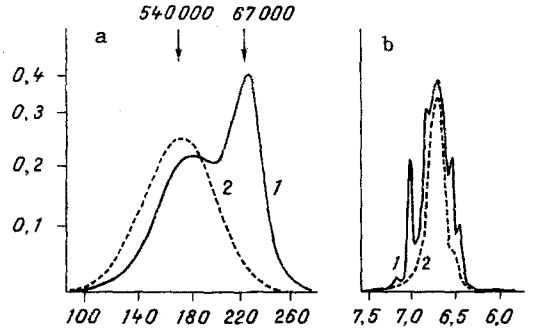

Fig. 1. Molecular-weight distribution and isoelectric characteristics of PH-PP and fPH. a) Gel-chromatography of PH-PP (1) and of fPH (2) (carrier: sepharose CL-6B, K 26/100, eluent 0.85% NaCl, containing 0.02% NaN_3, rate of elution 20 ml/h); abscissa, volume of eluate (in ml); ordinate, optical density at 280 nm (OD_{280}). b) Isoelectric focusing of PH-PP and fPH (legend as above); abscissa, values of pI; ordinate, optical density at 630 nm (OD_{630}).

circulation, specific interaction of PH-PP with plasma proteins, depending on the degree of its heterogeneity also has been observed [1]. This last observation may be of great importance for the discovery of factors determining the biological compatibility of modified hemoglobin with the recipient.

The aim of this investigation was to examine in more detail the character of heterogeneity of PH-PP by comparative analysis of solutions of native purified Hb, of PH-PP, and its fraction (fPH), using methods of gel-filtration, isoelectric focusing, and immunoelectrophoresis.

EXPERIMENTAL METHOD

PH-PP was obtained by modification of Hb by glutaraldehyde and pyridoxal-5'-phosphate by the method described previously [3, 9] (the degree of polymerization was ∿50%). The PH-PP fraction was isolated by preparative ion-exchange chromatography on DEAE-Sephacel, followed by ultrafiltration on H10P100 membranes (100 kD, from "Amicon," The Netherlands) by concentration and dialysis on a "Pellicon" PTMC cassette system (10 kD, from "Millipore," USA). Gel-filtration was carried out on a column with sepharose 6B. Isoelectric focusing of the specimens was carried out on plates with ampholines of pH 3.5-9.5 (from LKB, Sweden) with a voltage of 1000 V; the shape of the pH gradient was determined with the aid of reference proteins: the plates were stained in a solution of Coomassie brilliant blue G-250 ("Serva"). The gels were scanned on a laser densitometer (LKB, Sweden) at a wavelength of 630 nm. Rabbits were immunized with solutions of Hb and PH-PP in order to obtain antisera, by the following schedule: the first injection consisted of 1 ml of a solution with protein content of 10 mg/ml + 1 ml of Freund's complete adjuvant, subcutaneously into the region of lymph nodes; after 10 days and for the next 3 weeks, a weekly injection was given of 1 ml of solutions with protein concentration rising from 20 to 80 mg/ml, into the auricular vein. The booster dose was injected 2 weeks after the last injection of the series (20 mg protein with adjuvant, subcutaneously). Blood was taken 2 weeks after the booster injection. To increase the titer of antibodies to native Hb at the end of the schedule described above, four additional injections of protein together with adjuvant were given at intervals of 14 days (the protein concentration in the solution was increased uniformly from 20 to 100 mg/ml). Immunoelectrophoresis was carried out with antisera obtained to Hb and PH-PP on a "Multiphore" apparatus (LKB, Sweden) in a layer of 1% agarose at pH 8.6 (barbital buffer, ionic strength 0.02). Rocket immunophoresis was carried out for 3 h with a voltage of 10 V/cm; two-way immunoelectrophoresis was carried out with a voltage of 10 V/cm in the first direction (3 h) and 2 V/cm in the second direction (18 h). In the latter case the agarose contained 15% of antiserum to PH-PP. The plates were stained by the standard method in Coomassie brilliant blue R-250 solution.

