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Comparative Study of Estrogen Action

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SUMMARY

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By comparing 16 estradiol derivatives, it has been possible to assess the relative influence of distribution, metabolism, uterine uptake, and plasma and tissue binding on uterotrophic activity in the rat. This comparison has revealed that all active compounds are bound *in vivo* to the 8 S uterus cytosol receptor, but that their activity cannot be related to binding *in vitro*, owing to the many factors acting on the compound *in vivo* prior to the triggering of the biological response. It is suggested that only the free molecule in the plasma is active on the uterus and that consequently activity is modulated by plasma binding.

INTRODUCTION

The pharmacological approach to the development of active compounds is increasingly based on the determination of a correlation between structure and activity. To be able to draw such a correlation, it is necessary, as suggested by Ariëns (1), to develop a scheme to investigate the mechanism of action, which covers the study both of the conditions for induction of a response at the active site and of the conditions influencing the pharmacokinetics of the compound (uptake, distribution, and elimination). Any investigation restricted to only one or other of these two aspects has the inherent danger of inferring illusory correlations. For instance, Hansch and Deutsch (2) brought to light several mathematical relationships between physicochemical parameters and biological activity, while neglecting the site of interaction. Korenman (3) and Shutt and Cox (4), on the other hand, focused their attention on steroid-receptor interactions without considering factors such as transport, distribution, and metabolism and could thus only determine the specificity and geometry of the active site *in vitro* from the known structure and conformation of the intact substrate.

The present paper illustrates the pharmacological approach as applied to the mechanism of action of estrogens, a subject which has been intensively studied during the last decade. Well-documented summaries of available information are given in the authoritative reviews by Jensen and DeSombre (5), Kellie (6), and Williams-Ashman and Reddi (7), which consider both the metabolism of estrogens and the mechanism of their action at the cellular level.

Metabolic studies have yielded impressive data on catabolic pathways, inactivation by the liver, and formation of conjugates and excretion products. A minute amount of

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estrogen in relation to that metabolized and excreted, however, induces the hormonal response, which, according to Kellie (6), is elicited by a three-stage process: "The first stage involves the interaction of estrogen with a tissue component, the second stage involves primary biochemical events caused by this action, and the final stage amplifies these events into gross tissue changes." Particular emphasis has been laid on the first stage of this sequence, i.e., on the study of binding to cytosol and nuclear "receptors" (8): interaction with a cytosol component, dependence of nuclear binding on cytosol, and properties and purification of the receptor proteins (9–11).

These two lines of study, metabolic degradation and cellular action, appear to have little common ground, and it is now well accepted that estrogen action at the cellular level is not linked to steroid metabolism. Nevertheless, prior to the specific interaction with tissue proteins which triggers the biological response, other factors, in particular the pharmacokinetics of the compound, may intervene to modulate this response (12). In order to evaluate the importance of these factors, and especially of plasma binding, the relationships among the structure, uptake, metabolism, biological activity, and interaction with specific and/or nonspecific plasma- and cytosol-binding proteins of 16 chemically closely related steroids, all derivatives of estradiol, were studied according to the scheme illustrated in Fig. 1. Since attention was focused on prerequisites for activity, the effect of minimizing catabolic degradation by steric hindrance (introduction of a 17-ethynyl group) and of blocking one or both functional hydroxyl groups by methylation was investigated and compared with the effect of methoxylation in position 11, which is known to yield a highly potent steroid (13, 14).

MATERIALS AND METHODS

Animals. Female, 19–21-day-old Wistar rats (35–40 g) bred at the Roussel-Uclaf Research Center were used. They were given food and water ad libitum and maintained in air-conditioned surroundings under controlled lighting conditions.

Steroids. The steroids in Fig. 2, which correspond to all possible structural combinations of 11β-methoxy and 3- and/or 17-methylated derivatives of either estradiol or

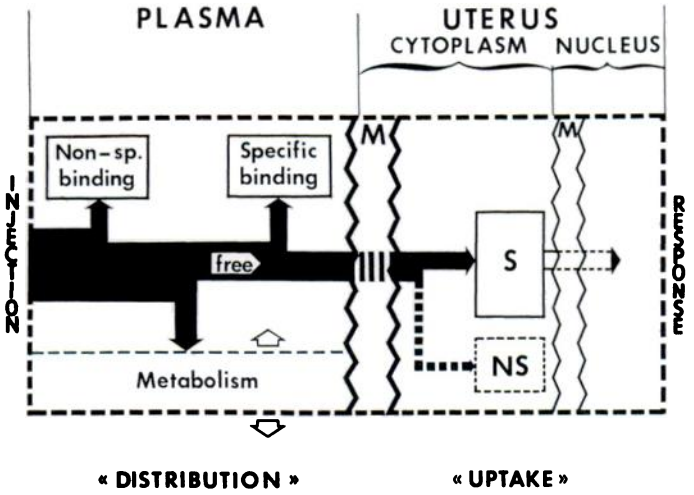

FIG. 1. *Pharmacodynamic scheme*

The injected steroid diffuses through the plasma either in free or bound (specific or nonspecific) form and/or is metabolized to active (upward arrow) or inactive (downward arrow) compounds. The unbound steroid penetrates the cellular membrane more or less freely, depending upon its lipophilicity, emerges into the cytoplasm, where it interacts with a specific (S) cytoplasmic binding protein, and is then translocated to the nucleus, where it generates the biological response.

