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16α,17α-Cycloalkane Derivatives of Progesterone Intensively Bind to a Rat Serum Protein

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Abstract—The interaction of 6α-methyl-[1,2-^{3}H]16α,17α-cyclohexanoprogesterone with rat serum proteins has been studied. Specific binding of this ligand characterized by $K_d = 0.36 \pm 0.10$ μM and concentration of binding sites (B_{max}) of about 1 μM (27.8 ± 12.5 pmol/mg total protein) was found. According to competitive analysis, the affinity of the studied progestins to a protein that differs from transcortin was to some extent correlated with their hydrophobicity. The dissociation kinetics of ^{3}H-ligand–protein complexes were biphasic, the binding sites forming stable and labile complexes with ^{3}H-ligand being eluted in the same region during ion-exchange chromatography. In overall properties, the serum protein differs from the progesterone receptor and the pregna-D′-pentarane-specific protein from rat uterus. It is suggested that the revealed protein may provide high progestagenic activity of 6α-methyl-16α,17α-cyclohexanoprogesterone by prolonging its retention in the bloodstream.

Key words: progestins, steroids, progesterone analogs, transport proteins, binding kinetics

Pentacyclic progesterone derivatives (pregna-D′-pentaranes) are promising compounds for clinical applications. First, many of them, unlike the natural hormone progesterone, retain their hormonal activity on oral administration. Second, some pregna-D′-pentaranes show properties of partial agonists/antagonists that selectively reproduce some effects of progesterone and block others [1, 2]. Studies on interactions of these compounds with progesterone receptor (PR) showed that the kinetic parameters of their binding to PR are comparable to those of progesterone itself and that these parameters do not correlate with the type of biological activity of the compound [3-6]. The use of tritium-labeled pregna-D′-pentaranes revealed in rat uterus an additional (different from PR) protein that specifically binds pregna-D′-pentaranes; its function is unknown, but it is probably related to the effects of these steroids [6, 7]. Because of the inevitability of contamination of tissue extracts with admixtures from blood, the question arose whether the revealed protein in uterus is a transport protein of the blood. The results of the present study on the interactions of pregna-D′-pentaranes with serum proteins answer this question negatively. The data on high binding capacity and moderate affinity of the serum protein for pregna-D′-pentaranes can explain in part the high biological activity of these compounds by their prolonged presence in the blood stream due to their complexing with the revealed serum protein.

MATERIALS AND METHODS

All reagents used were of analytical grade. [1,2,6,7-^{3}H]Progesterone with specific radioactivity of 86 Ci/mmol (St. Petersburg, Russia) and 6α-methyl-[1,2-^{3}H]16α,17α-cyclohexanoprogesterone (43 Ci/mmol) synthesized by us [8] were used. Unlabeled pregna-D′-pentaranes were synthesized as described previously [1, 9]. Promegestone (R5020) was purchased from New England Nuclear (USA). Activated charcoal was from

Abbreviations: PR) progesterone receptor; RBA) relative binding affinity.

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Serva (Germany), Dextran-70 from Fluka (Switzerland), and BSA from Dia-M (Moscow, Russia). Other unlabeled steroids and reagents were purchased from Sigma (USA). Buffer solutions with pH 7.5 at 20°C were as follows: buffer A (10 mM Tris-HCl, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 30% glycerol (v/v)); buffer B (10 mM Tris-HCl, 10 mM KCl, 1.5 mM EDTA, 10% glycerol (v/v)).

Blood serum from adult female rats weighting 200 to 250 g was used. Blood was collected on decapitation, kept for 1 h at room temperature, chilled to 0-4°C, and after 1 h centrifuged at 3,000*g* for 10 min. The serum was stored at −20°C for up to 2 months. For experiments on steroid ligand binding, the serum was diluted four-fold with buffer solution A. For chromatography, undiluted serum and buffer solution B as eluent were used. Equilibrium parameters of ligand–protein interactions were analyzed as described previously [6, 7] or by Scatchard plots [10]. The relative binding affinity (RBA) values were calculated as the ratio of K_d values for ^{3}H-ligand and the tested compound. The kinetics of dissociation of specific ligand–protein complexes was studied by isotope dilution with following analysis of the results as the sum of two exponentials [11]. The amount of specifically bound ^{3}H-ligand was determined as the difference in amount of radioactivity in the absence or presence of an excess (5.2 μM) of unlabeled counterpart.

