AOA were more pronounced in the dermis than in the epidermis.

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Chemically Modified Hemoglobin-Based Oxygen-Carrying Blood Substitute

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UDC 615.31:544.96.2.4].015.4.07

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 115, No. 4, pp. 364–366, April, 1993
Original article submitted November 11, 1992

Key Words: *hemoglobin; chemical modification; blood substitute*

There are various chemically modified hemoglobins which differ in their fractional composition and which contain pyridoxal-5-phosphate (MH-PP) as the regulator of reversible oxygenation. These hemoglobins are considered to be the most promising all-purpose oxygen carriers [2,7]. However, such compounds are nonuniform with respect to their structure and the functional groups which they contain [3,8]. Moreover, as their structure and functional groups are what determine their rheological and antigenic properties, this nonuniformity is one of the main obstacles to creating the corresponding medicinal preparations [6,8].

In order to select substances with optimal physicochemical and oxygen-carrying properties we obtained and studied a series of model MH-PP derivatives with different concentrations of the polymeric fraction. Various compositions of MH-PP were investigated with the aid of gel chromatography and SDS electrophoresis. In addition, an analysis was made of their functional characteristics, viscosity, colloidal and oncotic properties, and degree of immunogenicity.

MATERIALS AND METHODS

Samples of MH-PP were prepared by treating hemoglobin with glutaraldehyde and pyridoxal-5-phosphate according to a known procedure [1]. By varying the reaction conditions we obtained a series of hemoglobin derivatives containing from 5-10 to 70-75% of the polymeric fraction.

The distribution of MH-PP samples according to their molecular weight was determined by separation on a TSK-G-3000SW high-performance liquid chromatographic column (7.5×3000 mm) (LKB, Sweden), with an eluant flow rate of 0.5 ml/min and a 0.01 M phosphate buffer at pH 6.5. Detection was carried out at 400 nm, and the concentration of vari-

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0007-4888/93/0004-0381$12.50

TABLE 1. Properties of Hemoglobin Polymers

Sample №	Concentration of polymeric fraction, %	O_2 affinity, mm Hg	$M_n \times 10^{-3}$	$M_w \times 10^{-3}$
MM–PP–1	2–3	28–31	–	–
MM–PP–2	25–30	27–29	110±30	90±10
MM–PP–3	50–55	26–30	230±37	200±35
MM–PP–4	70–75	28–30	650±55	620±60
Blood	–	26–28	–	–

ous molecular-weight fractions was determined with the aid of LKB 2220 integrator (Sweden).

Oxygen-dissociation curves (ODC) for the samples investigated were recorded with a Hem-O-Scan apparatus under the following physiological conditions: 37°C, pH 7.4, pCO_2 40 mm Hg, and 0.15 M NaCl. A quantitative analysis of ODC was carried out on the basis of the calculated value of p_{50}; this value corresponds to the partial pressure of oxygen at 50% hemoglobin saturation and indicates the O_2-affinity of MH-PP. The relative viscosity of the MH-PP solutions was measured at 37°C in the concentration range of 2 to 12% with the aid of a U-shaped Oswald viscometer (capillary 0.8 mm in diameter; the time required for the water flow, 26.5 sec). The oncotic pressure of the MH-PP solutions was measured with the aid of an IL 186 oncometer and by using a membrane for 30 kD.

The effect of the modified hemoglobin solutions on the erythrocyte sedimentation rate (ESR) was determined by adding fresh donor blood (stabilized with 3.8% sodium citrate in an amount equal to that of the solution being analyzed. The magnitude of ESR (ml/h) was determined by the standard capillary method. Antibodies in antiserum were titrated by the radial reverse immunodiffusion method. Into a Petri dish was poured 10 μl of bacterioagar containing 22.5 mg/ml of the antigen solutions. Aliquots of the corresponding antiserum which had been diluted twice consecutively were introduced into the wells (10 ml). Precipitation was carried out at room temperature for 18 hours. The average molecular weight (M_w) of the samples was determined by the sedimentation analysis method with the aid of an analytical centrifuge (Beckman), and the average numerical molecular weight (M_n) with the aid of a membrane osmometer (Knauer). The data obtained were processed on an HP85 computer with General Statistics software.