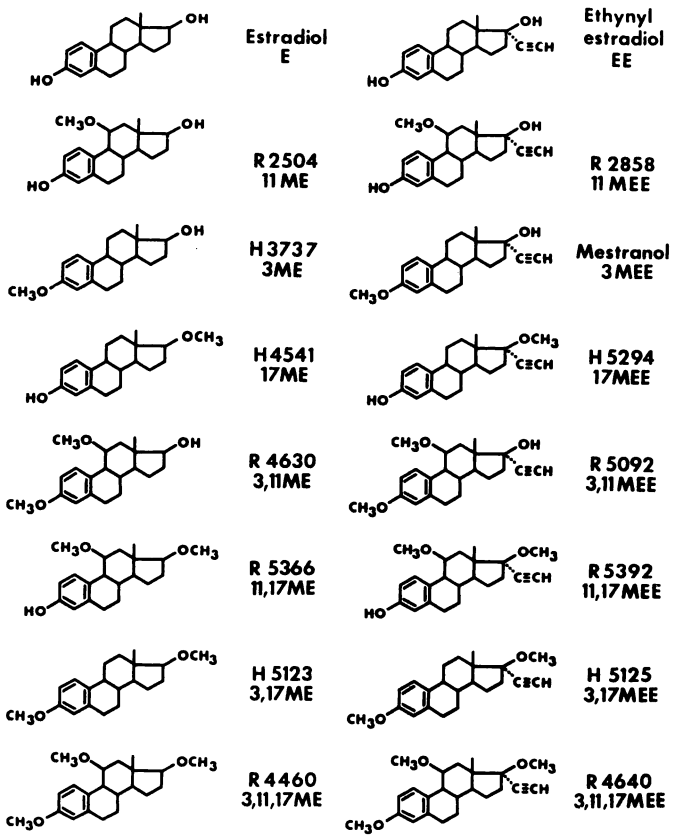

FIG. 2. *Formulae, names or reference numbers, and abbreviations of test steroids*

ethynylestradiol,[1] were synthesized in our laboratories and labeled in positions 6 and 7 with tritium (specific activity, approximately 50 Ci/mmole). [6,7-^{3}H]Estradiol was prepared by the catalytic reduction of 6-dehydroestradiol with carrier-free tritium gas. Tritiated 11β-methoxy derivatives were similarly prepared from 11β-methoxyestrone (15) after a five-step reduction to 11β-methoxy-6-dihydroestradiol. Tritiated 3- and 3,17-methoxy derivatives were obtained by direct methylation with methyl sulfate (weak base solution) and methyl iodide (strong base solution), respectively. Tritiated 17-methoxy derivatives were also prepared by direct methylation, but following protection of the hydroxyl at position 3 by reaction with pyranyl. The purity of the labeled steroids (more than 98%) was checked by thin-layer chromatography in benzene–ethyl acetate (9:1, v/v).

Proteins. Soluble tissue proteins were recovered in the supernatant fraction from rat uteri crushed in an all-glass homogenizer in either 0.88 M sucrose (pH 7.0) or 0.01 M Tris-HCl–1.5 mM EDTA buffer (pH 7.4) and then centrifuged at 105,000 × *g* for 1.5 hr at 4° in a Spinco ultracentrifuge. Plasma was obtained from the blood of the same animals. The blood was collected in heparinized tubes and centrifuged at 3000 rpm for 10 min at 4°. Bovine serum albumin was purchased from Armour Pharmaceutical Company, Chicago. Protein concentration was measured by the method of Lowry *et al.* (16).

Radioactivity measurements. Following dis-

[1] For the sake of simplicity, they will be referred to in the text by the following abbreviations: E, estradiol; EE, ethynylestradiol; M, methoxy or methyl substitution. The positions of the substituents are indicated by figures preceding the abbreviations; e.g., 3,11ME is 3-methyl ether 11-methoxyestradiol.

solution of plasma and homogenate samples in methoxyethanol–toluene (2:3, v/v) containing naphthalene (8%, w/v) and butyl PBD [2-(4′-terbutyl phenyl) 5-(4″-diphenyl), 1,3,4 oxadiazole] (0.4% w/v), and of organic solutions in toluene containing only butyl PBD (0.4%, w/v), radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (model 3375) with a standard error of counting of less than 1%. All counts were corrected to 100% efficiency by external standardization.

Determination of uterotrophic activity in rats (17). Groups of five rats received subcutaneous injections of the test compounds in 0.1 ml of a 5% solution of benzyl alcohol in sesame oil, daily for 3 days, and were killed 24 hr after the last administration. The uteri were excised and weighed. The increase in uterine weight is a measure of uterotrophic activity.

Determination of "dynamic" uterotrophic activity (18) and radioactivity uptake by rat uteri. Groups of five rats received an intravenous injection of 0.1 μg (about 3 μCi) of labeled steroid in 0.5 ml of a 10% solution of ethyl alcohol in 0.9% NaCl and were decapitated at various times during the 72 hr following injection. The uteri were rapidly excised, weighed, and crushed in 0.5 ml of NaCl in a conical all-glass homogenizer at 0°. The samples were washed three times with scintillator, and total tissue radioactivity was counted.

Metabolic studies. The plasma and crushed uteri from groups of five rats that had received intravenous injections of 0.1 μg (about 15 μCi) of labeled compound in 0.5 ml of a 10% solution of ethyl alcohol in 0.9% NaCl were pooled, and the volume of the latter was made up to 3 ml with NaCl. Total radioactivity was determined on a 1-ml aliquot, whereas 20 μg of unlabeled compound were added to a 2-ml sample, which was then extracted twice with 10 ml of chloroform. The chloroform extracts were then subjected to thin-layer chromatography in a cyclohexane–acetone (7:3, v/v) or benzene–ethyl acetate (7:3, v/v) solvent system. A control plate enabled the test compound and its main metabolites to be identified by their R_F values. The plates were scraped and eluted with methanol, and the radioactivity of each fraction was counted, then corrected by a loss factor.

Sucrose gradient ultracentrifugation. Either 1 ml of cytosol prepared from rat uteri homogenized in 0.01 M Tris-HCl–1.5 mM EDTA buffer (pH 7.4) containining 10 mg of protein or 1 ml of 1:10 diluted plasma was incubated for 2 hr at 4° with the radioactive steroid and then layered on a linear sucrose gradient [5–20% (w/v) sucrose dissolved in the same buffer]. The gradients were centrifuged at 40,000 rpm for 18 hr at 4° in a Spinco model L65 centrifuge, using an SW 41 rotor. The radioactivity of 4-drop fractions, collected from the bottoms of the tubes, was measured. In experiments *in vivo* the animals received, 1 hr before death, 0.2 nmole (about 11 μCi) of labeled steroid in 0.5 ml of a 10% solution of ethanol in 0.9% NaCl. The cytosol from the excised uteri was layered and centrifuged as above.

