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Characterization of Steroid-Binding Sites by Affinity Labeling. Further Studies of the Interaction between 4-Mercuri-17β-estradiol and Specific Estrogen-Binding Proteins in the Rat Uterus*

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ABSTRACT: The nature and extent of binding of 4-mercuri-17β-estradiol to a specific 8S rat uterine cytosol fraction and the subsequent transfer of this complex into the particulate fraction were investigated. When different amounts of the mercury steroid are incubated with cytosol samples, a constant level of steroid becomes bound in such a manner that it cannot be extracted with organic solvents. Preincubation with 17β-estradiol decreases the level of nonextractable steroid by an amount approximating that which sediments on sucrose density gradients as a complex with 8S protein. Incubation of cytosol with 4-mercuri-17β-estradiol eliminates subsequent binding of 17β-estradiol in the 8S region, and the sucrose gradient pattern resembles that observed when 17β-estradiol is incubated with preheated cytosol. Transfer of the mercury steroid from its 8S-complexed cytosol form into the particulate fraction does not occur under conditions where specific nuclear uptake of 17β-estradiol is demonstrable. The amount of 17β-estradiol which is specifically bound to 4–5S nuclear components is decreased proportionately when subsaturation levels of 4-mercuri-17β-estradiol are incubated with cytosol prior to the introduction of the 17β-estradiol. The results suggest that the previously observed estrogenic activity of the mercury derivative is not the result of intranuclear steroid–protein interaction, in contrast to the generally proposed mechanism of action of 17β-estradiol.

Uptake of estradiol by rat uterine tissue *in vitro* is accompanied by interaction of the steroid with a specific cytosol receptor protein having a sedimentation coefficient of 8 S (Toft and Gorski, 1966; Jensen *et al.*, 1968; Rochefort and Baulieu, 1969). Transfer of estradiol into the particulate fraction is occasioned by appearance of specific binding to a 5S component (Shyamala and Gorski, 1969; Puca and Bresciani, 1968; Jensen *et al.*, 1969) and to a 4S component (Jensen *et al.*, 1969; Musliner *et al.*, 1970). Although nuclear receptor complex formation is dependent on integrity of the cytosol receptor system (Jensen *et al.*, 1968; Brecher *et al.*, 1967), it has not been demonstrated clearly whether or not dissociation of the cytosol steroid–protein complex precedes nuclear uptake of estradiol.

The synthesis and affinity-labeling capability of 4-mercuri-17β-estradiol (4ME)[1] for estrogen-sensitive enzymes were described in the first paper of this series (Chin and Warren, 1968). Subsequent work established the inherent estrogenic activity of 4ME and its ability to interact with the uterine cytosol 8S estrogen receptor protein in the rat in a manner consistent with an affinity-labeling mechanism (Muldoon and Warren, 1969). The studies presented herein show that 4ME, in contrast to estradiol, interacts with the 8S receptor in an irreversible manner, and that the stable complex thus formed is not transferred to the particulate fraction. The fact that 4ME is estrogenic suggests that the primary intracellular events which precede manifestation of biological activity are different from those observed with the native hormone.

Materials and Methods

Female Holtzman rats, 21–23 days of age, were sacrificed by cervical dislocation. Uterine horns were excised, trimmed, rinsed in cold hypotonic Tris–EDTA buffer (0.01 M Tris and 0.0015 M Na_2EDTA, pH 7.4), and homogenized in the same buffer at a concentration of 3 uteri/ml. The homogenate was centrifuged for 90 min at 105,000*g* in a Spinco Model L2-65 ultracentrifuge. The resultant supernatant is referred to as the

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[1] The abbreviation 4ME is used to designate compounds in solution arising from added 4-acetatomercuri-17β-estradiol, with the understanding that the mercury steroid may exist in the free form or in combination with solution anions other than acetate.

