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# COMPARATIVE ANALYSIS OF THE MEMBRANE-ACTIVE PROPERTIES OF GOSSYPOL DERIVATIVES AND THEIR POLYMERIC COMPLEXES

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*It has been shown that water-soluble complexes of gossypol derivatives obtained by their immobilization on polyvinylpyrrolidone possess a lower membrane activity than the initial substances. The degree of interaction of the substances under investigation with biomembranes has been determined from the change in the intensity of the fluorescence of 1-anilinonaphthalene-8-sulfonic acid (ANS).*

Some gossypol (GP) derivatives obtained by condensation through its aldehyde groups [1, 2] possess a broad spectrum of pharmacological activity [3], one of the possible mechanisms of the realization of which is interaction with biological membranes.

The membrane activity of GP derivatives is determined by the nature of the substituents at the aldehyde groups [4]. Thus, the product of the interaction of GP with barbituric acid (compound (**1**)) obtained by Knoevenagel condensation inhibits $Ca^{2+}$-ATPase more effectively and increases the permeability of the membranes of the sarcoplasmic reticulum (SR) of the skeletal muscles of the rabbit to a greater degree than the Schiff base obtained from GP and sodium $\alpha$-aminoethanesulfonate (compound (**2**)).

The water-insolubility of compounds (**1**) and (**2**) restricts their use as physiologically active substances. In order to convert them into water-soluble forms, they have been immobilized on polyvinylpyrrolidone (PVP) with the formation of complexes containing 9.0-10.0% of active substance.

We have investigated the influence of the complex of compound (**1**) with PVP (compound (**3**)) and the complex of compound (**2**) with PVP (compound (**4**)) on the functional parameters of the transport of $Ca^{2+}$ in the SR of the skeletal muscles of the rabbit, on the release of $Ca^{2+}$ from vesicles of SR and phosphatidylcholine (PC) liposomes loaded with calcium, on the hemolysis of erythrocytes, and on the osmotic properties of rat liver mitochondria. The membranotropic properties of the complex compounds were studied in comparison with those of the initial preparations. The interaction of these preparations with the membranes was evaluated from the change in the intensity of the fluorescence of 1-anilinonaphthalene-8-sulfonic acid (ANS).

Figure 1 shows the dependence of the degree of inhibition of $Ca^{2+}$-ATPase of the SR of rabbit skeletal muscles (structures responsible for the regulation of the concentration of $Ca^{2+}$ ions in the cytoplasm) on the amount of the substances investigated: 50% inhibition of the activity of the enzyme was found at 80 and 170 nmole/mg of SR protein, respectively, for compounds (**1**) and (**2**). For the polymeric complexes of these preparations their inhibiting action on the enzyme was appreciably decreased. Thus, 50% inhibition of the activity of $Ca^{2+}$-ATPase was observed for compound (**3**) at 120 nmole/mg of protein, and for compound (**4**) at 370 nmole/mg of protein.

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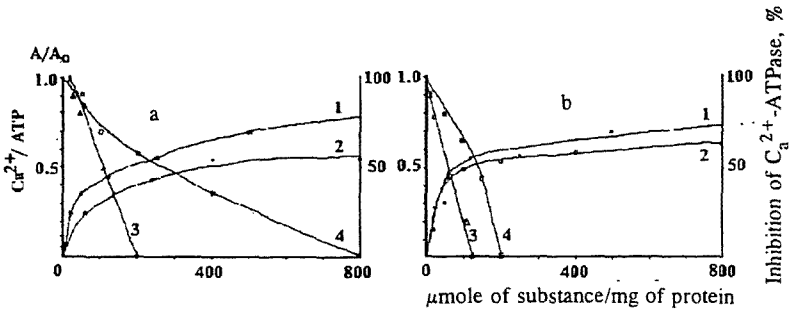


Fig. 1. Influence of compounds (**1**)-(**4**) on the value of the coefficient $Ca^{2+}$/ATP and on the activity of the $Ca^{2+}$-ATPase of the sarcoplasmic reticulum: a) 1, 3) compound (2); 2, 4) compound (**4**); b) 1, 3) compound (**1**); 2, 4) compound (**3**). The coefficient $Ca^{2+}$/ATP is represented as the ratio of the values in the experiments ($A$) and the control ($A_0$).

