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SPECTROPHOTOMETRIC STUDY OF PROGESTERONE BINDING TO UTEROGLOBIN

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SUMMARY

A spectrophotometric method is proposed for studying the interaction of progesterone with purified rabbit uteroglobin. The method exploits the depression in the absorbance of the α,β-unsaturated keto-group of the steroid which takes place upon binding to uteroglobin. The magnitude of this depression at around 260 nm is correlated to the binding affinity and can be used to calculate the apparent equilibrium association constant and the number of steroid binding sites per protein molecule. The results obtained demonstrate that reduction of the disulphide bonds of native uteroglobin is a prerequisite for progesterone binding, and that the sulphydryl groups are not directly implicated in the interaction with the steroid. The only tyrosine residue of the uteroglobin subunit changes its absorption spectrum as a consequence of steroid binding, and the binding reaction is accompanied by a positive entropy change. These findings suggest an association of progesterone binding with conformational changes of the polypeptide structure of uteroglobin.

INTRODUCTION

Uteroglobin is a small globular protein which is present in large quantities in the uterine secretion of the rabbit during the preimplantation phase of pregnancy [1]. This protein, which has also been called blastokinin [2], exhibits the interesting property of binding the same steroid which induces its biosynthesis, namely progesterone [3, 4, 5]. In previous reports, we have described the quaternary structure of uteroglobin, which is composed of two identical subunits held together by disulphide bonds [6], and have studied its interaction with progesterone and other steroids by means of radioactive labelled ligands [7, 8].

In this paper we propose a spectrophotometric procedure for studying the interaction of progesterone with uteroglobin, based on the quenching of the absorption of the 4-ene-3-keto-structure of the steroid ring-A, a procedure that was originally developed for the study of steroid binding to serum proteins (for review see Ref. 9). In addition, we present spectrophotometric evidence suggesting that changes in the quaternary structure of the protein as well as in the environment of the tyrosine residue take place during the binding reaction.

EXPERIMENTAL

[1,2-^{3}H]-progesterone, specific radioactivity 48 Ci/mmol was obtained from New England Inc. The non-radioactive steroids were purchased from Sigma Chemicals Inc. with the exception of Norethynodrel (17α-ethynyl-17β-hydroxy-5(10)-estrene-3-one) which was kindly provided by Schering AG, Berlin. Activated charcoal was purchased from Mallinckrodt Inc.

Uteroglobin was purified to homogeneity from the uterine flushes of rabbits treated sequentially with estradiol-17β and progesterone as described [6]. In summary, the procedure includes a chromatography on Sephadex G-100 followed by ion exchange chromatography on carboxymethyl-cellulose. The final preparations of uteroglobin were homogeneous according to the following criteria: (1) a single band in polyacrylamide gel electrophoresis at alkaline and acidic pH, both in the absence and in the presence of 8M urea; (2) a single band in polyacrylamide gel electrophoresis containing sodium dodecylsulphate; (3) a single precipitation line in immunoelectrophoresis against an antiserum prepared in guinea pigs with either crude uterine proteins or purified uteroglobin; (4) an homogeneous behaviour in the analytical ultracentrifuge both in velocity sedimentation and in equilibrium centrifugation experiments [6].

The molecular weight of the native protein was calculated to be between 15,000 and 16,000, and was reduced to half by reduction with mercaptoethanol or dithioerythritol [6]. These findings are in agreement with the aminoacid analysis and the results of peptide mapping studies, which indicate that native uteroglobin is composed of two identical polypeptide chains of 75 aminoacids each held together by two disulphide bonds and other non-covalent interactions [6].

For most binding studies a buffer consisting of 64 mM Tris–HCl, pH 7.5, containing 135 mM NaCl was used (Tris–saline buffer). Binding of radioactive progesterone to uteroglobin was determined by the

