EFFECT OF LOW-MOLECULAR-WEIGHT PEPTIDES ON CONVERSION OF FIBRINOGEN INTO FIBRIN

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Numerous investigations have shown that among the natural anticoagulants there are products of enzymic protein hydrolysis [2, 8, 9]. Anticoagulant activity of peptides has been shown to depend on the presence of amino acids such as proline, arginine, and lysine in them [6, 7]; the presence of the Pro-Arg sequence, moreover, is one of the factors determining this activity [4].

This paper describes a study of the action of certain low-molecular-weight peptides (synthetic analogs of the N-end of the α-chain of fibrin) on fibrin formation. The main sequence in the peptides mentioned above is Gly-Pro-Arg. It is this tripeptide, which is an exact copy of the N-terminal region of the α-chain of fibrin, that exhibits appreciable anticlotting activity [11]. The authors cited stabilized the structure of this peptide by adding it to the C-end of proline. The tetrapeptide thus synthesized has higher anticoagulant potential. Considering previous data [10-12] showing that peptides of the Gly-Pro-Arg type act on the final stages of blood clotting, two aims were pursued: first, from a set of synthesized substances to discover those which were most active, and second, to attempt to show at what stage of clot formation these peptides act more effectively.

EXPERIMENTAL METHOD

Commercial preparations of fibrinogen and thrombin were used and were additionally purified by the method in [4]. Fibrin monomer (FM) was prepared by a modified method [5].

A 0.12% solution of FM in 0.02 M acetic acid was used. FM was converted into the polymer by the addition of 2.5 ml of 0.05 M phosphate buffer, pH 7.6, to 0.5 ml of the solution of FM. To investigate the effect of the peptides on fibrin formation, 0.1 ml of a solution of the peptide of the appropriate concentration, made up in phosphate buffer, was added with 2.4 ml of the same buffer to 0.5 ml of the FM solution. To study the action of the peptides on conversion of fibrinogen into fibrin under the influence of thrombin, 0.5 ml of fibrinogen

TABLE 1. Values of Characteristic Viscosity (η, dl/g) of FM Solutions in the Presence of Peptides in a Medium of 2.2 M Urea

Peptide	Ratio of peptide to FM	
	100:1	500:1
I	1,6±0,1	1,0±0,1
II	2,2±0,2	0,35±0,03
III	2,9±0,2	1,4±0,1
IV	1,5±0,1	0,35±0,03
V	6,6±0,4	5,6±0,4
VI	6,7±0,5	5,6±0,5
VII	6,6±0,4	5,5±0,4
VIII	6,1±0,4	4,3±0,3
FM in medium of 3,5 M urea	0,35±0,03	
FM in medium of 2,2 M urea	6,82±0,43	

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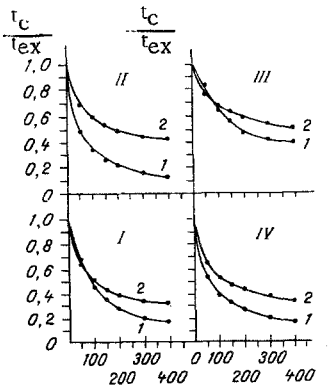

Fig. 1

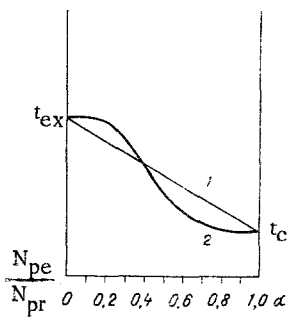

Fig. 2

Fig. 1. Dependence of reaction velocities of conversion of fibrinogen into fibrin under the influence of thrombin (1) and polymerization of FM (2) on peptide concentration. N_{pe}/N_{pr} Molar ratio of peptide to protein; t_c) duration of reaction without peptide; t_{ex}) duration of reaction in presence of peptides.

Fig. 2. Duration of FM polymerization reaction after addition of inhibitor at different stages of the process. Explanation in text.

solution was treated with 0.5 ml of 0.02 M acetic acid, 1.9 ml of 0.05 M phosphate buffer, pH 7.6 (or 0.1 ml of peptide of the appropriate concentration and 1.8 ml of buffer) and 0.1 ml of thrombin solution. The final fibrinogen concentration was 0.12%. The thrombin concentration was chosen so that the clotting time was about 120 sec.

