Our investigation shows that in the case of prolonged use of phencarol the rate of absorption of the preparation from the gastrointestinal tract, the nature of its distribution among the organs and tissues, and the pathways of elimination from the organism do not differ from these indices after a single administration of the preparation.

In the tissues of the lungs, liver, spleen, brain, in the skin and blood, the specific radioactivity and rate of elimination of the preparation (or products of metabolism) after single and prolonged use are practically the same. In the adipose tissue and kidney tissue, after repeated administrations of phencarol the specific radioactivity is higher than after a single administration. The possibility remains that prolonged administration of the preparation results in its deposition in the adipose tissue, while reabsorption of phencarol or products of its metabolism is increased in the kidneys.

However, as a result of the acceleration of the elimination of labeled products from these tissues (increase in elimination constant) 12 h after the beginning of the experiment, the specific radioactivity in the adipose tissue and kidneys becomes the same in animals that received the preparation once and over a longer period of time.

After repeated administrations, the elimination of the preparation with the feces and urine slows down somewhat: Thus, in 96 h of the experiment, after repeated administrations, 6.5% less radioactivity is eliminated with the feces and 3.7% less with the urine than in the case of a single administration. Thus, in 96 h, after repeated administrations of phencarol, only 10.1% less radioactivity is eliminated than after a single administration, i.e., there is no pronounced accumulation in the case of long-term use of the preparation.

LITERATURE CITED

1. M. É. Kaminka, R. A. Spryshkova, and I. V. Golovanova, Farmakol. Toksikol., No. 3, 279-283 (1978).
2. A. A. Golubev, E. I. Lyublina, N. A. Tolokontsev, et al., Qualitative Toxicology [in Russian], Leningrad (1973), pp. 108-109.

SPONTANEOUS INACTIVATION OF RITETRONIUM IN AQUEOUS BUFFER SOLUTIONS AND PHYSIOLOGICAL MEDIA

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UDC 615.216.5.015.25

It is known that most of the myorelaxants used are very slowly (hours and tens of hours) eliminated from the organism, mostly in the unchanged state [1-3]. The restoration of muscle contractions under the action of these myorelaxants is explained by the redistribution of the myorelaxant from the muscles to other organs and tissues. Under given conditions, the subsequent entry of the myorelaxant from these tissues into the blood can cause recurrence of paralysis of the skeleton muscles, up to complete stoppage of respiration — the recurarization [2]. In certain cases (during disturbances of the water—electrolyte balance, vascular hypotonia, potentiation of myoparalytic effect by fat-soluble anesthetics, disturbance of microcirculation, etc.), neuromuscular block, uncontrolled with respect to the reaction rate and duration, can also be caused by the inability of the myorelaxants received to be inactivated *in vivo*. The recurarization can be completely prevented only if the myorelaxant is inactivated in the organism at a rate commensurable with the time of operation. Of the antidepolarizing myorelaxants used in practice, only AN-8165 has such property [4]. We believe that the disadvantage of this preparation is that its inactivation occurs as the result of the interaction with HYPER·H-dependent reductase of microsomal liver cells, whose activity may vary.

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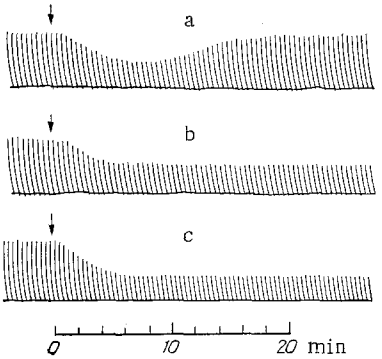


Fig. 1. A 50% blockade of diaphragm contractions of a rat, caused by ritetronium and its transformation product imidoamide (II). a) In Krebs solution at pH 7.4, concentrated solution of ritetronium at pH 4.0: concentration of ritetronium (in brackets — minutes) 100% (0), 70% (8), 57% (17), 50% (20); initial concentration (C_0) $1.6 \cdot 10^{-5}$ M; b) in Krebs solution at pH 8.2, concentrated solution of ritetronium at pH 8.2: 100% of imidoamide II; c = $1.8 \cdot 10^{-4}$ M; c) in Krebs solution at pH 7.4, concentrated solution of ritetronium at pH 7.4; 13% of ritetronium — $1 \cdot 10^{-5}$ M; 87% of imidoamide (II) — $7 \cdot 10^{-5}$ M.