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charcoal adsorption technique [7, 8]. Reduction and S-carboxymethylation of purified uteroglobin with iodoacetate, was carried out in a nitrogen atmosphere at constant pH as previously described [6]. Briefly, the protein was dissolved in 0.2 M Tris–HCl, pH 8.3, containing 8 M urea and 0.2% $EDTANa_2$, and reduced with a 100-fold molar excess of 2-mercaptoethanol in a nitrogen atmosphere. After 4 h at room temperature iodoacetate was added in the dark (95% of the 2-mercaptoethanol concentration, in 0.25 M Tris, 0.5 M NaOH and 0.2% $EDTANa_2$), and the pH maintained at 8.2 with 2 M NaOH. Twenty minutes after completion of the reaction, excess of 2-mercaptoethanol was added, and the pH brought to 4.0 with 2 M acetic acid. The protein was separated from other components of the reaction mixture by passing through a Bio-Gel P2 column equilibrated in the dark with 0.2 M acetic acid, and lyophilized. Quantitation of uteroglobin was carried out photometrically assuming a ϵ_{260} of 2000 and 1700 for reduced uteroglobin and S-carboxymethylated uteroglobin respectively. These values were calculated using different procedure for the determination of protein concentration which all gave similar results [6].

Spectrophotometric measurements were carried out in a Beckman Acta CII double beam spectrophotometer, equipped with a thermoregulated cuvette holder. Differential spectra were obtained using matched split-compartment cells [10] with a path length of 0.4375 cm in each compartment. For most spectral measurements a bandwidth of 1 nm at 260 nm was used.

RESULTS

The absorption spectra in the wavelength region 280–240 nm of free progesterone against buffer, and of progesterone in the presence of 10-fold molar excess of reduced uteroglobin against the protein are shown in Fig. 1. The free steroid shows an absorption maximum at 249 nm which is characteristic of α,β-unsaturated ketosteroids with $\epsilon_{249} = 16{,}700$. In the presence of an excess of uteroglobin, there is not only a shift in the absorption maximum to shorter wavelengths, but also a marked depression of absorption. The difference spectrum depicted in the inset of Fig. 1, shows that maximal depression takes place at wave lengths around 260 nm. A similar but less pronounced quenching of the absorption of the 4-ene-3-keto function has been reported for the interaction of various α,β-unsaturated ketosteroids with serum albumin and other serum proteins [9, 11–16]. As a control, the inset of Fig. 1 also shows that an excess of reduced uteroglobin has no effect on the absorbance of cortisol, a ketosteroid which has been shown not to compete with [^{3}H]-progesterone for binding to uteroglobin [8]. These findings suggest that the depression of the steroid absorbance is a consequence of its binding to the protein.

Westphal[9, 11] has shown that the extent of quenching of the steroid absorbance is proportional to the binding affinity, and therefore can be used to determine the apparent association constant and the number of binding sites per protein molecule. Knowing the extinction coefficient of free and bound steroid, E_1 and E_2 respectively, the fraction of free progesterone, α, at any given concentration of total steroid can be calculated according to the following expression:

$$\alpha = E_{app} - E_2/E_1 - E_2.$$

When the values are expressed as percentage of E_1, E_{app} represents the percent absorption at a given concentration of the steroid, and E_2 can be calculated from a plot similar to that depicted in Fig. 2, by extrapolation to the ordinate. At 10°, E_2 is 22% of the absorbance of free progesterone at 260 nm (E_1).

Using this procedure to calculate the concentration of free and protein-bound steroid reproducible satu-

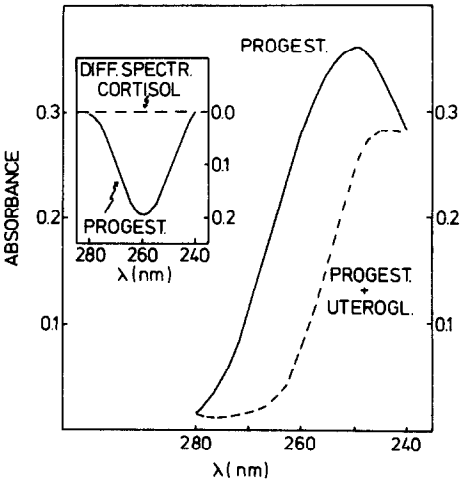

Fig. 1. Ultraviolet spectra of free progesterone and of progesterone bound to uteroglobin. A 20 μM solution of progesterone was prepared by adding 10 μl of a 2 mM ethanolic solution of the steroid to 0.99 ml Tris–saline buffer (64 mM Tris–HCl, pH 7.5, containing 135 mM NaCl), and a spectrum was run at 10°, using as reference 1 ml Tris–saline buffer containing 10 μl ethanol (——). For the determination of the spectrum of progesterone bound to uteroglobin, a 200 μM solution of purified uteroglobin (3 mg/ml in Tris–saline buffer) was reduced at 37° for 15 min with 5 mM dithioerythritol. After cooling to 10°, 0.99 ml aliquots were pipetted in matched cuvettes. To the sample cuvette were added 10 μl of a 2 mM solution of progesterone in ethanol, and the reference cuvette received an equivalent volume of ethanol. After 15 min, a spectrum was run at 10° (-----).