Gel filtration was performed at 4-6°C using 1.6 × 75 cm column of Sepharose 6B and elution with buffer solution B at rate 0.9 ml/min. Aliquots (100 μl) of collected fractions were incubated with [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone (about 5 MBq) in a final volume of 200 μl for 1 h at 0-4°C. Protein-bound and free ligand were separated with dextran-coated activated charcoal (final concentrations 0.13 and 0.67%) for 5 min with following centrifugation at 3,000*g* for 5 min. The amount of radioactivity in aliquots of the supernatant was then measured (the counting efficiency with scintillation liquid according Bray [12] was 18%).

Ion-exchange chromatography was performed using a 0.8 × 30 cm column of DEAE-Toyopearl 650 M at elution rate 0.9 ml/min. Just after the sample, a linear NaCl concentration gradient (10-60 mM) in buffer solution B of total volume 100 ml was applied. The distribution of ligand-binding activity was determined as above. Elution profiles of proteins forming slowly and rapidly dissociating complexes with ^{3}H-ligand were determined in parallel experiment when to the samples after their incubation with [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone an excess of unlabeled counterpart (final concentration of 5.2 μM) was added and 25 min later an amount of protein-bound radioactivity was measured.

Serum proteins were fractionated by ammonium sulfate precipitation by addition of calculated amounts of crystalline $(NH_4)_2SO_4$ to 4-fold diluted serum and then mixing for 30 min at 0°C and centrifugation at 50,000*g* for 10 min. The resulting precipitate was dissolved in the initial volume of buffer solution, the centrifugation was repeated, and specific ^{3}H-ligand-binding activity in the supernatant was measured.

The protein content was determined by Coomassie brilliant blue staining [13]. Electrophoresis in 7.5% polyacrylamide gel with SDS was performed according Laemmli [14] using BSA and ovalbumin as standards. All experiments were repeated at least three times. Two parallel determinations were used for kinetic analysis of ligand–protein interactions.

RESULTS OF INVESTIGATION

By equilibrium K_d value, [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone binding in serum (Fig. 1, table)

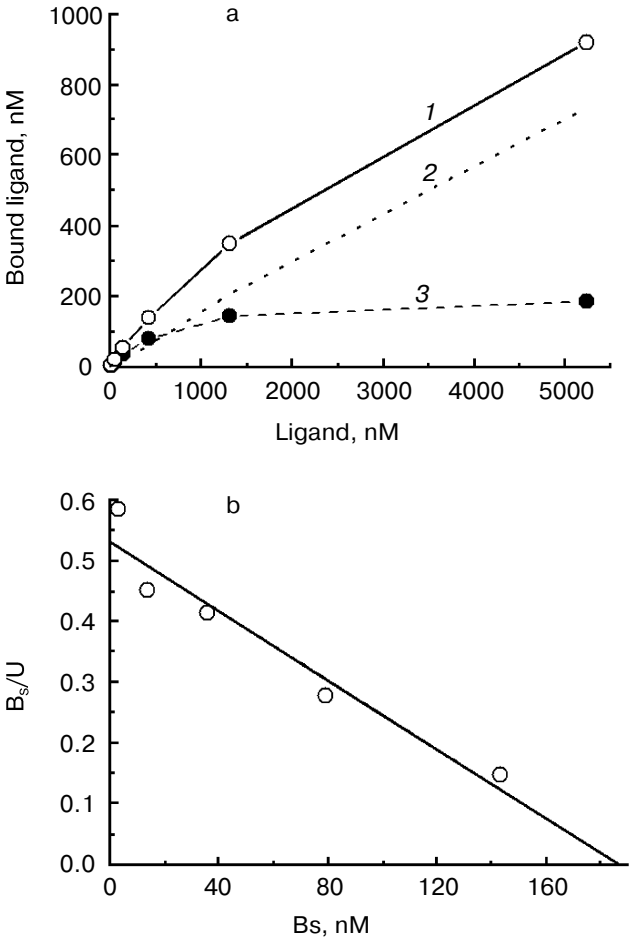

Fig. 1. Analysis of equilibrium binding of [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone to rat serum proteins. a) Dependence of total (*1*), nonspecific (*2*), and specific (*3*) ligand binding on the ligand concentration; b) Scatchard plot for specific ligand binding. B_s, specifically bound ligand; U, unbound ligand. Calculated values for equilibrium K_d and binding site concentration B_{max} were 351 and 186 nM (34 pmol/mg protein), respectively.