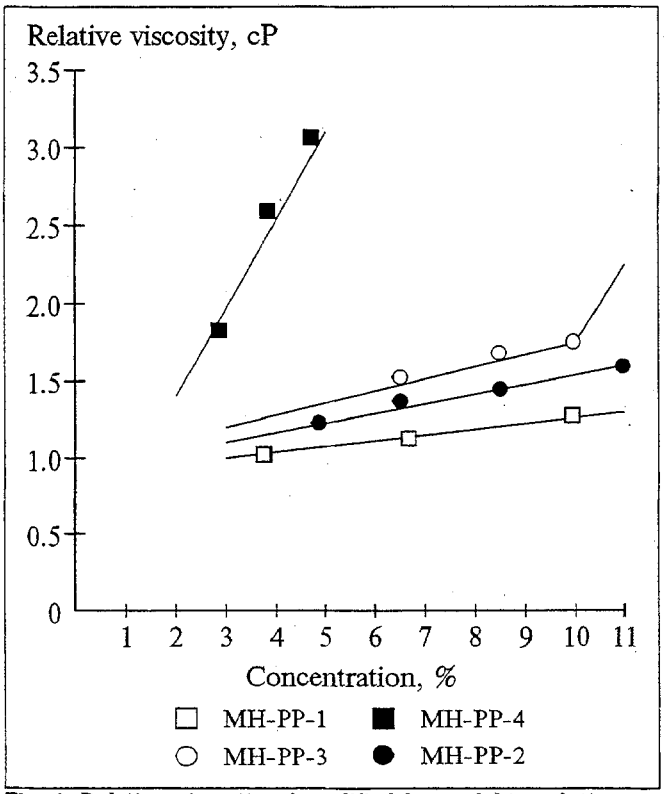

Fig. 1. Relative viscosity of modified hemoglobin solutions at 37°C as a function of the concentration of the polymeric fraction.

RESULTS

We regarded as the main characteristics of the MH-PP obtained the concentration of the polymeric fraction and the molecular weight of the compounds, as well as their affinity to oxygen.

The data in Table 1 show that an increase in the concentration of the polymeric fraction has practically no effect on the functional effectiveness of the polyhemoglobins. Thus, the affinity of the compounds under investigation to oxygen is determined mainly by the nature of the hemoglobin modification due to MH-PP. This affinity is preserved in the pressure range of 27-30 mm Hg when measured under physiological conditions (for fresh donor blood the corresponding figure is 26-28 mm Hg).

However, the biocompatibility of the hemoglobin derivatives is determined not only by their functional effectiveness, but also by several other parameters. Among these are the rheological properties and the colloidal and oncotic pressure of the MH-PP solutions and their antigenicity.

The relative viscosity of the MH-PP solutions as a function of the concentration of the polymeric fraction is shown in Fig. 1. It can be seen that the relative viscosity of each of the samples is greater than that of the native hemoglobin and increases with an

TABLE 2. Dynamics of Formation of Antibodies during Immunization of Rabbits with Solutions of Native Protein, MM–PP–2, and MM–PP–3

Antigen	Immunization period, days			
	0	14	28	56
Native protein	–	1:2	1:4	1:8
MM–PP–2	–	1:4	1:8	1:16
MM–PP–3	–	1:8	1:32	1:128

increase in the degree of polymerization. But even then, the viscosity of the MH-PP solution exceeds that of the blood (4.7-5.7 cP, at 5-6% concentration) only when the concentration of the polymeric fraction is greater than 70%. The relative viscosity of the other modified hemoglobin solutions was considerably lower than that of the blood at all the concentrations investigated.

For an assessment of the rheological properties of MH-PP solutions as possible blood substitutes it is not enough to know their relative viscosity. We also need to find out whether they cause aggregation of regular blood constituents. For this purpose we measured the ESR in donor blood that had been diluted to 1:1 by volume with the hemoglobin derivatives under consideration. As can be seen from Fig. 2, an increase in the concentration of the polymeric fraction to 25-30% resulted in an increase in the ESR (from 15 to 25 ml/h), which practically does not exceed the physiological values. Any further increase in the degree of polymerization led to a sharp acceleration of the ESR to 55-60 ml/h. This rate is impermissibly high, and MH-PP with such a degree of polymerization obviously cannot be used *in vivo*.

Another important physiological characteristic of MH-PP solutions is their colloidal and oncotic pressure. It has been reported [2,9] that the introduction of unpolymerized hemoglobin into the bloodstream produces excessive oncotic pressure, which has a negative effect on the cardiovascular system. In the case of 8-10% solutions of native hemoglobin and of an MH-PP solution containing 2-3% of the polymeric fraction the oncotic pressure is 22-27 torr. With an increase in the concentration of the polymeric fraction the oncotic pressure decreased proportionally, reaching 10-20 torr at 50% of polyhemoglobin. Thus, the polymerization of hemoglobin makes it possible, as hemoglobin is introduced into the bloodstream of experimental animals, to bring the oncotic pressure close to that observed during the transfusion of the erythrocyte mass.