The inactivation of the myorelaxant ritetronium (I) described below is related to a more persistent factor. Ritetronium is a new highly active nondepolarizing myorelaxant of the d-tubocurarine type [5]. When studying its physicochemical properties, we found that in aqueous solutions, at pH close to the physiological, the compound undergoes a certain transformation. It has been assumed that the preparation can undergo a similar transformation *in vivo* in the blood plasma and in the intercellular fluid (pH 7.4). Preliminary investigations have shown that the rate of the transformation of ritetronium *in vitro* in the blood serum of rabbits, in the blood plasma of dog and in man, is commensurable with the duration of the myoparalytic block *in vivo* (~30 min); the blocking effect, determined on an isolated diaphragm of a rat, placed in Krebs solution at pH 7.4, spontaneously decreased with time (Fig. 1a), while the magnitude of the blocking action caused by d-tubocurarine remained constant. The restoration of the contractions of an isolated diaphragm of a rat in the presence of ritetronium is clearly due to its transformation into a less active compound. Since it became clear that the phenomenon discovered of the inactivation of the preparation must be taken into account in practice, the nature of the process and factors influencing its rate, as well as the structure of the transformation products and their curare activity, had to be clarified. We believe that the present article will give a fairly good answer to these problems.

The transformation of ritetronium *in vitro* in the blood plasma at pH 7.4 and at 37°C was controlled by the change in its UV spectrum. The half-transformation time of the preparation in the plasma and in physiologically balanced salt system glucosol [6] was found to be the same, and the UV spectra of the preparation incubated up to completion of the transformation in the blood plasma, glucosol, andphosphate buffer systems were found to be authentic (Fig. 2). This indicated the absence of the influence of plasma enzymes on transformation of ritetronium and made it possible to use buffer systems for studying the process.

Myorelaxant I is a derivative of the diimide of 1,4,5,8-naphthalenetetracarboxylic acid. Under the given conditions, for a compound with such a structure two types of transformations are possible. The first type is hydrolysis at the imide bonds, with the formation of an imidoamide (II) and (or) diamide (III). Further hydrolysis of the amides formed can lead to di- tri- and tetracarboxylic acids. The second type of transformation, the dequaternization, i.e., splitting of ethyl groups from the ammonium group nitrogen atom as the result