Ligand binding specificity of the rat serum protein based on analysis of competition of steroids with [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone

Compound	K_d, μM (mean ± SE)	RBA (mean ± SE)*
6α-Methyl-16α,17α-cyclohexanoprogesterone	0.357 ± 0.097 (7)**	1
16α,17α-Cyclohexanoprogesterone	1.458 ± 0.464 (3)	0.243 ± 0.053 (3)
16α,17α-Cyclohex-2′-enoprogesterone	2.150 (2)	0.140 (2)
16α,17α-Cyclopentanoprogesterone	4.140 (2)	0.073 (2)
16α,17α-Cyclopropanoprogesterone	7.000 ± 1.069 (3)	0.043 ± 0.003 (3)
Promegeston (R5020)	8.600 ± 0.833 (3)	0.035 ± 0.007 (3)
Progesterone	9.767 ± 1.065 (3)	0.033 ± 0.005 (3)

* Relative binding affinity (RBA) of 6α-methyl-16α,17α-cyclohexanoprogesterone was taken as 1.
** Number of independent measurements appears in parentheses.

is weaker than its binding to rat uterine cytosol proteins, including progesterone receptor and a protein specific for pregna-D′-pentaranes [5]. The ligand specificity of the serum protein also differs from that of the mentioned uterine proteins (Fig. 2, table): in the cases of progesterone receptor and the protein specific for pregna-D′-pentaranes from uterus, 6α-methyl-16α,17α-cyclohexanoprogesterone had the lowest affinity among the tested steroids [3-7], while in the case of serum this analog showed the highest affinity for the protein. Hydrocortisone at concentration 3 μM did not compete with [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone for serum protein binding sites, suggesting thus nonidentity of this protein to transcortin, which is known to bind progestins.

When the kinetics of dissociation of [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone complexes with the serum protein were analyzed, two phases were found, the ratio of fast and slowly dissociating complexes being close to 1 : 1 (Fig. 3). From three experiments, the dissociation velocity constant value (k_{-1}, sec^{-1}) for the slow phase was $(7.20 \pm 0.93) \cdot 10^{-5}$. The correct evaluation of k_{-1} value for the fast phase was complicated by the comparability of the time for separation of free and bound ligand and the half-life of the labile complexes. The order of the formal k_{-1} value for the fast phase was of 10^{-2} sec^{-1}. The measured k_{-1} value for the slow phase statistically differs from that measured when uterine cytosol was used [5].

To further characterize the serum protein that binds 6α-methyl-16α,17α-cyclohexanoprogesterone, standard methods of protein fractionation were used. On ammonium sulfate precipitation, ligand-binding activity retains in general the precipitation profile of total serum protein with a maximum revealed in a fraction precipitated in $(NH_4)_2SO_4$ solution close to saturation (Fig. 4). Some lag in ligand-binding activity precipitation from precipitation of total protein on increasing $(NH_4)_2SO_4$ concentration gives the possibility to use stepped precipitation for partial purification of the pregna-D′-pentarane-binding protein. The profile of serum protein precipitation differs significantly from that for progesterone receptor, which precipitates at salt concentration of about 30-35% saturation [15], and from the pregna-D′-pentarane-specific protein from uterus that, from our preliminary data, loses its ligand-binding activity in the presence of high salt concentrations.

On fractionation of serum proteins by gel filtration, the profile of elution of ^{3}H-ligand-binding activity almost completely follows the profile of total serum protein (Fig. 5) with a maximum corresponding to the elution of BSA (molecular weight of 67 kD). For an unknown reason, the ^{3}H-ligand-binding activity in the collected fractions quickly declines (to about 10% of the initial level after 18 h).