From the spectrophotometric investigation a "Specord uv vis" instrument was used, at a fixed wavelength of 310 nm, in centimeter quartz cuvettes at 25°C. Fibrin formation was accompanied by an increase in turbidity of the solution. The reaction time was measured until an increase took place in the scattering of light, corresponding to the beginning of fibrin aggregation.

Experiments to determine the characteristic viscosity of the solutions were carried out on Ostwald viscosimeters in a medium of 2.2 M urea, containing protein in a concentration of 1 mg/ml. The molar ratios of peptide to protein were 100:1 and 500:1. The following peptides were used:

Gly-Pro-Arg-Pro (I)	Gly-Lys-Pro-Arg-Pro (V)
Gly-Pro-Arg-Pro-Lys (II)	Gly-Pro-Lys-Arg-Pro (VI)
Gly-Pro-Arg-Pro-lys-Boc (III)	Gly-Pro-Arg-Lys-Pro (VII)
Gly-Pro-Arg-Pro-Arg (IV)	Lys-Gly-Pro-Arg-Pro (VIII)

The peptides were synthesized by the method described previously [3] at Leningrad University.

EXPERIMENTAL RESULTS

Determination of the clotting time showed that peptides V-VIII (in the chosen concentrations) did not affect the rate of fibrin formation. This conclusion also was confirmed by the viscosimetric data (Table 1). In urea in concentrations of 3.5 M, protein was found in the monomer form. Reduction of the molarity of the urea to 2.2 M led to growth of fibrin polymer chains, characterized by an increase in viscosity from 0.35 to 6.82 dl/g. Peptides V-VIII reduced this parameter very slightly, only in high concentrations, evidence that their action is nonspecific. Peptides I-IV actively intervened in polymerization, reducing the characteristic viscosity of the FM solutions even in low concentrations (Table 1). If the ratio of peptide to protein was 500:1 the characteristic viscosity of the protein solutions in 2.2 M urea in the presence of these inhibitors was virtually identical with the viscosity of the FM solution in 3.5 M urea, evidence of a shift of equilibrium in the reaction FM $\rightleftarrows$ FP toward the monomer. On the basis of these data it can be postulated that the manifestation of anti-

polymerization activity requires the presence of the natural sequence Gly-Pro-Arg in the peptide (and the glycyl group, moreover, must occupy the first position), in agreement with the results of other investigations [10]. We used peptides I-IV for the later investigations.

An increase in the concentration of the peptides led to intensification of their inhibitory effect both on the reaction of RM polymerization and on conversion of fibrinogen into fibrin under the influence of thrombin (Fig. 1). Comparison of the action of pentapeptides II and IV on these reactions with the action of tetrapeptide I described previously [12] shows that the additional εNH_2-group of lysine reduced the affinity of the peptide for FM, with the result that peptide II exhibited rather weaker antipolymerization activity. Meanwhile, the presence of lysine at the C-end increased the inhibitory action of this preparation on the enzymic stage of clotting (Fig. 1: I, II). Blocking the εNH_2-group of lysine reduced the activity of the peptide in both cases (Fig. 1: III). Replacement of lysine by arginine in position 5 had the result that peptide IV preserved the same antipolymerization potential as the tetrapeptide, but its effect on the enzymic stage was increased (Fig. 1: IV). Evidently, the presence of a free εNH_2-group in the pentapeptides II and IV increased their affinity for fibrinogen.

The study of the effect of the peptides on fibrin self-assembly followed the method in [1]. At definite time intervals from the beginning of the reaction of fibrin formation a solution of peptide was added to the reaction mixture. The molar ratio of peptide to FM in all experiments was 100:1. The results of these experiments are shown in Fig. 2. The value of α, plotted along the abscissa, is the ratio of the time from the beginning of the reaction to the time of addition of the inhibitor and t_c, where t_c is the region of the polymerization reaction time until the appearance of the first fibrin aggregates without the inhibitor, and t_{ex} is the time of formation of visible fibrin aggregates. The theoretical straight line (Fig. 2: I) corresponds to the situation when all stages of self-assembly are equally sensitive to the action of the peptides. In reality, however, the influence of the peptides tested on fibrin formation was nonlinear in character (Fig. 2: 2). The same dependence was obtained for all the active peptides, and only the values of t_{ex} differed.