EXPERIMENTAL RESULTS

Comparison of the elution profiles of PH-PP and fPH (Fig. 1a) shows that PH-PP is a polydispersed polymer containing molecules with mol. wt. from 65 to 600 kD, whereas the fraction, which is homogeneous in composition, contains no low-molecular-weight components and has mol. wt. of about 500 kD.

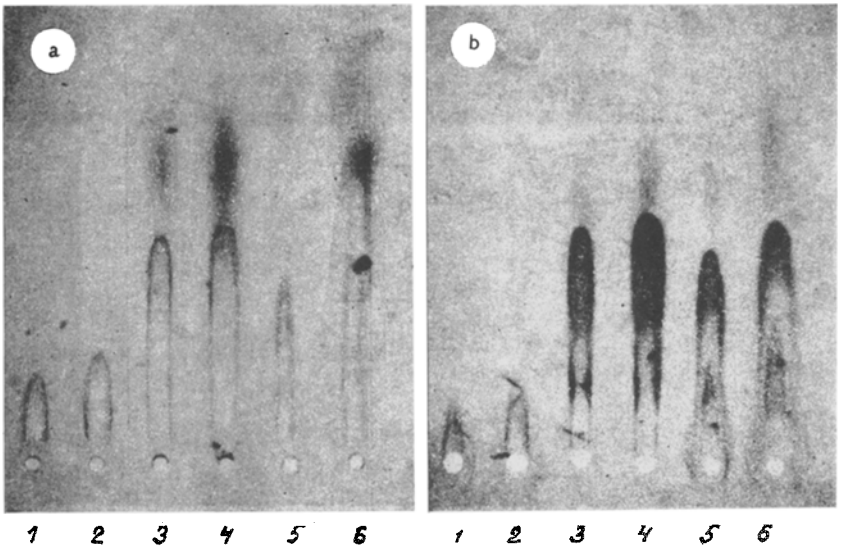

Fig. 2. Investigation of Hb and its derivatives by rocket immunoelectrophoresis. a) Against rabbit antiserum to original Hb; b) against rabbit antiserum to PH-PP. Wells contain: 1, 2) Hb (75 and 150 μg respectively); 3, 4) fPH; 5, 6) PH-PP in the same amounts.

A considerable degree of heterogeneity of PH-PP with respect to molecular weight suggests that it may also have analogous heterogeneity with respect to the total surface charge on the molecules, having regard to the different degrees of modification of the original Hb by glutaraldehyde and by pyridoxal-5'-phosphate. Analysis of the total surface charge on the molecules of the compounds studied by isoelectric focusing shows (Fig. 1b) that covering of the amino groups on the surface of the original Hb molecule by modifying agents does not lead to any significant change in the isoelectric properties of PH-PP and fPH compared with native Hb. For instance, values of pI for PH-PP lie within the range from 6.6 to 7.2, those for fPH between 6.5 and 7.0, whereas for native Hb the range of values is 6.9 to 7.2 (the great homogeneity of fPH with respect to its isoelectric properties must be noted). Thus, heterogeneity of PH-PP is manifested mainly as polydispersion by molecular weight and, to a lesser degree, by a change in the surface properties of the molecule.

To assess structural differences between PH-PP and the original Hb, we also used an immunochemical approach, enabling preservation and modification of antigenic determinants on the protein surface as a result of its chemical modification to be determined. By rocket immunoelectrophoresis against antisera to native Hb and to PH-PP it was shown that both PH-PP and fPH form precipitates with antibodies to the original Hb. This is evidence of preservation of the corresponding antigenic determinants in the course of its modification (Fig. 2a). Meanwhile, during interaction between the test compounds and serum to PH-PP significant differences were found in the antigenic properties of Hb, PH-PP, and fPH (Fig. 2b). For the last two substances, several peaks were formed (more clearly defined in the case of fPH), possible evidence of the appearance of new antigenic determinants, as a result of the modification, leading to the formation of antibodies which were not present in antiserum to native Hb. This is clear if Figs. 2a and 2b are compared. Comparison of the peaks obtained for Hb, PH-PP, and fPH reveals the effect of the degree of modification of Hb on its immunogenic properties and of the stronger antigenicity of high-molecular-weight Hb derivatives than of the native protein.