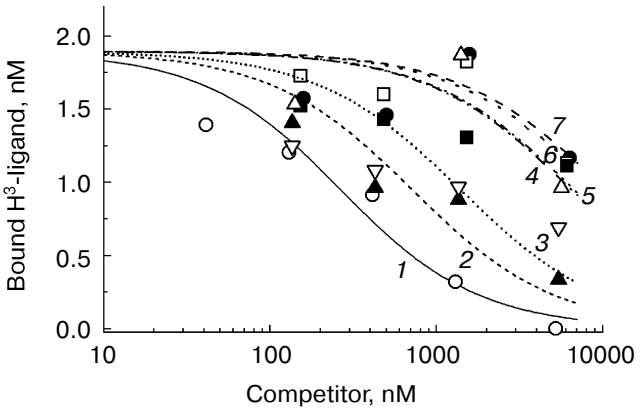

Fig. 2. Ligand binding specificity of the rat serum protein that binds [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone. Unlabeled competitors: *1*) 6α-methyl-16α,17α-cyclohexanoprogesterone; *2*) 16α,17α-cyclohexanoprogesterone; *3*) 16α,17α-cyclohex-2′-enoprogesterone; *4*) 16α,17α-cyclopropanoprogesterone; *5*) 16α,17α-cyclopentanoprogesterone; *6*) promegeston (R5020); *7*) progesterone. The final protein concentration was of 7.0 mg/ml. Calculated concentration of binding sites B_{max} was of 45 nM. Calculated values for equilibrium K_d (nM) and relative binding affinity RBA (in parts), respectively, were: *1*) 190 and 1; *2*) 545 and 0.349; *3*) 1080 and 0.176; *4*) 5100 and 0.037; *5*) 5300 and 0.036; *6*) 7400 and 0.026; *7*) 8200 and 0.023.

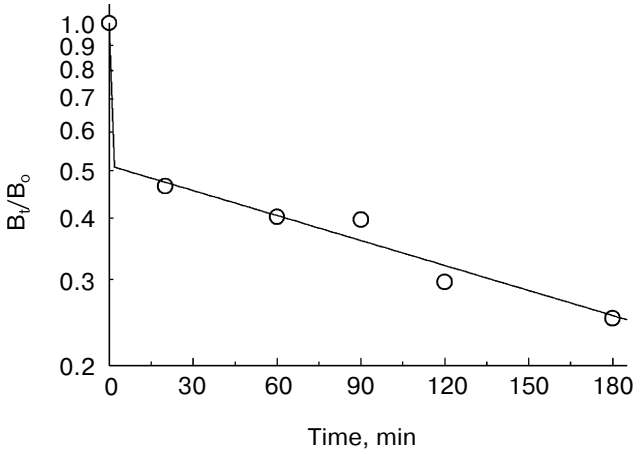

Fig. 3. Kinetics of dissociation of complexes between [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone and the rat serum protein. B_t and B_o, current and initial concentrations of specifically bound ^{3}H-ligand. The final protein concentration was 6.8 mg/ml. The concentration of added [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone was 7.2 nM, and B_o was 1.0 nM. The calculated value of the dissociation velocity constant k_{-1} (sec^{-1}) for the slow phase was $6.5 \cdot 10^{-5}$. The initial ratio of fast to slowly dissociating complexes was 49 : 51.

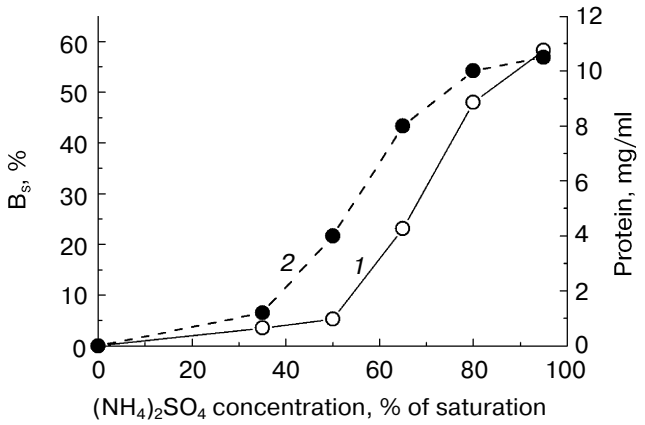

Fig. 4. Precipitation of rat serum proteins with ammonium sulfate: *1*) specifically bound [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone (B_s); the binding in nonfractionated serum (15,400 cpm on addition of 40,200 cpm of ^{3}H-ligand) was taken as 100%; *2*) total protein (the concentration in nonfractionated serum was of 10.5 mg/ml).

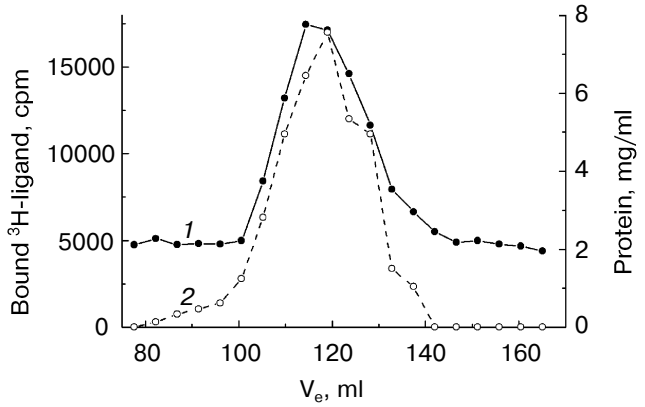

Fig. 5. Gel filtration of rat serum proteins on Sepharose 6B column: *1*) totally bound [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone (on addition of 47,200 cpm of ^{3}H-ligand) (left scale); *2*) total protein (right scale). V_e, elution volume.