The quantitative characteristic of the antigenic activity of hemoglobin derivatives MH-PP-2 and MH-PP-3 were determined by comparing their immunogenicity with that of native hemoglobin. For this purpose rabbits were immunized with the hemoglobin derivatives. The activity of the sera at different immunization periods was investigated by the radial immunodiffusion method. As the titer of antibodies was taken the maximum dilution of the serum at which the antigen-antibody reaction still needed to be taken into account.

It can be seen from the data in Table 2 that the antigenic activity of the samples containing 25-30% of the polymeric fraction shows a small increase as compared with that of the native protein. At the same time, in the case of MH-PP-3 (containing approximately 50% of the polymeric fraction) the titer of antibodies for the last immunization period increases tenfold on the average as compared with hemoglobin.

These results show that there is a correlation between the antigenic properties of the polymeric derivatives of hemoglobin and their molecular weight. This indicates that polyhemoglobins containing more than 30% of the polymeric fraction can have a negative effect on the organism.

An analysis of the results obtained makes it possible to conclude that hemoglobin polymers with a 25-30% degree of polymerization have optimal oxygen-carrying properties. This was confirmed by screening tests carried out on dogs according to the model of acute lethal hemorrhage [4] and the model of hemorrhage shock [5]. The tests show that a properly selected MH-PP preparation can have a marked therapeutic effect without causing visible side effects.

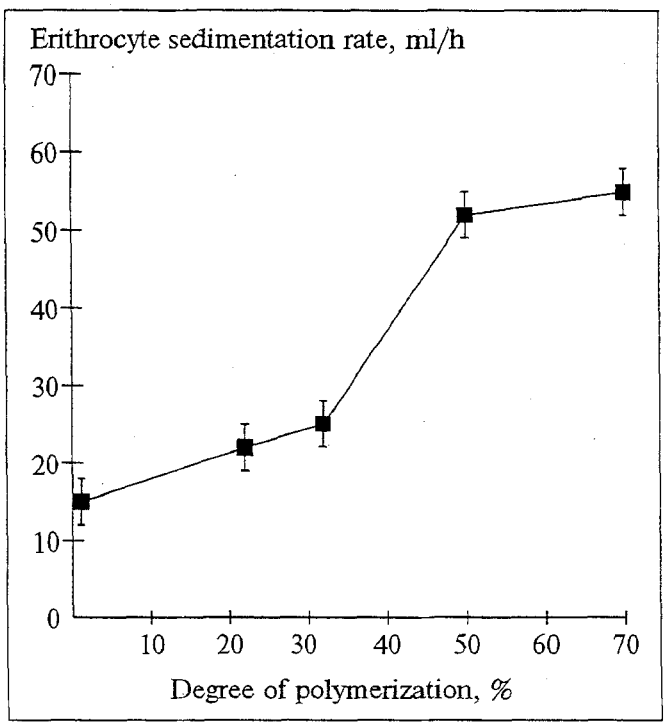

Fig. 2. Effect of the degree of polymerization of hemoglobin on the suspension properties of blood. Volume ratio of blood:MH–PP solution 1:1; concentration of hemoglobin in solutions 9±0.5%.

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PHARMACOLOGY

Antihypoxic Effects of Some 3-Hydroxypyridine Derivatives in Isolated Rat Myocardium

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UDC 612.015.3+612.014.4641:612.17.084

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 115, № 4, pp. 366–368, April, 1993
Original article submitted November 23, 1992

Key Words: *hypoxia; heart; contractile function; bioenergetics, antihypoxants; succinate*

The electron-transporting function of the respiratory chain is known to be disturbed at its substrate-binding site in the early stages of hypoxia and ischemia [2,3,8]. Its first enzymatic complex is the limiting stage of this process. It is thus thought to be possible to activate compensatory metabolic pathways (alternative to the main NAD-dependent mitochondrial oxidation) acting as mechanisms of emergency adaptation to these pathological processes during hypoxia. A succinate oxidase pathway with oxidative thermodynamic advantages in the mitochondrial respiratory chain at low pO_2 values is the first of these [1]. Heterocyclic compounds are thought to facilitate succinate entry into the cell and its subsequent oxidation in the respiratory chain, this determining the antihypoxic properties of these compounds. The aim of the present study was to investigate the possibility of using mexidol (a succinate-containing 3-hydroxypyridine derivative) as an antihypoxant with a direct energizing effect [4].

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MATERIALS AND METHODS

Experiments were carried out on male albino rats weighing 180-200 g which were divided in a pressure chamber into rats with high resistance (HR) and with low resistance (LR) to hypoxia. The antihypoxic properties of the hydroxypyridine derivatives emoxypine and mexidol were studied on an isolated per-

0007-4888/93/0004-0384$12.50