The *inset* shows difference spectra of the steroid bound to uteroglobin versus free steroid obtained by using matched split compartment cells. Each cuvette contained in one compartment 1 ml of a 20 μM solution of the steroid in Tris–saline buffer, and in the other compartment 1 ml of 200 μM solution of uteroglobin in the same buffer (pretreated with 5 mM dithioerythritol at 37° for 15 min). The material in the sample cuvette was thoroughly mixed and, after 15 min at 10°, a spectrum was taken. The values are corrected for a 1 cm light path, and represent the difference spectra obtained with progesterone (——) and cortisol (– – –) under identical conditions.

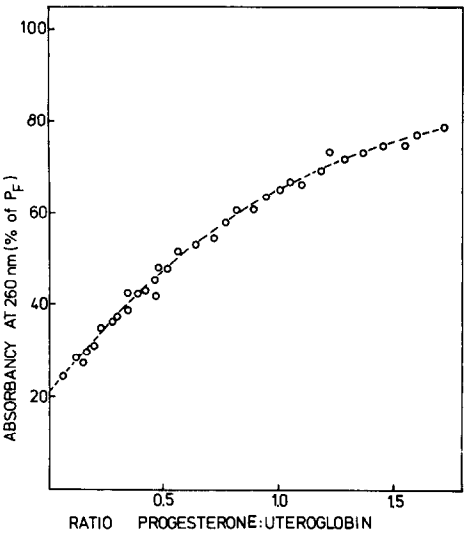

Fig. 2. Effect of uteroglobin on the absorbance of progesterone at 260 nm. Matched 4 ml cuvettes (1 cm light path) were filled with 3 ml of either Tris–saline buffer (reference) or a 100 μM solution of progesterone in the same buffer (sample). After measuring the absorbance at 260 nm and 10°, aliquots of a concentrated solution of reduced uteroglobin (1 mM in Tris–saline buffer) were added to both cuvettes and the absorption at 260 nm measured. The values were corrected for dilution, and plotted as percentage of the absorbance of free progesterone (P_F) against the progesterone–uteroglobin molar ratio.

ration curves are obtained (Fig. 3). At 10° and after treatment with 5 mM dithioerythritol, the apparent equilibrium association constant, K, for the binding of progesterone to uteroglobin, calculated from the Scatchard plot (Fig. 3, inset), is $4 \times 10^5\ M^{-1}$ (range $2.7–7.5 \times 10^5$) and the number of binding sites per uteroglobin molecule, r, is 0.42 ($\pm$0.13). This value has been calculated in Tris–saline buffer assuming a molecular weight of 15,000 for native uteroglobin, which is the size of the binding species of the protein (Fig. 5). However, the value of r changes considerably depending on the conditions of uteroglobin preparation and storage (see Discussion).

As previously reported, reduction of uteroglobin with dithioerythritol is required for the detection of optimal binding of progesterone to the protein [7]. The results presented in Fig. 4, show that dithiothreitol is also an efficient reducing agent, and that the native form of uteroglobin has only a few sites with the affinity of the reduced form. The majority of the unreduced uteroglobin molecules bind progesterone with much lower affinity, of the order of $4 \times 10^4\ M^{-1}$ at 10° [7]. Also included in Fig. 4 is a plot of the binding data obtained with reduced and S-carboxymethylated uteroglobin, showing that it has an affinity for progesterone even higher than the reduced protein (range $5.2–12.7 \times 10^5\ M^{-1}$ at 10°). It is, therefore, clear that although reduction of the disulphide bonds is required for steroid binding, the sulphydryl groups are not directly involved in the interaction with progesterone.