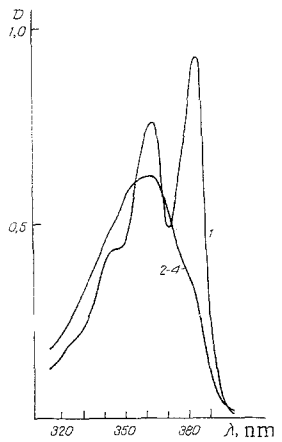


Fig. 2

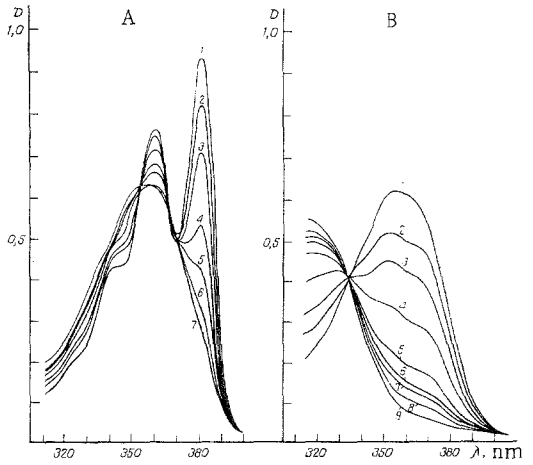


Fig. 3

Fig. 2. UV spectra of ritetronium (c = $2.6 \cdot 10^{-5}$ M). 1) In water at pH 4.0; 2-4) in buffer at pH 7.4. Spectra 2-4 were obtained after incubation at 37°C of concentrated solutions of ritetronium in a phosphate buffer, glucosol, and blood plasma at pH 7.4.

Fig. 3. UV spectra of ritetronium in phosphate buffer solutions after incubation at 37°C. A) Number of compound (in brackets—pH): 1 (4.0), 2 (6.0), 3 (6.35), 4 (6.76), 5 (7.05), 6 (7.41), 7 (8.1); B) correspondingly 1 (8.1-8.3), 2 (9.2), 3 (9.5), 4 (9.85), 5 (10.2), 6 (10.35), 7 (10.45), 8 (10.6), 9 (10.9).

of transalkylation with blood plasma proteins, should be excluded, since no influence of enzymes was observed, while the UV spectra of dequaternized derivatives differ greatly from the UV spectra of the transformation products.

The potentially possible hydrolysis at the amide bonds should be accompanied by separation of amine (IV). We therefore developed a spectrophotometric procedure for the determination of this amine according to the formation of a Schiff base with p-dimethylaminobenzaldehyde (V) in DMSO. When this method was used to check the buffer solutions of ritetronium in the pH range of 6.0-8.0, incubated at 37°C up to completion of the transformation, for the presence of amine, the results were negative. No amine was formed during the incubation of the preparation at higher pH values (up to 10.5) also.

Thus, the absence of amine IV after completion of the transformation of the myorelaxant excludes the possibility of its hydrolytic splitting, and hence, as the result of the hydrolysis of ritetronium, only products II and III, or their mixture, or a mixture of these products with starting compound I, can be formed. In other words, the reaction mixture can be one-, two-, or three-component.

We assumed that the hydrolysis of the preparation should be accompanied by consumption of alkali and carried out a potentiometric titration of an aqueous solution of ritetronium at 37°C. The potentiometric titration curve had two jumps corresponding to the addition of 1 and 2 equivalents of alkali (pK_{a_1} = 6.6 and pK_{a_2} = 9.6). The curves of direct and back-titration coincided, which indicated the absence of side reactions. At the same time, we ran the UV spectra of the solutions at different pH values, obtained in the course of the titration after the addition of portions of alkali or acid. The UV spectra of the solutions at the same pH values, obtained as the result of direct and back-titration, also coincided, which indicated the reversibility of the process.

The UV spectra of the preparation in buffer solutions at different pH values formed two families of curves (Fig. 3): one in the pH range of 4.0-8.2, and the second in the 8.2-10.2 pH range. Each family of curves has not fewer than two isobestic points, which confirms the data obtained during the potentiometric titration and makes it possible to assume the existence of a two-component system in each pH range [7]. The composition of the solutions calculated by the method in [8] coincided with that calculated according to

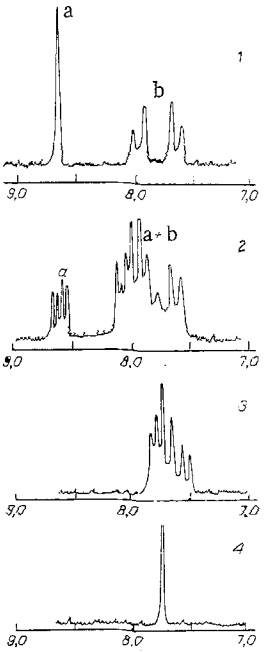
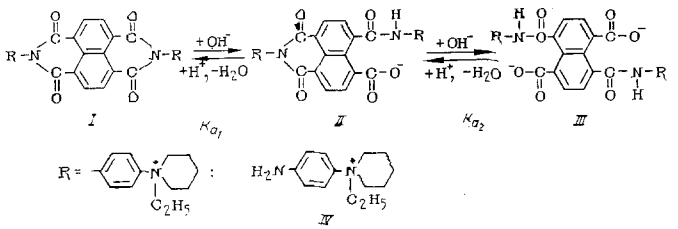