To investigate the antigenic spectrum of PH-PP and its electrophoretic mobility, the method of two-way immunoelectrophoresis was used against serum obtained by immunizing rabbits with PH-PP. Separation of PH-PP by electrophoretic mobility, corresponding to the first direction of immunoelectrophoresis, shown in Fig. 3 by comparison with mobility of the original Hb leads to the conclusion that PH-PP contains no unmodified Hb in its composition. Comparison of the data for electrophoretic mobility with the results of gel-chromatography of PH-PP given above (Fig. 1a) shows that 50% of the unpolymerized Hb in the composition of the test product consists not of native, but of structurally modified protein. The result of two-way

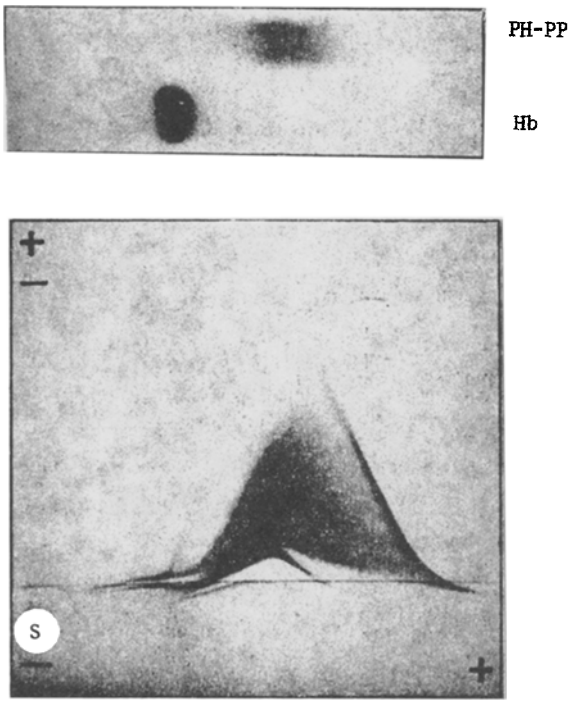

Fig. 3. Two-way immunoelectrophoresis of PH-PP. S) Starting well for sample, 100 μg of antigen (PH-PP) introduced; agarose for second direction of immunoelectrophoresis contains 15% of antiserum to PH-PP. Above — electrophoresis of Hb and PH-PP under conditions corresponding to the first direction of two-way immunoelectrophoresis.

immunoelectrophoresis indicates that PH-PP possesses a broad antigenic spectrum, due partially to the structural heterogeneity of the antigen. It has to be pointed out that the absence of clear-cut boundaries between the majority of peaks corresponds to the nondispersed character of heterogeneity of PH-PP. It is essential to note that low-molecular-weight fractions of PH-PP cause the formation of a very small quantity of precipitate, most of which is located in the region corresponding to higher molecular weights. The results are evidence that an increase of molecular weight has an effect on the antigenic properties of the modified Hb and they are in agreement with the results of serologic studies of Hb and its polymer [6].

Modification of Hb by glutaraldehyde and pyridoxal-5'-phosphate thus leads to the formation of a product heterogeneous mainly for molecular weight, and polydispersed only to a very slight degree with respect to isoelectric properties. A qualitative difference between the antigenic properties of the original Hb and of PH-PP, reflecting structural changes taking place with Hb as a result of its polymerization, was demonstrated. The results suggest that the antigenic activity of PH-PP can be reduced by reducing the fraction of high-molecular-weight components in its composition.

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