The need for reduction of the disulphide bonds suggested that the uteroglobin subunit could be the progesterone binding species. However, when reduced and S-carboxymethylated uteroglobin is incubated with [^{3}H]-progesterone and submitted to chromatography on a column of Sephadex G-50 (Fig. 5), the radioactive complex of protein and steroid elutes ahead of cytochrome C, in a position corresponding to the molecular weight of native uteroglobin (15,000). Subsequent determination of the progesterone binding activity in the eluate also shows, that it is limited to the position of the dimeric protein (Fig. 5). It is, therefore, obvious that, reduction of the disulphide bridges is not sufficient to separate the uteroglobin subunits, and that the progesterone binding species of uteroglobin is probably the reduced dimeric protein, in which the two subunits are held together by non-covalent interactions. In fact, we know that 6 M guanidium hydrochloride is required to achieve separ-

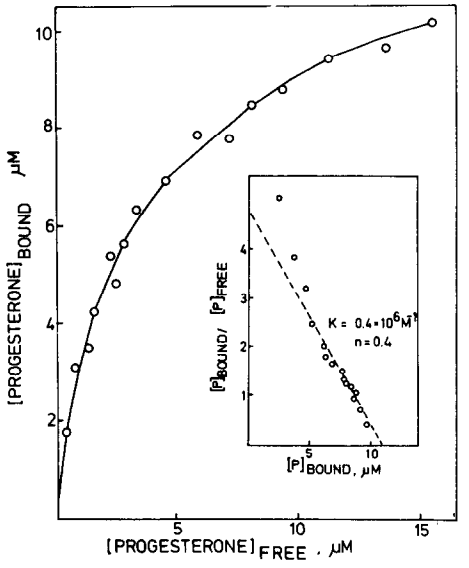

Fig. 3. Equilibrium saturation curve for the binding of progesterone to reduced uteroglobin. Three matched cuvettes, one of which served as reference, were filled with 3 ml Tris–saline buffer containing 5 mM dithioerythritol, and two additional cuvettes with 3 ml of a 27.5 μM solution of reduced uteroglobin in the same buffer (treated with 5 mM dithioerythritol at 37° for 15 min). After 20 min at 10°, the absorbance at 260 nm was registered and the four sample cuvettes received 1 μl aliquots of a 2 mM solution of progesterone in ethanol, whereas to the reference cuvette 1 μl ethanol was added. The absorbance of each cuvette was measured 5 min after the additions and the whole procedure repeated 15 times. The total concentration of progesterone was determined from the duplicate values of the buffer cuvettes, using a $\epsilon_{260} = 12{,}500$. The absorbance at a given concentration of progesterone, E_{app}, was determined from the uteroglobin cuvettes, after subtracting the protein absorbance. With this value, the fraction of free progesterone, α, was determined as described in the text, using $E_2 = 22\%$. The figure represents a plot of the concentration of uteroglobin-bound progesterone against the concentration of free steroid.

The *inset* shows a representation of the data according to Scatchard [20]: $(P)_{Bound}$, concentration of protein-bound progesterone, $(P)_{Free}$, concentration of free steroid.

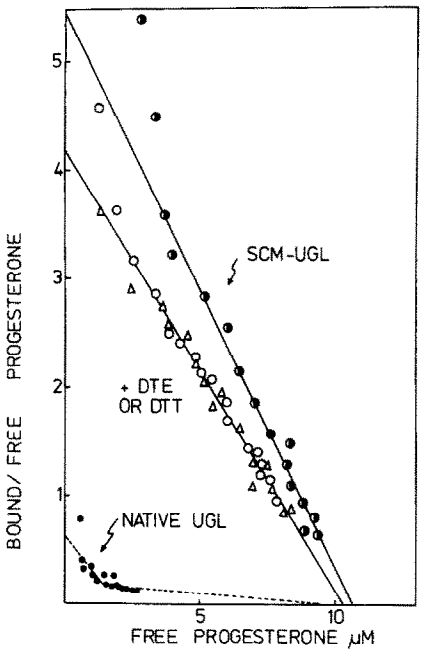

Fig. 4. Effect of reduction and S-carboxymethylation of uteroglobin on its ability to bind progesterone. The binding of progesterone to various preparations of uteroglobin (27.5 μM in Tris–saline buffer) was determined as described in the legend to Fig. 3, and the values are plotted according to Scatchard [20]. (●---●) native untreated uteroglobin; (○——○) uteroglogin treated for 15 min at 37° with 5 mM dithioerythritol; (△——△) uteroglobin treated under the same conditions with 20 mM dithiothritol; (◑——◑) uteroglobin reduced and S-carboxymethylated with iodoacetate as previously described [6].

ation of the uteroglobin subunits after reduction and alkylation [6].