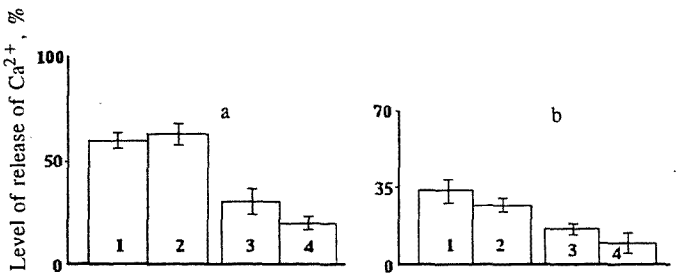


Fig. 2. Level of release of $Ca^{2+}$ from vesicles of the sarcoplasmic reticulum (a) and liposomes (b) loaded with $Ca^{2+}$ under the action of compounds (**1**)-(**4**) (1 μM), measured from the fluorescence of chlorotetracycline (CTC): 1) compound (**1**); 2) compound (**3**); 3) compound (**2**); 4) compound (**4**).

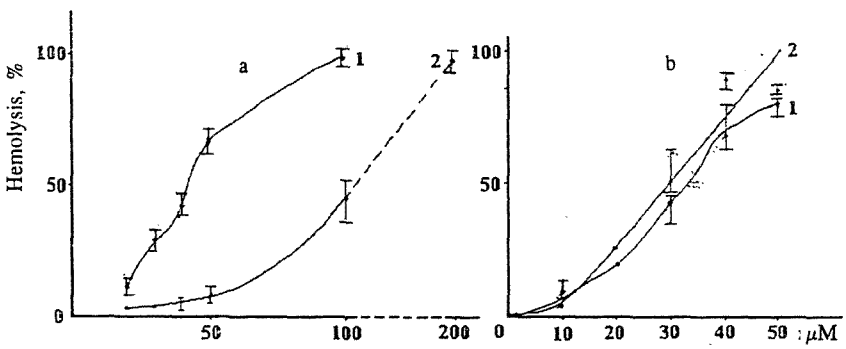


Fig. 3. Dependence of the degree of homolysis of erythrocytes on the concentrations of compounds (**1**)-(**4**): a) 1) compound (**2**); 2) compound (**4**); b) 1) compound (**1**); 2) compound (**3**).

The compounds under consideration lowered to different extents the ratio $Ca^{2+}$/ATP which characterizes the efficacy of the active transport of $Ca^{2+}$ as the ratio of the $Ca^{2+}$ absorbed by the SR membranes to the amount of ATP hydrolyzed (see Fig. 1). The compounds under investigation lowered $Ca^{2+}$/ATP by one half in the following amounts: compound (**1**) — 60; (**3**) — 130; (**2**) — 107; and (**4**) — 260 nmole/mg of protein.

Thus, the results presented show that, in comparison with the initial preparations, the water-soluble analogues of compounds (**1**) and (**2**) act approximately twice as feebly both on the permeability of the SR membranes and on the activity of the SR $Ca^{2+}$-ATPase.

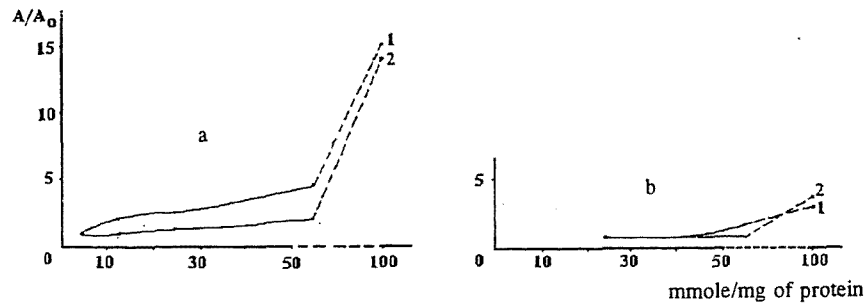


Fig. 4. Influence of compounds (**1**) and (**2**) (a) and of compounds (**3**) and (**4**) (b) on the change in permeability for sucrose of deenergized mitochondria: a) 1) compound (**1**); 2) compound (**2**); b) 1) compound (**3**); 2) compound (**4**). $A/A_0$ — ratio of the rates of swelling of the mitochondria ($\Delta E_{520}$/min) in the experiment ($A$) and the control ($A_0$).