Fig. 4. PMR spectra in D_2O of ritetronium (1-3), and naphthalene-1,4,5,8-tetracarboxylic acid (4). 1) pH 4.0, a/b = 4/8; 2) pH 8.2, a/a + b = 2/10; 3) pH 10.2; 4) pH 11.5, a and b are proton signals of naphthalene and benzene rings, respectively.

the data of the potentiometric titration, which served to confirm our assumption. The UV spectra of the ritetronium solutions at pH 8.2-8.3 and 10.2-10.5 were identical with those of the solutions of the preparation after the addition of 1 and 2 equivalents of alkali and coincide with the spectra of the individual compounds II and III. This conclusion is conclusively confirmed by the proton magnetic resonance spectra (PMR) of these solutions in the absorption region of aromatic protons (Fig. 4).

Hence, the transformation of ritetronium in buffer and physiological solutions is a reversible hydrolysis reaction at the imide bonds only.

At pH 4.0 and below, ritetronium is not appreciably hydrolyzed; in the pH 6.0-8.3 range, a mixture of the diimide (I) with the imidoamide (II) exists in the solution, and in the pH range of 8.3-10.2, a mixture of the imidoamide (II) and diamide (III). At pH 7.4, under the equilibrium conditions, ritetronium exists in the form of a mixture of 13% of diimide (I) and 87% of imidoamide (II).



The myoparalytic activity of imidoamide (II) was found to be one order of magnitude lower than that of ritetronium. In experiments on isolated diaphragm of a rat, ritetronium blocked the contractions at a concentration of $1 \cdot 10^{-5}$ M, and the imidoamide II, at $1.8 \cdot 10^{-4}$ M (Fig. 1). Ritetronium in a dose of 0.17 μmole/kg blocked the muscle contractions of a cat, and the imidoamide II, in a dose of 1.8 μmole/kg.

To clarify the factors influencing the rate of the hydrolysis reaction of ritetronium, we studied the kinetics of this reaction under pseudomonomolecular conditions. In the

organism, the preparation is distributed between the intercellular fluid and the blood plasma, in which the principal inorganic anions are the diphosphate ion and bicarbonate ion, respectively. The reaction kinetics in the phosphate buffer systems in the pH 6.0-8.0 range at 37°C was of the first order type, and is described by Eq. (1), and the rate constant by Eq. (2):

$$D_t = D_0 \cdot e^{-K_{obs}\ t} \tag{1}$$

$$K_{obs} = \frac{2,3}{t} \lg \frac{D_0 - D_\infty}{D_t - D_\infty}, \tag{2}$$

where D_0, D_t, and D_∞ are the optical densities at the initial and current moments of time, and after equilibrium is reached, respectively. The linear dependences of the reaction rates on the hydroxyl and diphosphate ion concentration show that the two anions participate in the hydrolysis reaction. From these linear dependences, we found the catalytic constants of the second order $k_{OH^-} = 360\ M^{-1}/liter \cdot sec^{-1}$ and $k_{HPO_4^{-2}} = 7.4 \cdot 10^{-3}\ M^{-1}/liter \cdot sec^{-1}$. The contribution of water to the hydrolysis reaction was small