Equilibrium dialysis. A Nojax dialysis bag containing 1 ml of either tissue protein (1.5 mg/ml), dilute plasma (1:20), or BSA (3 mg/ml) was introduced into 15 ml of 0.1 nM labeled steroid or 0.2–1000 nM unlabeled steroid in 0.1 M phosphate buffer (pH 7.4). After magnetic stirring at 4° for 20 hr (tissue) or 48 hr (plasma), the radioactivity of three 0.2-ml samples from inside and outside the bag was determined. The results obtained were represented in a proportion graph; the intrinsic association constant and number of binding sites were evaluated as described previously (19–21). In competition experiments, various concentrations of unlabeled competitor were added to a tracer concentration (0.15 nM) of radioactive steroid. The percentage decrease in bound radioactive steroid was recorded.

Kinetic studies. To determine the association rate (22), 1 nM labeled steroid (approximately 0.66 μCi) was added to 15 ml of a 1.5 mg/ml rat uterus cytosol protein solution in phosphate buffer (pH 7.4) maintained at 0°. Every 5 min for 1 hr, a 0.5-ml aliquot was transferred to a flask containing 0.5 μg of unlabeled steroid to stop the reaction. Immediately afterward 0.5 ml of cold dextran-coated charcoal suspension (0.5% Norit A and 0.05% dextran in the same

buffer) was introduced. The contents of the flask were shaken overnight at 4° and then centrifuged for 10 min at 3000 rpm. The radioactivity of two 0.2-ml aliquots of the supernatant fraction was counted.

RESULTS

Uterotrophic acitivity. Dose- and time-response curves have been plotted in Figs. 3 and 4, respectively, and show that the introduction of a methoxy group in position 11 very markedly increases activity with respect to EE and, in particular, to E. Methylation of the hydroxyl groups in positions 3 and 17 decreases it. The positive effect of the 11M substituent is also reflected in the activity of the di- and tri-substituted steroids. As a statistical analysis of the results to deduce a potency ratio has not been possible, since nonparallelism and curvature cannot be eliminated even though logarithmic transformation renders the original values homogeneous, active doses (index A) have been arbitrarily taken as the dose which increases the uterine weight either 2.5-fold for the estradiol derivatives or 5-fold for the ethynylestradiol derivatives.

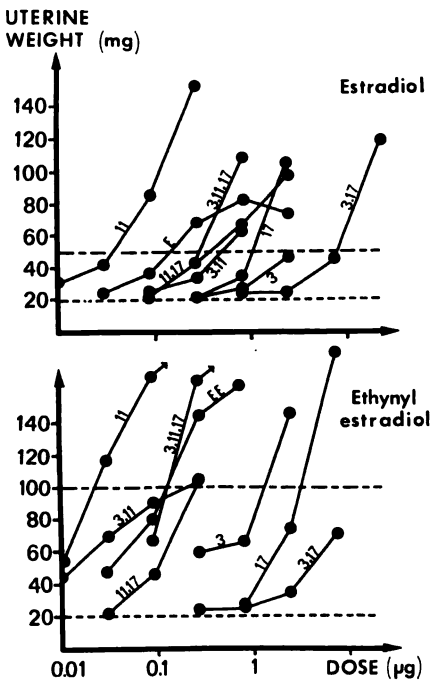

FIG. 3. *Dose-response curves (subcutaneous)*
Each value is the mean of five determinations. The lower broken line indicates the uterine weight of the controls. The upper line is an arbitrary one, drawn to enable the determination of the comparative uterotrophic activities from the points of intersection with the dose-response curves. It corresponds to a 2.5-fold increase in uterine weight for the E series and a 5-fold increase for the EE series.

Derivatives of EE are on the whole more active than those of E, primarily as a result of differences in metabolism explained by the impossibility of oxidizing tertiary alcohols to the less active ketones.

If the area beneath the uterotrophic activity curve between 0 and 72 hr is taken as a measure of the response and is expressed as an index P, the value for estradiol being taken as unity, it can be shown that good correlation exists between the active dose (index A) and index P (Table 1).

Uptake and biological response. As a high concentration of radioactivity in a given tissue does not imply per se that the tissue plays a physiological role, it is necessary to observe the uterotrophic activity and the

TABLE 1

Uterotrophic activity and radioactivity uptake
Uterotrophic activity results are expressed as the total dose (micrograms) (index A) required to increase uterine weight either 5-fold for the EE series or 2.5-fold for the E series, since a 5-fold increase cannot be obtained with estradiol itself. This dose is determined graphically from the dose-response curves as illustrated in Fig. 3. They are also expressed as an index P calculated from the area beneath the curve, corresponding to the increase in uterine weight between 0 and 72 hr (Fig. 4). The value of P for estradiol is taken as unity. Radioactivity uptake is expressed as an index I similarly calculated. The value of I for estradiol is taken as unity.

Substituent	E series			EE series		
	A	*P*	*I*	*A*	*P*	*I*
	0.15	1	1	0.15	1.4	1.7
3	3.0	0.2	0.4	1.3	0.5	0.7
11	0.04	2.1	1.7	0.02	2.5	3.9
17	1	0.1	0.4	3	0.1	0.6
3,11	0.5	0.4	0.5	0.2	2.3	2.9
11,17	0.4	0.8	0.9	0.25	2.4	3.3
3,17	8	0.1	0.3	15	0.2	0.3
3,11,17	0.3	0.5	0.6	0.15	2.0	2.1