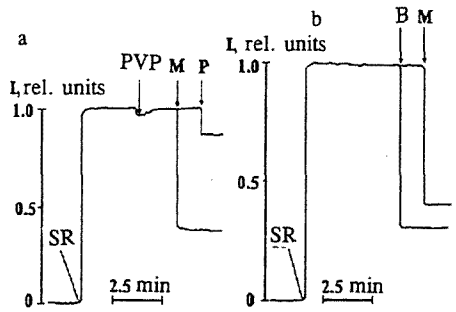


Fig. 5. Interaction of compounds (**1**)-(**4**) with the membranes of the sarcoplasmic reticulum, measured from the changes in the intensity of the fluorescence of ANS. The arrows show the moments of adding the sarcoplasmic reticulum (SR, 100 $\mu$g/ml of recording medium): a) compound (**2**) (M, 5 $\mu$M); compound (**4**) (P, 5 $\mu$M); polyvinylpyrrolidone (PVP, 5 $\mu$M); b) compound (**1**) (B, 5 $\mu$M); compound (**3**) (M, 5 $\mu$M). I) Intensity of fluorescence of ANS, rel. units.

An investigation of the influence of compounds (**3**) and (**4**) on the calcium permeability of SR membranes and PC liposomes loaded with $Ca^{2+}$ (Fig. 2) by the fluorescence of chlorotetracycline (CTC) likewise showed a fall in the membrane activity of these compounds in comparison with the initial preparations, the observed effect being more pronounced for compound (**4**). Thus, at a concentration of 1 $\mu$M, the release of $Ca^{2+}$ from SR vesicles amounted to 60 $\pm$ 2% and 64 $\pm$ 3.5%, respectively, for compounds (**1**) and (**3**), and 35.5 $\pm$ 4.5% for 23.5 $\pm$ 2.5%, respectively, for compounds (**2**) and (**4**).

When these substances were added in a concentration of 1 $\mu$M to a suspension of liposomes, the release of $Ca^{2+}$ into the medium was as follows: for compounds (**1**) and (**3**) 36 $\pm$ 5% and 29 $\pm$ 2.5%, respectively, for compounds (**2**) and (**4**) 17.5 $\pm$ 1.5% and 11 $\pm$ 3%, respectively.

In view of the changes in the membrane activity of polymeric complexes of gossypol derivatives in comparison with the initial compounds that we had detected, it appeared of interest to study their influence on the integrity of the membranes of erythrocytes, as the cells most sensitive to the introduction of pharmacological agents into the organism. Figure 3 shows the dependence of the degree of hemolysis of erythrocytes on the concentration of the substances under investigation. Compounds (**1**) and (**2**) caused 50% hemolysis of the erythrocytes at 33 and 42 $\mu$M, and compounds (**3**) and (**4**) at 30 and 140 $\mu$M, respectively. Thus, a fall in hemolytic activity was observed only for compound (**4**).

Mitochondria form a highly sensitive test system for revealing toxic effects of various compounds. An investigation of the energy-dependent swelling of mitochondria in an isoosmotic solution of sucrose enabled us to study the detergent properties of the preparations. In this connection, we examined the influence of the polymeric analogues of compounds (1) and (2) in comparison with the initial substances on the permeability for sucrose of the internal membranes of deenergized mitochondria.

According to Fig. 4, compounds (1) and (2) (100 nmole/mg of protein) increased the permeability of the mitochondrial membranes for sucrose approximately 15-fold. When similar amounts of the polymeric complexes were used, 3- to 4-fold falls in the detergent effects were observed.