$$k_{H_2O} = 6 \cdot 10^{-5}\ sec^{-1}.$$

The catalytic rate constant of the hydrolysis by bicarbonate was estimated indirectly, by comparing the rate of hydrolysis of ritetronium in glucosol with the rate calculated from the values of the k_{OH^-} and $k_{HPO_4^{-2}}$ constants found. The experimentally determined rate was found to be twice as high. This difference may be due to the contribution of catalysis by the bicarbonate ion to the observed rate of hydrolysis, since this ion is the principal component of glucosol. The value of $k_{HCO_3^-} = 13 \cdot 10^{-3}\ M^{-1}/liter \cdot sec^{-1}$ obtained is comparable with $k_{HPO_4^{-2}}$, which agrees with the data on the nucleophilicity of these anions [9]. Thus, the expression for the rate of hydrolysis of ritetronium in physiological media, taking into account all the principal contributions, can be described by the equation:

$$k_{hydr} = 6 \cdot 10^{-5} + 7.4 \cdot 10^{-3} \left[HPO_4^{-2} \right] + 1.3 \cdot 10^{-2} \left[HCO_3^- \right] + 360 \cdot [OH^-]. \tag{3}$$

In the pH 6.4-8.0 range, the rate of the reverse reaction, i.e., the cyclization of the imidoamide II into ritetronium, is low, but at pH < 5.0 it dominates over the hydrolysis and is described by the kinetic equation:

$$k_{cycl} = 6 \cdot 10^{-5} + 0.61 \cdot H^+ + k_{AH^+} \cdot [AH^+], \tag{4}$$

where k_{AH^+} is the catalytic constant of cyclization of a conjugated acid AH^+; for acetic acid it corresponds to $2.7 \cdot 10^{-3}\ M^{-1}/liter \cdot sec^{-1}$ at 37°C. In the pH range of 6.4-8.0, k_{cycl} is independent of pH, and its numerical value is close to k_{H_2O} and is equal to $6 \cdot 10^{-5}\ sec^{-1}$.

Thus, from the experiments *in vitro*, we can conclude that the ritetronium introduced into the organism, in blood and intercellular fluid, will be hydrolyzed by the action of OH^-, HCO_3^-, and HPO_4^{-2} ions. Twenty minutes after administration, the preparation will be hydrolyzed to a minimum of 50%, and after 2 h, the content of slightly active imidoamide II reaches 87%. Since the curare activity of imidoamide II is lower by one order of magnitude than that of ritetronium, we can conclude that when this preparation is used, recurarization is not very probable.

EXPERIMENTAL (CHEMICAL)

The PMR spectra were run on the "Varian HA 100" spectrometer in H_2O. Tertiary butyl alcohol served as the internal standard. The UV spectra were recorded on the SF-8 spectrophotometer. For the kinetic measurements, we used the SF-4A spectrophotometer. The potentiometric measurements were carried out by the pH 673 pH-meter. The ritetronium compound used was analytically pure; control methods have been described in [10]. Chemically pure sodium acetate, chemically pure potassium monophosphate, and chemically pure sodium diphosphate were recrystallized from water; chemically pure acetic acid and chemically pure potassium chloride were used without additional purification. In the investigations we used monodistilled water. The rabbit serum and the dog and human plasma were obtained from the Leningrad Blood Transfusion Institute. In the pH 6.4-7.9 range, we used 0.15 M phosphate buffer solutions, and in the pH 3.8-5.15 range — 0.2 M acetate buffer solutions. In all the solutions the ionic strength was maintained at the 0.5 M level by the addition of potassium chloride.

Hydrolysis of Ritetronium in Glucosol, Blood Serum, or Blood Plasma. The hydrolysis was carried out in a thermostated cell at 37°C. To the solution at pH 7.45 ± 0.05, a concen-

trated solution of the substrate was introduced, and the working concentration was about 10^{-3} M. The pH was established by passing carbon dioxide with argon (5% of CO_2). An aliquot portion of the reaction mixture was diluted by a pH 4.0 buffer (thus the course of the reaction was interrupted), and a spectrophotometric determination was carried out. Invariability of the spectrum during 2-3 h was accepted as an indication that the reaction was concluded.