Preliminary sequence data indicate that one of the two disulphide bonds of uteroglobin is located close to the aminoterminal end, which also contains the only tyrosine residue of the uteroglobin subunit (Ponstingl, Nieto and Beato, unpublished). It was therefore, interesting to know whether the tyrosine residue is involved in the steroid binding. Difference spectra of free versus progesterone bound uteroglobin in the absorption range of the tyrosine chromophore (Fig. 6a) show marked peaks around 287–288 nm and 281–282 nm which can be attributed to perturbations of the tyrosine phenolic group [16–18]. As in the case of the depression at 260 nm, this perturbation is more pronounced with S-carboxymethylated uteroglobin than with the reduced protein (Fig. 6a), and it does not take place after incubation with cortisol (Fig. 6b). A precise quantitation of the progesterone-induced perturbations in the aromatic region of the spectrum is hindered by the overlapping with the much more pronounced depression of the absorbance around 260 nm (Fig. 1). To avoid this interference, we have used the synthetic steroid norethynodrel, which binds very tightly to uteroglobin [8] and has an own absorbance lower than progesterone with a maximum at 244 nm (ϵ = 1,225). A difference spectrum of free versus norethynodrel bound uteroglobin exhibits similar perturbations in the aromatic region as observed with progesterone, but without the pronounced depression at lower wave lengths (Fig. 6b). In addition, the perturbation of the tyrosine chromatophore was proportional to the concentration of norethynodrel, over a range 0.1–1.0 in the steroid to protein ratio. All these data indicate that the environment of the tyrosine residue is altered as a consequence of steroid binding.

The influence of temperature on the apparent association constant of progesterone to S-carboxymethylated uteroglobin is shown in Fig. 7 in the form of a van't Hoff plot. It is clear that the affinity decreases with increasing temperature, as described for other steroid binding proteins, with the exception of serum albumin [9, 19]. The thermodynamic parameters of the binding reaction can be calculated from Fig. 1. Besides a negative value of the enthalpy change ($\Delta H^\circ = -3.46$ kcal mol^{-1}), which is evident from the slope of the van't Hoff plot, there is also a positive change of entropy ($\Delta S^\circ = 14.4$ cal × K^{-1} × mol^{-1}). Similar changes have been described for the interaction of glucocorticoids with their hepatic receptor [19].

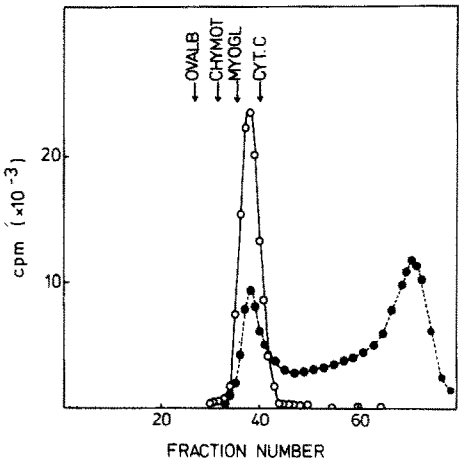

Fig. 5. Gel filtration of reduced and carboxymethylated uteroglobin on Sephadex G-50: binding of [^{3}H]-progesterone. Reduced and S-carboxymethylated uteroglobin (500 μg in 0.3 ml Tris–saline buffer) was incubated at 0° for 15 min with 30 μl of 2 μM [^{3}H]-progesterone. After addition of cytochrome *C* (100 μg in 50 μl Tris–saline buffer) the sample was applied to a column of Sephadex G-50 fine (1.4 × 60 cm) equilibrated with Tris–saline buffer. The column was eluted with the same buffer at 0–2°. The position of the internal marker, cytochrome *C*, was determined by measuring the absorbance at 405 nm in the eluate. The position of the other marker proteins, ovalbumine, chymotrypsinogen and myoglobin, was determined in a parallel run. Aliquots, 100 μl, of each eluate fraction, were taken to determine the total radioactivity (●---●), and the progesterone binding capacity (○---○). The later test was carried out by incubation at 0° for 60 min with 0.1 μM [^{3}H]-progesterone, followed by adsorption of the free steroid to charcoal [7, 8]. The blank values, obtained in the absence of protein, have been substracted.

