CHROMATOGRAPHIC STUDY OF HUMORAL HEMOLYTIC FACTORS AFTER BLOOD LOSS

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Acute blood loss is accompanied by a stereotyped response of the erythron, namely erythrodiaeresis, preceding activation of erythropoiesis and linked, in particular, with increased hemolytic activity of the blood serum [6, 8, 10, 11]. Despite the universal nature of this phenomenon, there is only sporadic information on the role of antierythrocytic antibodies, lysolecithin, and tissue hemolysins in posthemorrhagic hemolysis [1, 4, 5]. The mechanism of the increased hemolytic activity of the blood serum in the posthemorrhagic period remains unexplained.

This paper describes an attempt to isolate hemolytic factors, on a model of acute unreplaced blood loss with the aid of chromatographic methods.

EXPERIMENTAL METHOD

Experiments were carried out on 118 Wistar rats weighing 180-200 g. Acute blood loss was produced by taking blood from the jugular vein of the animals in a volume of 35-40% of the circulating blood volume, determined with the aid of the dye T-1824. Bilateral nephrectomy was performed under pentobarbital anesthesia. The dose-dependent effect of hemolytic factors was studied in vivo 15, 60, and 120 min after intravenous infusion of the test fraction in doses of 4.87, 9.75, and 19.5 activity units/μg protein/100 g body weight [24]. Free serum hemoglobin was measured colorimetrically by the cyanhemoglobin method, and the protein concentration in the serum samples and eluate was determined by the aid of Coomassie Blue G [9]. The acid resistance of the erythrocytes was estimated as an integral parameter of the acid erythrograms, namely the total acid resistance of the erythrocytes (TARE) [2]. Serum hemolytic activity (SHA) was determined quantitatively after incubation of the blood (+37°C, 3 h) with standard (syngeneic) erythrocytes in the ratio of 2:1, and was estimated in activity units (AU) by the formula:

$$\mathrm{SHA(AU)} = \frac{(\mathrm{TARE} - \mathrm{TARE}) \cdot 100}{\mathrm{TARE}},$$

where TARE s, e, c denotes total acid resistance of standard erythrocytes, and of erythrocytes after incubation in experimental and control samples [3] respectively. SHA also was calculated in activity units per unit of protein in the test samples (AU/μg). The composition of the blood serum samples from the experimental animals was studied by methods of high pressure liquid chromatography (HPLC). Preparative ion-exchange chromatography was carried out on DEAE-Toyopearl (7.5 × 75 mm), with elution by 0.04 M Tris-HCl, pH 7.8, containing 0.1% NaN_3 in a 0-1 M NaCl gradient; the elution time was 40 min. To determine molecular weight, a TSK-G-3000 SW column (7.5 × 300 mm) and 0.1 M phosphate buffer, pH 6.56, containing 0.02% NaN_3 and 0.1% of the detergent 7X were used. Detection was carried out at 280 nm. The percent-

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TABLE 1. Hemolytic Activity of Posthemorrhagic Blood Serum of Animals after Bilateral Nephrectomy, and with Intact Kidneys (AU)

Series of experiments	n	SHA in posthemorrhagic period, h	
		1	24
Blood loss	28	46,6±1,74	58,6±2,12
Bilateral nephrectomy + blood loss	26	15,1±1,21	13,4±3,24
p 1-2		<0,001	<0,001

TABLE 2. Effect of Dose of Hemolytic Component of Hemorrhagic Serum on Intensity of Hemolysis in Vivo

Time of observation	Parameter	Dose (AU/μg protein/100 g)			
		4.87	9.75	19.5	
0	TARE (% min)	473,7±8,2	230,3±15,9	290,1±27,0	358,0±24,6
	Hb (mg %)	3,2±0,2	3,1±0,1	4,0±0,2	3,1±0,1
15 min	TARE	435,0±22,7	314,6±10,4**	270,0±3,5	453,0±14,7*
1 h	TARE	482,0±4,6	274,6±5,2*	456,0±15,7**	442,0±8,3*
2 h	TARE	338,0±11,5***	412,3±17,1***	170,0±12,6**	358,4±18,7
	Hb	5,5±0,49*	10,0±0,29***	32,3±0,81***	3,4±0,27

Legend. *p < 0.05; **p < 0.01; ***p < 0.001 Compared with 0.

age composition of the fractions with different molecular weights was determined by means of a 2220 integrator (LKB). Fractions 1 ml in volume were taken with the aid of a SuperRack 2211 fraction collector (LKB). The experimental results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

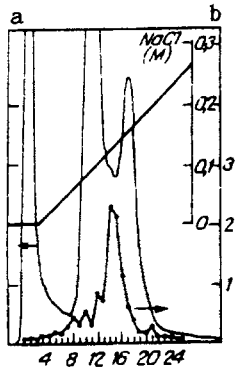
The study of dependence of the blood serum hemolytic activity of animals in the posthemorrhagic period on liver function showed an increase in SHA compared with the corresponding parameter in nephrectomized rats. The results obtained in the course of 24 h are given in Table 1.