Potentiometric titration was carried out in a thermostated cell at 37°C. The constants were calculated by the usual procedure [11].

The kinetic measurements were carried out spectrophotometrically according to decrease (during opening of the imide ring) or increase (during cyclization of imidoamide II) of the absorption at 382 nm and 37°C ± 0.1°C. The concentrated solution of the substrate (10-15 μliter) was introduced into cuvette with a cover with 3 ml of a buffer solution, placed in a thermostated block. All the experiments were carried out up to a steady value of optical density, which was taken as D_∞. The observed rate constant of the pseudo-first order (k_{obs}) was found from the slope of the linear dependence of $-\log (D_t - D_\infty)$ for the hydrolysis of the diimide I, or $-\log (D_\infty - D_t)$ for the cyclization of the imidoamide II with time. The linearity was retained up to 5 half-transformation times. In the calculation, only those values of k_{obs} were accepted if the scattering between them did not exceed 5%.

Reaction of Amine IV with Aldehyde V. A 0.3 ml portion of the aqueous solution of amine IV at a concentration of $5\cdot10^{-4}$-$5\cdot10^{-3}$ M was added to 3 ml of a 1 M solution of V in DMSO. After 10 min from the development of color, the solution was determined photometrically at 450 nm. From the linear dependence of D on the concentration of amine V, it was possible to determine amine IV up to a concentration of $2\cdot10^{-4}$ M, i.e., from 100 to 40% of amine IV, if one of one amide group of compound II is hydrolyzed, or from 50 to 20% if two groups of compound III are hydrolyzed.

EXPERIMENTAL (PHARMACOLOGICAL)

The myoparalytic activity of ritetronium and imidoamide II was determined on a frenico-diaphragmal preparation of a rat and on a muscle of a cat, as described in [12, 13]. The left hand-side part of the diaphragm with the diaphragmal nerve was placed into a 50-ml bath containing Krebs solution (37°C), through which carbon dioxide was passed. The constancy of the pH was controlled by a pH 673 pH-meter. The compounds were introduced into the bath with the muscle in an amount of not more than 0.2 ml in the form of a concentrated solution of the preparation. The concentration of the preparation was chosen so that the isometric contractions of the muscle caused by irritation of the nerve decreased by 50% (EC_{50}). The action of ritetronium as a function of pH was studied on a diaphragm of a rat in three series of experiments, five experiments in each series (Fig. 1).

In the first series, the concentrated solution at pH 4.0 contained ritetronium and Krebs solution at pH 7.4. In contrast to d-tubocurarine, the decreased muscle contractions were restored to the initial level without washing out of the preparation. In the second series, a concentrated solution at pH 8.2 contained imidoamide II and Krebs solution at pH 8.2; EC_{50} was $(1.8 \pm 0.2)\cdot10^{-4}$ M. In the control experiments, the activity of d-tubocurarine did not change with change in pH from 7.4 to 8.2. In the third series, a concentrated solution at pH 7.4 contained 13% of ritetronium and 87% of imidoamide II and a Krebs solution at pH 7.4. The total EC_{50} was $(8.0 \pm 1.2)\ 10^{-5}$ M, i.e., the EC_{50} of ritetronium was equal to $1\cdot10^{-5}$ M and of the imidoamide II, $7\cdot10^{-5}$ M.

In experiments on cats, the ritetronium was estimated from its ability to block the isometric contractions of the forward tibial muscle, caused by irritation of the motive nerve. Ritetronium was administered intravenously in a dose of 0.2 ml from a solution at pH 4.0, and imidoamide II from a solution at pH 8.2. Ritetronium blocked the contractions by 50% in a dose of 0.17 ± 0.01 μmole/kg, and imidoamide II in a dose of 1.8 ± 0.2 μmole/kg (5 experiments). The duration of the action of ritetronium in the experiments on cats was close to that for tubocurarine.