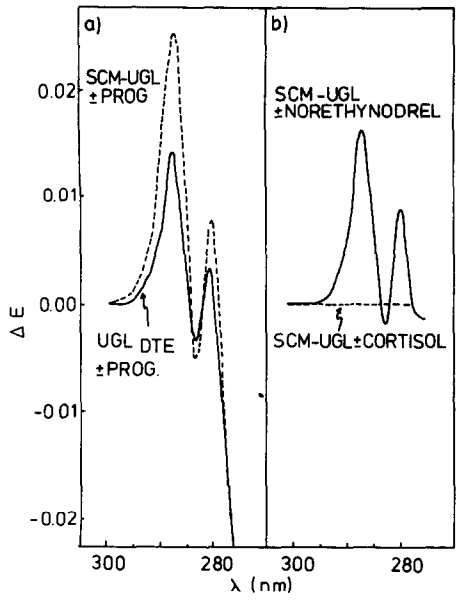

Fig. 6. Difference ultraviolet spectrum of uteroglobin complexed with steroid against free uteroglobin. One compartment of each split-compartment cell was filled with 1 ml of a 200 μM solution of uteroglobin in Tris-saline buffer, and the other compartment was filled with 1 ml of a 20 μM steroid solution in the same buffer. The sample cuvette was thoroughly mixed and both cuvettes were incubated at 10° for 20 min. The difference spectra were run at a starting bandwidth of 1 nm at 300 nm. (a) reduced uteroglobin (5 mM dithioerythritol at 37° for 15 min) incubated with progesterone (——); reduced and S-carboxymethylated uteroglobin incubated with progesterone (– – –); (b) reduced and carboxymethylated uteroglobin incubated with norethynodrel (——) and with cortisol (– – –).

DISCUSSION

The quenching of the absorbance of the 4-ene-3-keto-structure of the progesterone molecule is similar in nature to that described for the interaction of other ketosteroids with serum proteins [11, 16]. However, it appears to be specific for progesterone, as the absorption of cortisol is not changed by the presence of uteroglobin. In addition, the extent of quenching is much more pronounced than it is for the interaction of steroids with serum albumin, indicating a higher binding affinity. A similar quenching of the steroid chromophore has been observed when the steroid is transferred from water to ethanol [15, 16], and indicates that, upon binding, the A-ring of the steroid molecule changes to a more hydrophobic environment.

The usefulness of this spectrophotometric procedure for measuring the binding of progesterone to uteroglobin, resides in the fact that measurements can be done under true equilibrium conditions. There is no need for separating the free steroid from the protein-bound one, as required when radioactive ligand and the charcoal adsorption technique are used [7, 8]. The method has also advantages in comparison to equilibrium dialysis techniques, where problems can arise due to adsorption of steroid to the dialysis membrane or to Donnan effects. An additional important advantage of this procedure is that it allows one to follow the reaction as a function of time by using the techniques developed for enzymatic reactions, such as, stopped flow and temperature jump, which will yield an independent estimation of the affinity based on the ratio of the rate constants. On the other side, the limitation of the procedure is that only those steroids having a strong chromaphore can be investigated. Nevertheless, for the interaction of progesterone with uteroglobin the spectrophotometric method gives more precise information than the usual techniques with radioactive ligands [7, 8].