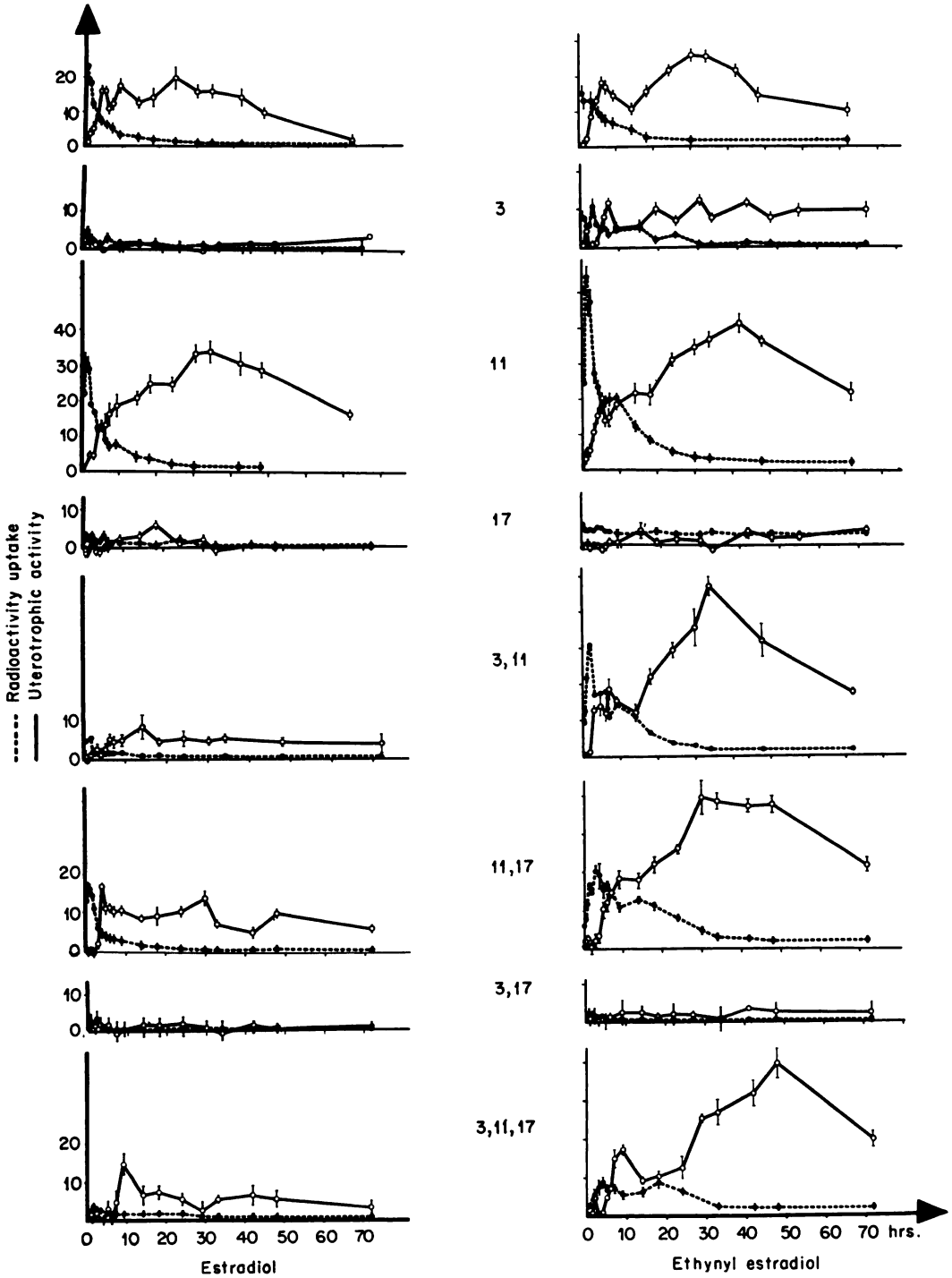

FIG. 4. *Dynamic uterotrophic activity and radioactivity uptake*

Following intravenous injection of 0.1 μg/rat of each of the 16 test steroids, the increase in uterine weight, in milligrams (——), and the radioactivity uptake, in 10^{-15} mole/mg (- - -) were represented as a function of time (hours). Each value is the mean and standard error of five determinations. The area beneath the curves between 0 and 72 hr was taken as a measure of uterotrophic activity (index *P*) and radioactivity uptake (index *I*). The figures in the center column indicate the position of the methoxy substituent in the estradiol (left-hand graphs) and ethynylestradiol (right-hand graphs) molecules. The topmost graphs refer to unsubstituted estradiol and ethynylestradiol.

radioactivity uptake in the uterus simultaneously in order to determine whether total uptake and biological response are correlated.

Figure 4 shows that the radioactivity concentration is highest for the compounds with the most pronounced activity, as can be seen by a comparison of the uterotrophic activity and radioactivity uptake indices (*P* and *I*, respectively) in Table 1. The uterotrophic activity increases as the radioactivity in the uterus increases; moreover, any delay in the radioactivity maximum, however slight, is reflected in a delay in the uterotrophic activity peak. Peak activity occurs between 25 and 45 hr. The introduction of an ethynyl or 11-methoxy substituent into the estradiol molecule appears to prolong action slightly, but cannot be said to give rise to a true long-acting or delayed effect.

The fraction of this total radioactivity uptake accounted for by the intact steroid and/or metabolite(s) has been determined. Total radioactivity in the uterus on injection of E, EE, 11ME, and 11MEE is due to unchanged compound only (Fig. 5), with a curve characteristic of retention rather