LITERATURE CITED

1. J. Maclagan, in: Neuromuscular Junction (E. Zaimis, ed.), New York (1976), pp. 421-486.
2. V. A. Kovanev, Ya. M. Khmelevskii, and F. F. Beloyartsev, Muscle Relaxants in Anesthesiology [in Russian], Moscow (1970), pp. 187-210.

3. C. Chagas, L. Sollergo, and G. Suarez-Kurtz, in: Neuromuscular Blocking and Stimulating Agents, Vol. 1 (J. Cheymol, ed.), Oxford (1972), pp. 409-423.
4. C. E. Blogg, R. T. Brittain, B. R. Simpson, et al., Brit. J. Pharmacol., 53, 446 (1975).
5. E. I. Krasnova, in: Pharmacology and Toxicology of New Products of Chemical Synthesis [in Russian], Minsk (1975), pp. 107-109.
6. D. Pol, Cells and Tissue Culture [Russian translation], Moscow (1963), p. 96.
7. I. Ya. Bershtein and Yu. L. Kaminskii, Spectrophotometric Analysis in Organic Chemistry [in Russian], Leningrad (1975), p. 34.
8. K. Vierordt, Use of Spectral Apparatus in Photometry of Absorption Spectra and in Quantitative Chemical Analysis [in German], Tübingen (1873).
9. P. William et al., J. Am. Chem. Soc., 82, 1778-1786 (1960).
10. G. M. Kheifets, N. V. Martyushina, T. A. Mikhailova, et al., Zh. Organ. Khim., 13, 1262-1269 (1977).
11. A. Albert and E. Sargent, Ionization Constants of Acids and Bases [Russian translation], Moscow–Leningrad (1964), pp. 21-40.
12. Pharmacological Experiments on Isolated Preparations, Edinburgh (1970), pp. 30-37.
13. Pharmacological Experiments on Intact Preparation, Edinburgh (1970), pp. 37-48.

STUDY OF THE MOLECULAR MECHANISM OF THE INHIBITION OF FIBRINOLYSIS BY ε-AMINOCAPROIC ACID

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Numerous experimental investigations have now shown that ω-amino acids are specific synthetic inhibitors of the fibrinolytic process. Among them, ε-aminocaproic acid (I) has found wide use in clinical practice.

Nonetheless, the question of the mechanism of the inhibition of fibrinolysis by synthetic substances, as before, remains open, since until recently there was no unanimity on this process. It has been shown [1-3] that at large concentrations, I modifies fibrin to a form resistant to the action of plasmin. However, the inhibition of fibrinolysis that is also observed at lower inhibitor concentrations cannot be explained on the basis of the postulated mechanism. A number of authors [4-6] believe that antifibrinolytics act at the stage of activation of plasminogen to plasmin. It was subsequently shown that synthetic inhibitors possess low antiactivator capacity [7]. The basic action of antifibrinolytics is associated with their interaction with plasmin. The influence of inhibitors on the hydrolytic activity of plasmin is explained by their binding to the latter on specific sites differing from the catalytic, which determines the inhibition of the entire process [8-10]. The contradictions in the factual material found by different authors are caused by the variety of methodological approaches that have been used in revealing the nature of the inhibiting action of ω-amino acids.

Definite successes in solving the problem indicated above can be achieved from the thermodynamic standpoint, using a complex of methods of microcalorimetry, UV, and IR spectroscopy, which ultimately permits an estimation of the nature of the intermolecular interactions of plasminogen and plasmin with I.

EXPERIMENTAL SECTION

For the experiments we used a commercial preparation of profibrinolysin (Belorussian Republic Blood Transfusion Station), which was additionally purified on a column (60 × 3.5 cm) with Sephadex G-75, equilibrated with 0.005 N HCl, pH 3.5. Plasminogen (150 mg) was dissolved in 10 ml of HCl of the same concentration and passed through the column at a rate

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