The self-assembly process consists [13] of at least two stages: initial polymerization of the FM molecule and subsequent lateral aggregation of the polymer strands with the formation of the definitive structure of fibrin. The results now obtained show (Fig. 2) that different stages of self-assembly of the FM molecules were unequally sensitive to the action of peptides. In the first stages of fibrin formation ($0 < \alpha < 0.15$) the antipolymerization activity of the peptides remained virtually unchanged and sufficiently high even compared with specific inhibitors of self-assembly such as fibrinogen and fragment D [1]. The effect of the peptides then gradually decreased, and at $\alpha \geqslant 0.75$ it disappeared completely. The peptides probably form dissociating complexes with FM [12]. In the course of self-assembly of the FM molecules the number of polymerization centers, with which peptide molecules form complexes, is reduced. During lengthening of the polymer chains, inhibitor molecules are displaced. After centrifugation of the clot thus formed, the supernatant contained the original peptide concentration, i.e., peptide was absent in the fibrin gel.

Peptides containing the Gly-Pro-Arg sequence thus affect both the enzymic stage of clotting and the stage of polymerization. Peptides probably prevent the hydrolytic action of thrombin due to binding with the central region of the fibrinogen molecule [14]. Inhibition of the polymerization process can be explained by interaction between the peptides and peripheral D-domains of the protein [11, 12]. Among the peptides investigated, pentapeptide IV had the highest activity.

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HEPATOTROPIC ACTION OF BENZOBAMIL IN CCl_4 POISONING

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Most of the known antiepileptic agents (phenobarbital, phenytoin, primidone, carbamazepine, sodium valproate) possess hepatotoxicity and are contraindicated by liver diseases [8]. The exception is benzobamil, a derivative of benzoylbarbituric acid, which not only does not disturb liver function, but can also stimulate regeneration of the liver as a mild inducer of the cytochrome P-450-dependent microsomal multipurpose oxidase system [6].

This paper gives data on the effect of benzobamil on metabolic parameters and liver function of rats with severe CCl_4 hepatitis.

EXPERIMENTAL METHOD

Experiments were carried out on 180 male albino rats weighing 200-220 g. For 4 days the animals received by intragastric injection a 50% solution of CCl_4 in olive oil in a dose of 2.5 ml/kg body weight, accompanied by an aqueous suspension of benzobamil in a dose of 75 mg/kg, equivalent to 0.05 LD_{50}. Control animals received CCl_4 and the same volume of distilled water. Activity of various enzymes, and concentrations of RNA, glycogen, protein, and lipids were determined in frozen sections of the liver by histochemical and cytophotometric methods [5]. In survey films stained with hematoxylin and eosin the number of necrotic hepatocytes was counted among 2000 cells in 40 fields of vision. The state of the liver function was judged from the retention of bromsulphthalein (BSP) 45 min after intravenous injection of the dye, and the duration of sleep after intraperitoneal injection of hexobarbital (80 mg/kg body weight). The total content and content of individual fractions of lipids and phospholipids in lipid extracts of the liver [11] were studied by one-layer chromatography on Silufol UF-245 plates (Czechoslovakia) [1], diene conjugates (DC) were determined as in [3], Schiff bases as in [4], and antiradical activity of the lipids as in [12]. The content of reduced glutathione [13] and the kinetics of malonic dialdehyde (MDA) formation for a period of 60 min during stimulation of lipid peroxidation *in vitro* by Fe^{++} and ascorbic acid or by an enzymic method [3] were determined in homogenates of the liver, perfused with KCl and Tris-buffer (pH 7.4). Activity of urocaninase [2], alkaline phosphates (AlP) [10], phospholipase A [7], lactate dehydrogenase (LDH) isozymes [9], aspartate-aminotransferase (AST), and alanine aminotransferase (ALT), and concentrations of total lipids, total and bound bilirubin, and low- and very low-density lipoproteins [4] were measured in the blood serum.

EXPERIMENTAL RESULTS

CCl_4 causes severe liver damage, affecting the bioenergetics and carbohydrate, lipids, and pigment metabolism. On the 4th day of poisoning activity of the cytoplasmic enzymes LDH

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