Previous reports suggested that reduction of the disulphide bonds of uteroglobin is required for optimal progesterone binding [7]. This finding has been confirmed with the spectrophotometric procedure described here, and in addition, we have shown that alkylation of the sulphydryl groups with iodoacetate does not prevent the binding of progesterone. On the contrary a higher affinity is observed after alkylation, which is probably due to stabilization of the reduced conformation. In fact, when a reductant such as dithioerythritol is used, a certain degree of reversion to the oxydized state is unavoidable during the binding reaction. In that respect it is important to point out, that preparations of uteroglobin which have been kept in the refrigerator for long periods of time show higher binding of progesterone in the absence of any reductant, indicating that changes in the quaternary structure of the protein take place during prolonged storage. On the other site, storage of reduced and carboxymethylated uteroglobin, even at −30°, results

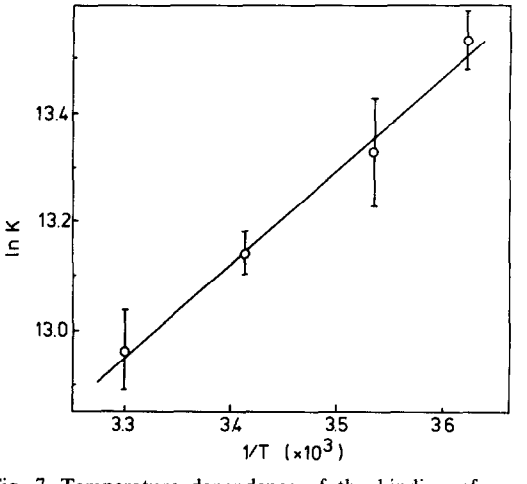

Fig. 7. Temperature dependence of the binding of progesterone to reduced and carboxymethylated uteroglobin. The apparent association constant, K, for the binding of progesterone to reduced and carboxymethylated uteroglobin (30 μM in Tris–saline buffer), was determined at 3°, 10°, 20° and 30° as described in the legend to Fig. 3. The data are depicted in form of a van't Hoff plot: the natural logarithm of K is plotted against the reverse of the temperature in grad Kelvin. The slope of the line equals $(-\Delta H°/R)$. The points represent the average of two determinations carried out under the same conditions and with the same preparation of uteroglobin.

in decreased affinity for progesterone and leads to changes in the shape of the saturation curve. These factors are responsible for variation in the calculated number of steroid binding sides per uteroglobin molecule, *r*. Of relevance for the quantitation of this number is also the procedure of uteroglobin preparation. Our procedure [6] includes various lyophilization steps with the corresponding freezing and thawing of the protein, a treatment that could reduce the progesterone binding capacity of uteroglobin. It is also possible that the low values of *r* are due to artefacts of the carboxymethylation procedure, which could lead to alkylation of sites other than the sulphydryl groups.

Freshly prepared native uteroglobin in the absence of reductants exhibits only very few binding sites for progesterone with the affinity of the reduced protein, and the majority of its binding sites show low affinity for progesterone, indicating that the steroid binding site is less accessible in the oxidized native state. In fact, conformational changes in the structure of the polypeptide chain are suggested by difference spectra of reduced versus native uteroglobin in the region around 235 nm [21]. This opens the possibility for a physiological regulation of the progesterone binding capacity of uteroglobin by means of natural reductants, such as glucagon and cystein. The concentration of such reducing agents could change between the uterine lumen and the blastocyst, and therefore a mechanism for carrying steroids from the maternal organism to the developing embryo, or *vice versa*, could be easily envisaged. Determinations of the concentrations of progesterone in the uterine lumen of the rabbit during the pre-implantation phase indicate that they are in the range which will cause a significant fraction of it to be bound to uteroglobin [22].

Besides the quenching of the steroid chromophore reported above, the spectrometric procedure yields also information concerning the involvement of the only tyrosine residue of the uteroglobin subunit in the binding of the steroid. As evidenced by the difference spectrum, the tyrosine chromophore changes its environment as a consequence of the binding of the steroids. The observed perturbations in the absorbance of the tyrosine phenolic group are similar to those found when moving from acidic to alkaline conditions, and can be interpreted as a consequence of changes in the ionization of the phenolic group. This indicates that either the phenolic group of tyrosine is directly involved in the binding of the steroid, or, that it is closed enough to the steroid binding site as to change its absorbance properties upon binding. It is of course possible that this change in the absorbance of the tyrosine residue is indirect and results from a conformational change of the polypeptide chain following binding of the steroid. The fact that the binding reaction is accompanied by a positive change of entropy could also be interpreted as indicative of a steroid induced local relaxation of the structure of uteroglobin leading to an increased degree of freedom. More generally, however, the positive changes of entropy in this type of reactions are due to displacement of the water molecules from the binding site of the steroid, or to hydration water associated with the protein molecule [23].

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