Thus, in various types of membranes (SR, liposomes, mitochondria, erythrocytes) a tendency has been traced to a fall in the membrane-active properties of low-molecular-mass compounds when they are immobilized on a polymeric support. However, for compound (3) this tendency was less pronounced (particularly on erythrocytes and the SR) than for compound (4).

With the aim of elucidating the cause of this effect, we made an estimate of the interaction of preparations with SR membranes recorded from the displacement by these compounds of the fluorescent probe ANS, which is localized in the surface region of a lipid bilayer [5]. When the probe is displaced from membranes and passes into an aqueous medium, the quantum yield of the fluorescence of the ANS falls sharply, and, as a result, so does the intensity of fluorescence. As can be seen from Fig. 5, compound (4) caused an approximately twofold weaker quenching of the intensity of the ANS than compound (2). Compound (3) caused almost the same fall in the intensity of fluorescence of the probe as compound (1).

It is likely that the decrease in the displacement of ANS by the polymeric analogues in comparison with the initial preparations is connected with the incomplete splitting out of the active principle from the polymeric support, as a result of which the amount of low-molecular-mass compound competing for ANS for a binding site in the membrane will be smaller. In this way, apparently, it is possible to explain the fall in the membrane activity of the polymeric analogs of compounds (1) and (2), as well. However, we have shown that for compound (4) the fall in membrane activity and the degree of displacement of ANS from the binding site in the membrane, as compared with its initial substance — compound (2) —, is more pronounced than for compound (3), which is close in its effects to compound (1). The observed difference can be explained by different lipotropicities of compounds (1) and (2), which are the active principles of compounds (3) and (4), as a result of which, on redistribution between the membrane and the PVP, the amount of compound (1) in the membrane will be higher than that of compound (2). This explanation is supported by results that we obtained previously by the ESR–spin probe method [6]. Thus, it was established that compound (1) induces structural rearrangements of the lipid matrix membranes to a greater degree than (2).

The differences found in the degrees of modifying action of compound (1) on the one hand, and (2), on the other hand, depend on the nature of the substituents in the aldehyde groups of the gossypol, since the hydrophobic sections of the two molecules are invariant. Compound (1) includes a fragment containing proton-donating and proton-accepting groups the positions of which in the geometry of the fragment itself make possible the simultaneous formation of two hydrogen bonds with the carbonyl groups of phospholipids which is perhaps the reason for the preferential "recognition" of just this derivative by membranes.

## EXPERIMENTAL

The UV spectra of the gossypol derivatives were taken on a CF-26 spectrophotometer ($c$ = 0.02%). IR spectra were recorded on a Specord 71 IR spectrophotometer in a mull of paraffin oil. TLC was conducted on a Silufol UV-254 plate.

**1,1′,6,6′,7,7′-Hexahydroxy-3,3′-dimethyl-5,5′-diisopropyl-8,8′-dinaphthylidene Dibarbituric Acid (Compound 1).** An alcoholic solution of 3.28 g (0.02 mole) of barbituric acid was added to a solution of 5.18 g (0.01 mole) of GP in 80 ml of ethyl alcohol heated to 70-80°C, and the heating of the mixture was continued for 2 h. The precipitate that deposited was filtered off, washed with ethyl alcohol, and dried. Yield 5.92 g (80.2%). Pulverulent red substance: mp >350°C; $R_f$ 0.80 (acetone). UV spectrum (DMSO–ethanol (1:9), $\lambda_{max}$, nm): 496 (log $\varepsilon$ 4.44). IR spectrum ($\nu$, $cm^{-1}$): 3530, 3440 (OH···OH); 1620 (C=O). Found %: N 7.60 $C_{38}H_{34}N_4O_2$. Calculated %: N 7.58.