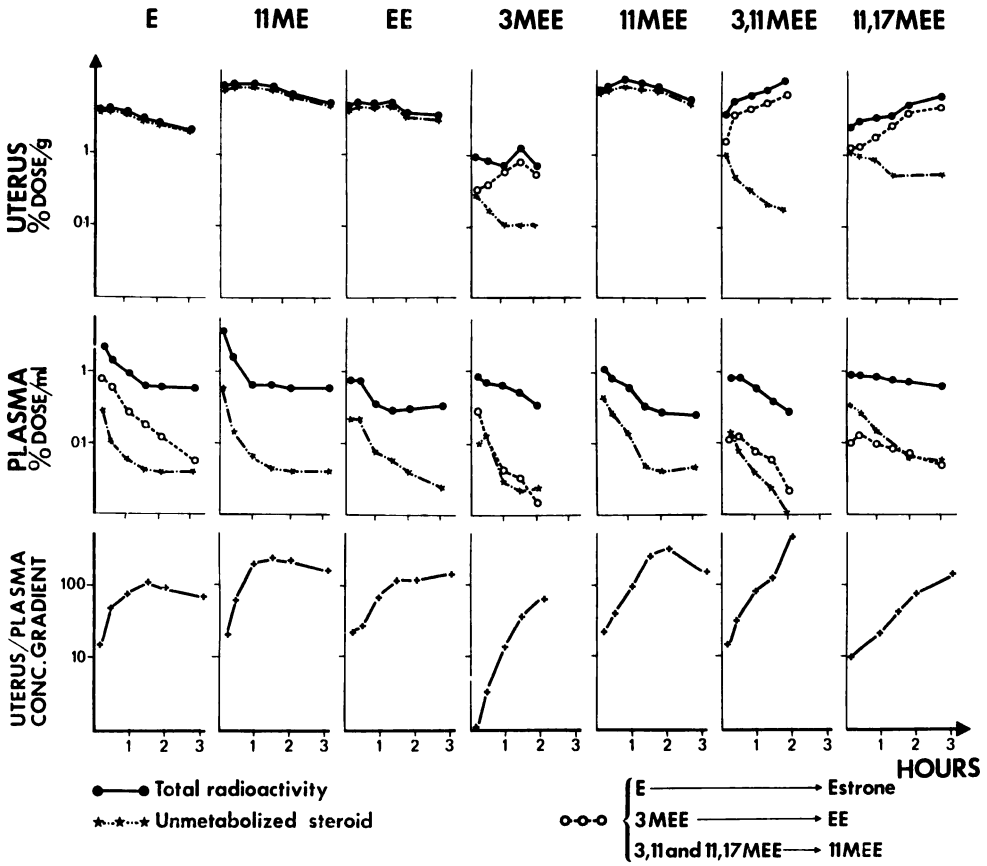

FIG. 5. *Metabolism of E, EE, and some of their derivatives in plasma and uterus*

The total radioactivity and that of the intact compound and some of its metabolites are expressed as a percentage of the dose per milliliter of plasma or per gram of tissue and plotted against time on a semilogarithmic scale. The metabolites were separated by thin-layer chromatography and identified by comparison with reference compounds, and the results were corrected by a loss factor. The only metabolites sought were estrone, EE, and its M derivatives. The unchanged steroid concentration gradient between uterus and plasma is illustrated in the bottom series of graphs. Beyond 3 hr, and in certain instances 2 hr, this ratio has no true significance in view of the low amount of unchanged steroid detected in the plasma.

than disappearance. Compounds substituted at position 3 undergo demethylation, as has already been demonstrated by Jensen *et al.* (23), as well as compounds substituted at position 17. In each case the tissue-stored forms are the metabolites EE, 11ME, and 11MEE. This observation reveals the difference between the stable 11-methoxy bond and the methyl ether bonds at positions 3 and 17, which may be cleaved, and has been confirmed for the other compounds of the EE series (20).

The compound giving rise to the highest concentration of radioactivity in the uterus is 11MEE, with a maximum occurring at 1 hr. However, if the 11MEE is a metabolite of 3- and/or 17M derivatives of 11MEE, the radioactivity curve no longer has a maximum during the first 3 hr, but a continuous upward slope. The percentage of the dose of 11MEE recovered is approximately the same whether 11MEE or a derivative is injected, but less EE is found on injection of a methyl ether derivative of EE than on injection of EE itself. This could explain the lower activity of 3- and/or 17MEE derivatives.

Of the compounds identified in the plasma (EE, its methoxy derivatives, and estrone), it was found that, for any one substrate, the same metabolites are recovered as in the uterus, except in the case of E, which is partially converted into estrone. In contrast to the uterus, however, the compounds are fairly extensively metabolized, as indicated by the marked difference between the total radioactivity level and that associated with the intact compound.

The unchanged steroid concentration gradient between uterus and plasma is illustrated in Fig. 5.

Specific binding in the uterus. Sucrose gradient ultracentrifugation was used to establish possible correlations among uptake, interaction with the estradiol-binding uterine cytosol protein, and activity. The study was restricted to the most potent compounds, i.e., E, EE, 11ME, and 11MEE (since all other active compounds are active as a result of demethylation only), and to the least potent compound, 3,17ME (for purposes of comparison).

The gradients for studies *in vivo* (Fig. 6) show that, for the particular dose injected, all active compounds are bound to a protein with a sedimentation coefficient of 8 S and that 3,17ME is not bound at all. The compounds with the highest peaks, the 11M derivatives, are also those found to exhibit highest uptake and highest activity (Table 1), and it thus appears that binding to the 8 S protein *in vivo* is directly related to activity.

The question arises whether a similar correlation might not also be valid for activity and binding *in vitro*. Figure 6 shows that, at a 5 nM concentration, all four active steroids are bound to the 8 S binding protein, that E and EE are also bound in the 4–5 S region, and that 3,17ME is not bound at all. The relative degree of binding of the various compounds has changed, however: whereas *in vivo* the 11M derivatives give the highest peaks, this is so for the nonsubstituted steroids *in vitro*.

The changes in the sucrose gradient density patterns resulting from the addition of nonradioactive compound have thrown further light on binding to the 4 S and 8 S proteins. Whereas any of the four active steroids at 10 nM displaces 1 nM estradiol from its 8 S peak to a 4 S peak (Fig. 7a), it displaces 2 nM 11MEE from the 8 S peak to the free zone, binding to the 4 S region being negligible (Fig. 7b).