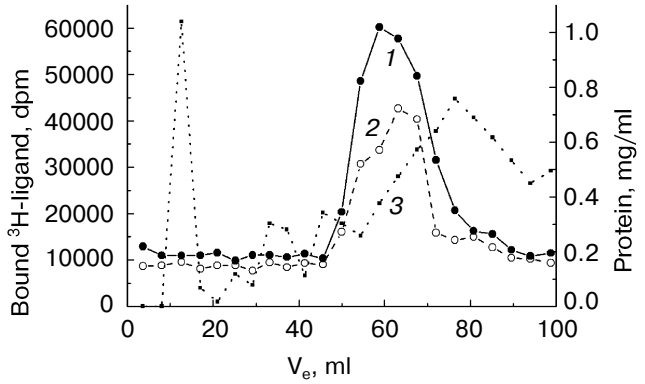

Fig. 6. Ion-exchange chromatography of serum proteins on DEAE-Toyopearl column: *1*) totally bound [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone (on addition of 71,000 dpm of ^{3}H-ligand); *2*) bound [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone 25 min after addition of an excess of unlabelled counterpart (left scale); *3*) total protein (right scale). V_e, elution volume.

^{3}H-Ligand-binding activity is succeeded to separate from the bulk of protein by ion-exchange chromatography of the serum (Fig. 6), profiles of elution of proteins forming fast and slowly dissociating complexes with 6α-methyl-16α,17α-cyclohexanoprogesterone coinciding.

Staining of the protein in a polyacrylamide gel after electrophoresis under denaturing conditions of ion-exchange chromatography fractions collected in the region V_e = 45-80 ml (see Fig. 6) showed three minor bands with molecular weights of 71, 82, and 96 kD. Their abundance corresponded to the elution profile of ^{3}H-ligand-binding activity and the purity of the preparation (concentration of binding sites (B_{max}) was about of 150 pmol/mg protein, which corresponds to the preparation purity of about 1% taking the molecular weight of the protein to be 80 kD and assuming that the protein has one ligand binding site per molecule). The mobility of the main protein band in collected fractions corresponds to BSA. The abundance of protein in this band does not correspond to the profile of elution of ^{3}H-ligand-binding activity but corresponds well with the profile of elution of total protein in a given region of the gradient.

DISCUSSION

Based on a number of studied characteristics (ligand-binding specificity (Fig. 2, table), velocity constant for dissociation of complexes (Fig. 3), behavior on

ammonium sulfate precipitation (Fig. 4)), the serum protein that binds 6α-methyl-16α,17α-cyclohexanoprogesterone differs from the protein from rat uterus that is specific for pregna-D′-pentarane-binding. This suggests that the observed specific binding of pregna-D′-pentaranes in uterine cytosol [6, 7] is not due to contamination of the preparation by the serum protein.

Among other pregna-D′-pentaranes studied here, 6α-methyl-16α,17α-cyclohexanoprogesterone is the most hydrophobic. The interaction of this ligand with serum proteins might be suggested to be due to nonspecific hydrophobic bonds. The data on gel filtration (Fig. 5) and, to some extent, the data on ammonium sulfate precipitation (Fig. 4) correspond to such an interpretation. However, the results of ligand-binding specificity analysis do not confirm this assumption: the hydrophobicity (as approximately evaluated by adsorption on glass of radioactive compounds from aqueous solution [16]) in the series of steroids 6α-methyl-16α,17α-cyclohexanoprogesterone > 16α,17α-cyclopentanoprogesterone > 16α,17α-cyclohexanoprogesterone > 16α,17α-cyclohex-2′-enoprogesterone > 16α,17α-cyclopropanoprogesterone > progesterone does not correspond to the affinity of these compounds for the serum protein (6α-methyl-16α,17α-cyclohexanoprogesterone > 16α,17α-cyclohexanoprogesterone > 16α,17α-cyclohex-2′-enoprogesterone > 16α,17α-cyclopentanoprogesterone > 16α,17α-cyclopropanoprogesterone > progesterone) (table). In addition, ion-exchange chromatography allows the separation of 6α-methyl-16α,17α-cyclohexanoprogesterone-binding activity from the bulk of total serum protein (Fig. 6), including the most abundant protein, albumin. Perhaps, hydrophobic interactions play an important though not decisive role in the binding of steroids to the revealed serum protein.