**Disodium Salt of bis-2,2′-{[(7,7′,8,8′-tetrahydro-1,1′,6,6′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-7,7′- dioxo)-2,2′-binaphthyl]-8,8′-methylenimino}ethanesulfonic Acid (Compound 2).** A solution of 15.0 g (0.37 mole) of caustic soda in 375 ml of absolute ethyl alcohol was treated with 47.2 g (0.37 mole) of β-aminoethanesulfonic acid, and the mixture was

heated for 3 h. A solution of 65.0 g (0.12 mole) of GP in 350 ml of absolute ethyl alcohol was added to the resulting solution, and the reaction mixture was heated in the water bath for 3 h. The precipitate that deposited was filtered off and was washed with ethyl alcohol and diethyl ether and dried. Yield 100.0 g (98.5%). Amorphous yellow powder: mp >360°C, $R_f$ (acetone–toluene (5:2)). UV spectrum (acetone–water (3:1), $\lambda_{max}$, nm): 385, 404 (log $\varepsilon$ 4.15, 4.14). IR spectrum ($\nu$, $cm^{-1}$): 3530, 3440 (OH···OH), 1625 (C=O), 1240, 1030 (O=S=O). Found %: N 3.75 $C_{34}H_{38}N_2O_{12}Na_2S_2$. Calculated %: N 3.46.

Compounds (**3**) and (**4**) were obtained as in [7].

**Compound 3.** Amorphous powder, from orange to red-orange, readily soluble in water and in the majority of organic solvents, mp 270-275°C, $R_f$ 0.80-0.82 (acetone). UV spectrum (DMSO–ethanol (1:9), $\lambda_{max}$, nm): 320, 480. UV spectrum ($H_2O$, $\lambda_{max}$, nm): 260, 500. IR spectrum ($\nu$, $cm^{-1}$) 3330, 3320 (OH···OH), 1650 (C=O). The proportion of compound (**1**) in the complex was determined spectrophotometrically as 9.00-9.2 wt.-%.

**Compound 4.** Yellow pulverulent substance, mp 185-187°C, $R_f$ 0.40 (acetone–toluene (5:2)). UV spectrum (acetone–water (3:1), $\lambda_{max}$, nm), 245, 385. IR spectrum ($\nu$, $cm^{-1}$) 3330, 3220 (OH···OH), 1645 (C=O). The proportion of compound (**1**) in the complex was determined spectrophotometrically as 9.0-9.4 wt.%.

Fragments of the SR were isolated from rabbit skeletal muscles by Ritov's method [8]. ATP-ase activity and the transport of $Ca^{2+}$ were investigated by the pH-metric method described previously [4]. Protein was determined by means of the biuret reaction [9]. Liposomes containing 10 mM $CaCl_2$ were obtained from 10% egg lecithin using the anion-exchange resin Dowex (1×1) for eliminating the detergent — cholic acid [10]. The accumulation of $Ca^{2+}$ in the SR membranes and the permeability of the SR membranes and liposomes for $Ca^{2+}$ were measured from the change in the intensity of fluorescence of chlorotetracycline (CTC). For general observations, see [4].

The hemolytic activities of the preparations were determined spectrophotometrically at a wavelength of 540 nm from the release of hemoglobin by erythrocytes resuspended in a medium containing 140 mM NaCl, 10 mM Tris citrate buffer, pH 7.2, and 1 mM EDTA. The optical density corresponding to 100% hemolysis was determined after the lysis of control samples of erythrocytes in distilled water. Mitochondria were isolated by Shneider's method [11] from rat liver. The detergent properties of the preparations were investigated by means of the energy-dependent swelling of the mitochondria in an isoosmotic solution of sucrose using a procedure described by Brierley [12]. The interaction of the preparations with the SR membranes was determined from the change in the fluorescence of ANS [5] on a SFR spectrofluorimeter (Russia) in a medium containing 100 mM KCl, 30 mM Tris-HCl, pH 7.0. The ANS concentration was 0.5 $\mu$M. The reticulum preparation was added in an amount of 100 $\mu$g of protein per 1 ml of incubation medium. The wavelength of the exciting light was 364 nm and that of the emitted light 480 nm.

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