Equilibrium dialysis experiments define more closely the physicochemical properties of the interaction and enable binding parameters to be calculated by analysis of the the curves obtained. As shown in Fig. 8, a pronounced S-shaped binding curve, typical of an S + NS system (19), was found for E, EE, 11ME, and 11MEE. The 3,17ME derivative gave a straight line, characteristic of an NS system, as did all the other steroids (20). Owing to the extent to which many of these compounds are nonspecifically bound, the absence of specific binding could only be established by competition studies with labeled 11MEE. At the lowest competitor concentration (3.33 nM), only E, EE, 11ME, and 11MEE gave rise to a marked decrease (30–50%) in specifically bound radioactive 11MEE. A similar decrease was observed on addition of a 33.3 nM concentration of the 17- and 11,17-methoxy deriva-

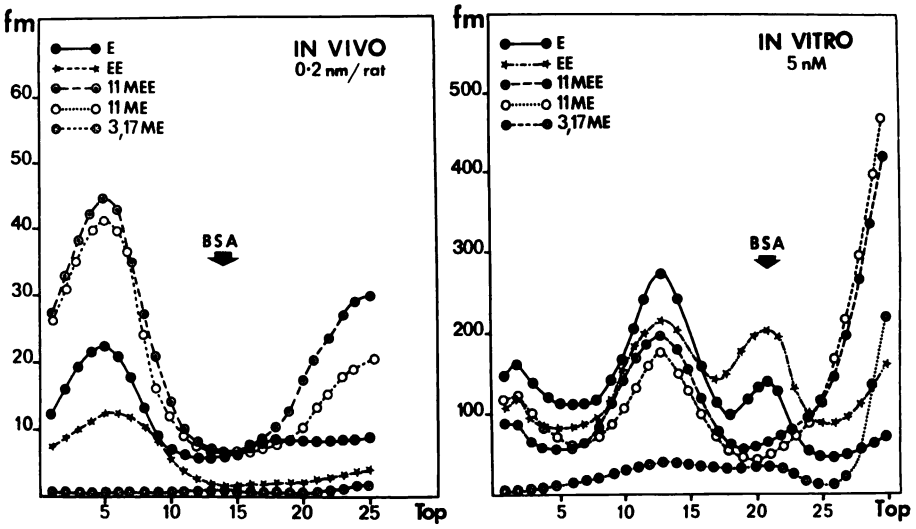

FIG. 6. *Comparison between binding in vivo and in vitro of E, EE, 11ME, 11HEE and 3,17ME*
In vivo: following injection of each labeled compound (0.2 nmole/rat), the uteri were excised and the cytosol was layered directly on a sucrose density gradient. *In vitro:* 1 ml of cytosol (10 mg of protein) was incubated for 1 hr at 4° with each labeled steroid and layered on a sucrose gradient. The amount of radioactive steroid (fm, 10^{-15} mole) present in each 4-drop fraction was determined. To identify the peaks, BSA (4.6 S) was run simultaneously. Whereas *in vivo* the 11-methoxy derivatives gave the highest 8 S peaks, the unsubstituted steroids did so *in vitro*. In both instances 3,17ME was not bound at all.

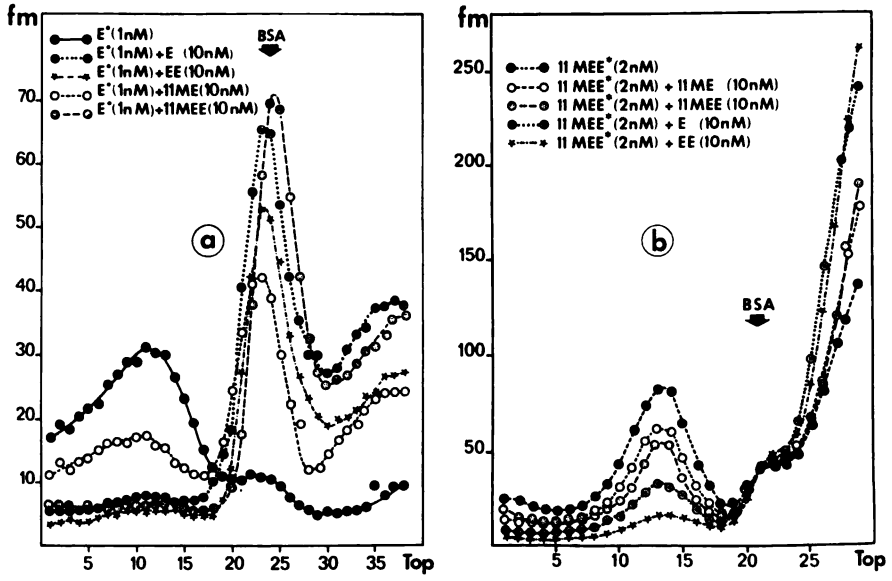

FIG. 7. *Competition studies with E, EE, 11ME, and 11MEE by ultracentrifugation*
Each of the four steroids (10 nM) was added to 1 μM radioactive E (a) or 2 nM radioactive 11MEE (b). Competition with E (a): any of the four steroids will displace E from the 8 S region. Competition with 11MEE (b): the radioactivity of 11MEE decreases in the 8 S region, but, instead of being displaced to the 4 S peak, reappears in the free zone. The ordinates are in units of 10^{-15} mole (fm).

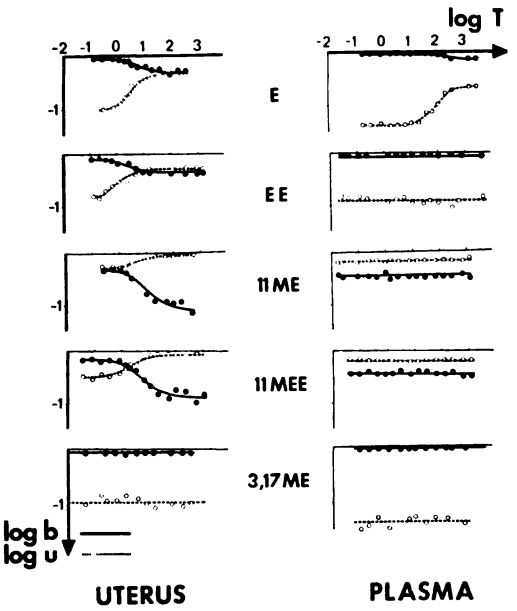

FIG. 8. *Proportion graphs representing binding to uterine cytosol proteins and to plasma*

The results of equilibrium dialysis experiments at 4° have been represented in proportion graphs in which the logarithms of the fraction of bound (*b*) and unbound (*u*) steroid are plotted against the logarithm of the total ligand concentration (*T*). E, EE, 11ME, and 11MEE are specifically bound to uterine cytosol (1.5 mg/ml). Only E is specifically bound to plasma (dilution, 1:20).

tives and a 333 nM concentration of the 3- and 3,11-methoxy derivatives. No 3,17-substituted compounds compete. It thus appears that all substitution affects binding: the 11-methoxy group produces a 3-fold decrease in affinity for the receptor, and methylation of the hydroxyl groups in positions 17 and 3 causes a 10-fold and 100-fold decrease, respectively. Binding parameters have been calculated from the proportion graphs for the four main compounds. Since the quantity of cytosol available for each experiment was limited, they were compared two by two: E with EE, 11ME with 11MEE, and, for confirmation, E with 11MEE. Table 2 shows that the number of binding sites, N_a, is the same in each case, and that the intrinsic association constant, K_a (affinity), varies in parallel with binding *in vitro* to the 8 S receptor, as shown in Fig. 7b. Affinity cannot therefore be directly correlated with the biological activity observed *in vivo*.