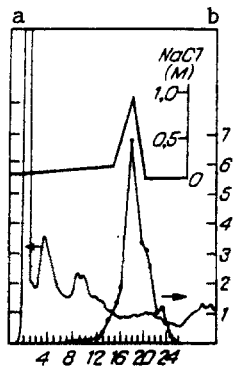
It follows from Table 1 that the hemolytic effect of the serum after blood loss was 3.1 times less in the nephrectomized animals than in animals with intact kidneys. The effect was intensified 24 h after blood loss.

The next step was to determine differences in the fractional composition of the sera with different SHA values and to select the fraction responsible for the hemolytic effect. For this purpose a chomatographic investigation was made of samples of posthemorrhagic serum with high SHA values by HPLC methods. The elution profile of a serum sample on DEAE-Toyopearl in a linear NaCl gradient is shown in Fig. 1. Specific hemolytic activity was established in fractions 14-15, with a peak value of 2.33 AU/μg protein. The change in the ionic strength gradient profile (Fig. 2) enabled more complete isolation of a component possessing SHA, whose maximal value reached 6.86 AU/μg protein in fraction 18. Moreover, 80.3% of the total SHA was contained in fractions 18 and 19. Losses of activity during fractionation amounted to 5-7%. To establish the molecular weight composition these fractions were concentrated, and subjected to further elution on a TSK-G-3000 SW column with parallel measurement of SHA. The resulting elution profile and the distribution by SHA are shown in Fig. 3, from which it follows that the component with molecular weight of between 80 and 100 kilodaltons possesses the greatest activity.

The biological activity of the hemolytic component of posthemorrhagic serum isolated by ion-exchange chromatography was studied in experiments in vivo by determining the presence of a dose-dependent hemolytic effect. The results are given in Table 2.

An increase in the dose of the ionizable hemolytic component is accompanied by a marked increase in plasma free hemoglobin concentration; values of TARE, moreover, vary with dose at different times of observation, and reach peak values with a dose of 19.5 AU/μg protein.





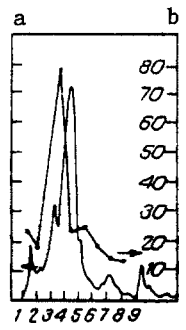


Fig. 1 Fig. 2 Fig. 3

Fig. 1. Elution profile of blood serum sample obtained on DEAE-Toyopearl column (7.5 × 75 mm) in 0.04 M Tris-HCl buffer, pH 7.8, in 0-1 M NaCl gradient, with elution rate of 1.0 ml/min (volume of sample 0.5 ml, protein content in sample 8.30 mg). Abscissa, serial No. of fraction; ordinate a) optical density at 280 nm, b) SHA in AU/μg protein.

Fig. 2. Elution profile of blood serum sample obtained on DEAE-Toyopearl column (7.5 × 75 mm) in 0.04 M Tris-HCl buffer, pH 7.8, in 0-1 M NaCl gradient, and with elution rate of 1.0 ml/min (volume of sample 0.6 ml, protein content in sample 10.55 mg). Legend as to Fig. 1.

Fig. 3. Elution profile of concentrate of fractions 18 and 19, obtained on TSK-G-3000 SW column (7.5 × 300 mm) in 0.1 M phosphate buffer, pH 6.5, with elution rate of 0.5 ml/min (volume of sample 0.024 ml, protein content in sample 0.38 mg). Abscissa, serial No. of fraction; ordinate: a) optical density at 280 nm, b) SHA in AU/μg protein $\times 10^2$.

Thus the experiments demonstrated a kidney-dependent hemolytic component of posthemorrhagic serum with a molecular weight of 80-100 kilodaltons and isolated it by chromatographic methods. Introduction of this component into the animals' blood stream demonstrated the presence of a dose-dependent hemolytic effect.

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