A question of interest is whether fast- and slow-dissociating complexes of ^{3}H-ligand are formed by the same or different proteins. The coincidence of elution profiles of ligand-binding activities determining the production of fast- and slow-dissociating complexes with 6α-methyl-16α,17α-cyclohexanoprogesterone on ion-exchange chromatography (Fig. 6) corresponds to the first assumption. The data on uniformity of serum binding sites in their affinity for ^{3}H-ligand at equilibrium (Fig. 1) are also in agreement with this assumption. The appears in chromatography fractions of a number of protein bands with elution profiles from the ion-exchanger similar to that of the ligand-binding activity neither contradicts nor confirms this assumption. Obviously, this issue will be resolved unequivocally only after full purification of the protein is achieved.

The presence of a serum protein that binds pregna-D′-pentaranes can to some extent explain certain discrepancies between biological efficacy and the affinity of these compounds for progesterone receptor. On oral administration, 6α-methyl-16α,17α-cyclohexanoprogesterone acts as one of the most potent progestins [2]. At the same time, this steroid possesses the lowest affinity for the progesterone receptor among studied analogs [3-7]. One can speculate that extensive binding of 6α-methyl-16α,17α-cyclohexanoprogesterone to the serum protein provides prolonged existence of the steroid in the circulation, inhibiting its metabolism and excretion without significant interference with its availability to a higher affinity system of reception. The high binding capacity of the serum protein (about 1 μM) seems to be enable to render the circulation of 6α-methyl-16α,17α-cyclohexanoprogesterone mainly as specific complexes with the protein.

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REFERENCES

1. Levina, I. S., Kamernitzky, A. V., Fanchenko, N. D., and Simonov, V. I. (1982) *Endokrinologie*, **80**, 266-274.
2. Kamernitzky, A. V., and Levina, I. S. (1991) *Khim.-Farm. Zh.*, **25**, No. 10, 4-16.
3. Smirnov, A. N., Pokrovskaya, E. V., Shevchenko, V. P., Levina, I. S., and Kamernitzky, A. V. (1998) *Probl. Endokrinologii*, **44**, No. 1, 37-40.
4. Smirnov, A. N., Pokrovskaya, E. V., Shevchenko, V. P., Levina, I. S., and Kamernitzky, A. V. (1998) *Byull. Exp. Biol. Med.*, **125**, 532-534.
5. Smirnov, A. N., Pokrovskaya, E. V., Shevchenko, V. P., Levina, I. S., and Kamernitzky, A. V. (1998) *Biochemistry (Moscow)*, **63**, 1090-1097.
6. Smirnov, A. N., Pokrovskaya, E. V., Shevchenko, V. P., Levina, I. S., and Kamernitzky, A. V. (1999) *Bioorg. Khim.*, **25**, 774-781.
7. Smirnov, A. N., Pokrovskaya, E. V., Kogteva, G. S., Shevchenko, V. P., Levina, I. S., Kulikova, L. E., and Kamernitzky, A. V. (2000) *Steroids*, **65**, 163-170.
8. Kamernitzky, A. V., Levina, I. S., Kulikova, L. E., Galakhova, T. N., Shevchenko, V. P., Nagaev, I. Yu., Myasoedov, N. F., Smirnov, A. N., Pokrovskaya, E. V., and Shchelkunova, T. A. (1997) *Izv. Akad. Nauk. Ser. Khim.*, **46**, 1468-1471.
9. Levina, I. S., and Kamernitzky, A. V. (1990) *Khim.-Farm. Zh.*, **24**, 31-39.
10. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.*, **51**, 660-672.
11. Weichman, B. M., and Notides, A. C. (1977) *J. Biol. Chem.*, **252**, 8856-8862.
12. Bray, G. (1960) *Analyt. Biochem.*, **1**, 279-285.
13. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
14. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
15. Toft, D., Moudgil, V., and Lohmar, P. (1976) in *Hormone–Receptor Interaction: Molecular Aspects* (Levey, G. S., ed.) Marcel Dekker Inc., New York, pp. 243-264.
16. Shevchenko, V. P., Nagaev, I. Yu., Myasoedov, N. F., Kamernitsky, A. V., Levina, I. S., Kulikova, L. E., Pokrovskaya, E. V., Shchelkunova, T. A., and Smirnov, A. N. (1999) *Bioorg. Chem.*, **27**, 207-213.