Although the activity of the compounds with no specific binding *in vitro* can be partly accounted for by their metabolism into specifically bound EE or 11MEE, the discrepancy between activity and affinity of the most active compounds remains unexplained. Rates of association are ranked in the same order as affinities (Fig. 9 and Table 2), thus implying that even this dynamic parameter, like the intrinsic association constant, cannot explain the ranking in biological potency.

Specific binding in plasma. The curves obtained by equilibrium dialysis for binding in the plasma (Fig. 8) show that only the natural hormone, E, is specifically bound to a plasma protein, called estradiol-binding plasma protein (21). Sucrose gradient ultracentrifugation studies confirm the absence of specific binding for 11ME and 11MEE (Fig. 10), but suggest that both E and EE are bound to a binding protein mixture with the same sedimentation coefficient (4–5 S) as BSA. Any EE-specific binding could be thought to be masked in the proportion graphs by nonspecific binding, but competition studies indicate that this specific binding is in fact quite negligible. Whereas unlabeled E competes with labeled E, unlabeled EE has little effect, and 11M derivatives have none at all. Thus only E exhibits pronounced specific binding in the plasma, and the EE peak is primarily accounted for by nonspecific binding. The K_a value, 3.10^8 M^{-1} (20, 21), is approximately 10 times lower than that of the cytosoluble receptor and roughly half that of sex steroid-binding protein (19, 24).

Nonspecific binding. The $(KN)_n$ products for nonspecific binding in the uterus, plasma, and to albumin have been calculated (Table 2). Binding is weakest in the case of dihydroxy steroids. Methylation increases binding, whereas the presence of a methoxy group at position 11 markedly decreases it. On the whole, parallel results are recorded for uterus, plasma, and albumin.

DISCUSSION

Among the 16 steroids studied, some have been found to be only very slightly active.

TABLE 2

Binding parameters

The intrinsic association constants (K_a), number of binding sites (N_a), and degree of nonspecific binding [$(KN)_{ns}$] were measured by equilibrium dialysis, using the proportion graph method. When the binding curves were flat, a rough estimate of the intrinsic association constant was obtained by competition with labeled 11MEE. The association rate (k_{+1}) was determined by the charcoal adsorption technique.

Steroid	Uterus (1.5 mg/ml)				Plasma (1:20)	BSA (3 mg/ml)
	K_a	N_a	k_{+1}	$(KN)_{ns}$	$(KN)_{ns}$	$(KN)_{ns}$
	nM^{-1}	nM	$10^5\ M^{-1}\ sec^{-1}$			
E	4.5 ± 2.0*[a]	1.0 ± 0.4	0.7	0.5	3.1	5.0
	6.6 ± 1.3§	0.8 ± 0.1				
11ME	0.7 ± 0.3‡	1.5 ± 0.6	0.2	0.1	0.5	0.3
3ME	~0.01			6.8	12.0	11.2
17ME	~0.1			2.6	11.7	20.5
3,11ME	<0.01			0.2	0.5	0.7
11,17ME	~0.1			0.9	1.1	8.7
3,17ME	<0.001			7.8	20.4	24.6
3,11,17ME	<0.001			1.1	2.6	14.9
EE	7.1 ± 1.4*	0.7 ± 0.1	0.8	0.9	6.9	8.4
11MEE	1.4 ± 0.2‡	1.4 ± 0.3	0.5	0.1	0.6	0.4
	2.2 ± 0.2§	0.8 ± 0.1				
3MEE	~0.01			6.9	18.3	12.3
17MEE	~0.1			5.0	14.7	17.1
3,11MEE	~0.01			0.6	1.2	0.5
11,17MEE	~0.1			3.2	3.8	3.6
3,17MEE	<0.001			4.6	13.0	10.5
3,11,17MEE	<0.001			3.9	8.2	8.9

[a] E was compared with EE (*), 11ME with 11MEE (‡), and, for confirmation, E with 11MEE (§).

Their study ought not to be neglected, however, since even an inactive member of a defined chemical series of compounds yields useful information on mechanism of action. Others, such as 3, 11, 17MEE or 3, 11MEE, which are not specifically bound *in vitro*, are active only as a result of demethylation *in vivo*. Finally, a few are highly potent and their activity has been explained by the proposed pharmacodynamic scheme, which takes into consideration all the parameters covered (Fig. 11). The low-activity compound 3,17ME has been included for comparison.

The ethynyl group decreases metabolism and increases the affinity for the uterine receptor, thereby enhancing activity. An 11-methoxy substituent results in virtually total absence of plasma binding, whether specific or nonspecific, in a change in lipophilicity (20), and consequently in a high plasma concentration of free steroid and in better distribution. In spite of decreased uterine receptor affinity, an increase in uterine steroid concentration, and consequently in activity, is nevertheless recorded. The positive elements of these two substitutions are combined in 11MEE, which is the most active compound of the series. Methylation of the hydroxyl groups at positions 3 and 17, on the other hand, results in virtually nonexistent affinity, and the compound has to be extensively metabolized to exhibit even weak activity.

The foregoing experiments have also yielded information on the importance of

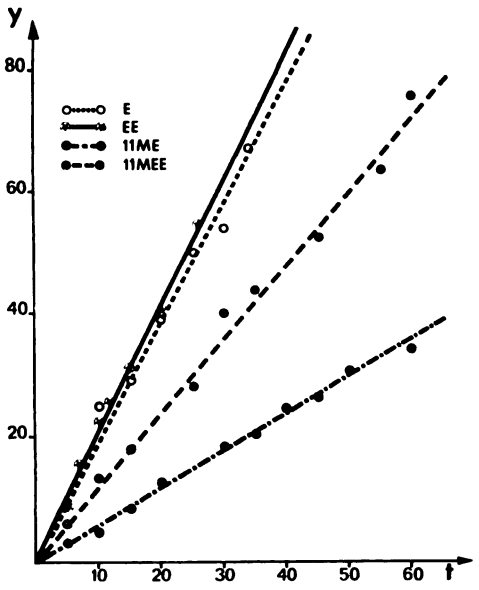

FIG. 9. *Rates of association of E, EE, 11ME, and 11MEE steroid-protein complexes*

The rate of association is given by the slope of the line.

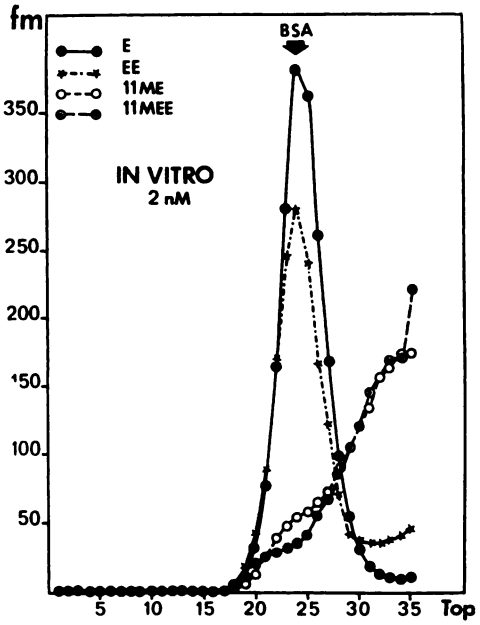

FIG. 10. *Binding of E, EE, 11ME, and 11MEE to rat plasma*

One milliliter of plasma (dilution 1:10) was incubated with 2 nM labeled steroid and then layered on a sucrose gradient. Both E and EE are bound in the 4 S region, whereas 11ME and 11MEE are found only in the free zone. The ordinate is in units of 10^{-15} mole (fm).

the steroid-protein interaction. All known estrogens are bound, to a greater or lesser extent, by cytosoluble uterine binding proteins, this being one of the earliest events in the mechanism governing the induction of the cell machinery response. It has been shown here that each and every active steroid, or in certain instances active metabolite, is bound to these proteins *in vivo* and, consequently, that binding to the cytosol 8 S protein is an unconditional requirement for activity.

They have moreover illustrated the difficulty encountered in interpreting and correlating results *in vitro* and *in vivo* when all factors which may influence activity are not taken into consideration. Although it has been possible to bring to light a relationship among biological activity, uptake, and uterine binding *in vivo*, it has not been possible to extend this relation to binding *in vitro*. The most active compounds are not necessarily those bound most tightly as shown by the fact that the 11M derivatives have markedly lower intrinsic association constants than E and EE.

It is partly the absence of specific binding to plasma proteins which explains the higher activity of 11-methoxy derivatives as compared to estradiol and ethynylestradiol, in spite of a lower affinity for the uterine 8 S receptor, thereby confirming that the formation of a steroid-protein complex in the plasma affects the biological activity recorded in the uterus (25). If it were necessary for the steroid to be bound to plasma proteins to cross the target cell membrane (26), the 11-methoxy derivatives would be inactive. It seems unlikely, therefore, that these proteins should act as transport proteins which carry the steroid from the plasma to the cells (27). They may, however, protect the steroids from metabolism (28), since if the dissociation of the complexes were slow, as in the case of specific plasma proteins, only the free fraction would be subject to enzymatic action. Whatever their role, it is certain that binding to plasma proteins is not a prerequisite for activity, but can modulate it (29).

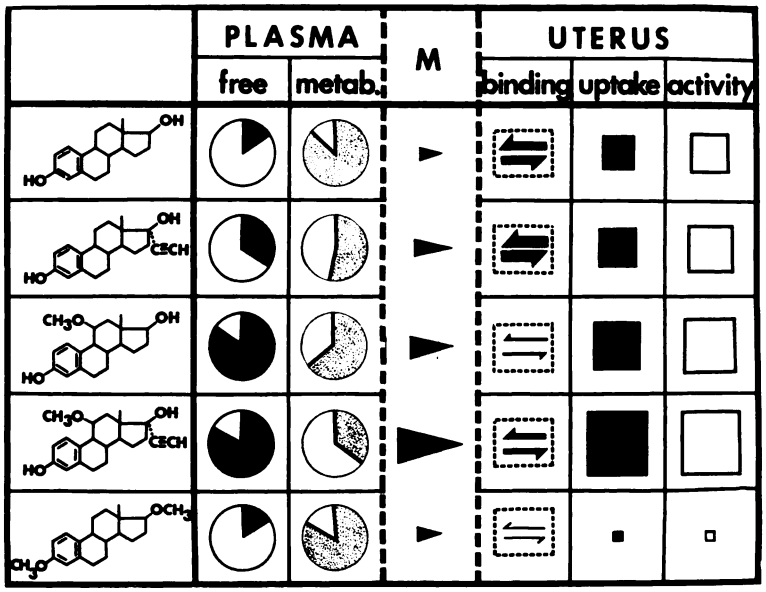

FIG. 11. *Application of the pharmacodynamic scheme*

The pharmacodynamic scheme outlined in the INTRODUCTION may be used to explain the biological activity of the test steroids as follows. Plasma: the filled and speckled sectors indicate the amounts of free and metabolized steroid, respectively. Membrane (M): the triangles represent the percentage of free steroid after metabolism. Uterus: the width of the reversible arrows depicts the magnitude of the intrinsic association constant of the steroid for the 8 S uterine binding protein. The black and white squares represent uptake and uterotrophic activity, respectively, the area for estradiol being taken as unity.

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