TABLE I: Solvent Extraction of 4ME and Estradiol from Cytosol.[a]

Treatment[b]	Benzene[c] Extract, No Pretreatment (cpm)	Benzene Extract, Pretreated Cytosol[d] (cpm)	Difference (cpm)
Estradiol-6,7-*t* (104,550 cpm)	101,828 ± 4744	103,247 ± 1053	1,419
4ME-6,7-*t* (325,537 cpm)	156,413 ± 4404	181,500 ± 3857	25,087
4ME-6,7-*t* (208,460 cpm)	48,628 ± 2417	72,048 ± 1911	23,420

[a] Incubations performed for 30 min at 4° using 0.5-ml aliquots of a single cytosol solution. Data are mean of three separate incubations plus and/or minus standard error. [b] Respective molar concentrations of labeled steroid are 5×10^{-9}, 1.5×10^{-7}, and 9.9×10^{-8} M. [c] Extraction with chloroform gave identical results. [d] Cytosol was preincubated with unlabeled estradiol (4×10^{-8} M) for 30 min prior to 30-min incubation with estradiol-6,7-*t* or 4ME-6,7-*t*.

cytosol fraction; the pellet, as the particulate fraction. The appropriate steroid was added to 0.5-ml aliquots of cytosol at final concentrations indicated in the text. The procedures of Jensen *et al.* (1968) were used for particulate fraction recombination and KCl extraction of the resedimented pellet.

With the exception of the 5-min recombinations at 37°, all procedures, including centrifugation, were performed at 2–4°. Glass-redistilled water was used throughout.

17β-Estradiol-6,7-*t* (50.0 Ci/mmole, New England Nuclear Corp.) was brought to greater than 98% purity by descending paper chromatography. Unlabeled 17β-estradiol was obtained from Sigma Chemical Co. and used without further purification. The 4ME-6,7-*t* was synthesized from 17β-estradiol-6,7-*t* as previously described (Muldoon and Warren, 1969). One preparation, used in the solvent extraction experiments, was synthesized from a mixture of tritiated and unlabeled estradiol and was purified by crystallization and paper chromatography to a specific activity of 5.0 Ci/mmole. A second preparation, used in the recombination experiments, was synthesized with much smaller amounts of carrier and was purified by repeated chromatography (25.1 Ci/mmole). Radiochemical purity was at least 97% in both samples. Unlabeled 4ME was prepared according to Chin and Warren (1968). Appropriate dilutions of stock solutions in absolute ethanol were made such that the total concentration of alcohol in the incubation systems never exceeded 1%.

Sucrose density gradient centrifugation was performed in a Spinco Model L2-65 ultracentrifuge for 13 hr at 220,000*g* using an SW-65 titanium rotor. Linear 5–20% sucrose gradients were prepared in Tris–EDTA (containing 0.3 M KCl for particulate extracts) and samples of 200 μl were applied. Armour standard bovine serum albumin (5 mg/ml in Tris–EDTA) was centrifuged simultaneously as reference standard. The tubes were punctured and 100-μl fractions were collected by gravity. Albumin was quantified by the method of Lowry *et al.* (1951). Radioactivity was measured in a Packard Model 3375 liquid scintillation spectrometer using the medium described by Bray (1960) with 30% efficiency for tritium. Counting was performed at a level which permitted an error of ±2.5% at 95% confidence limits.

Steroid extraction was effected with either benzene or chloroform, both reagent grade, redistilled prior to use. Cytosol samples were incubated for 30 min after addition of steroid and then extracted with a total of six volumes of organic solvent. The organic phases were pooled and evaporated to dryness in scintillation vials. The residue was redissolved in 0.2 ml of ethanol and the scintillation mixture was added.

The *in vivo* studies were performed on two groups (6 animals/group) of 23-day-old rats. All rats were injected intraluminally with 0.1 μg of tritiated 4ME (25.1 Ci/mmole), dissolved in 50 μl of 1.8% saline, and divided evenly between the two uterine horns (Muldoon and Warren, 1969). The first group was sacrificed 2 hr after injection; the second group, 6 hr after injection. Uteri from each group were excised, slit longitudinally, pooled, and rinsed three times in a total of nine volumes of cold Tris–EDTA buffer. The tissues were then homogenized (3 uteri/ml of Tris–EDTA buffer) and the cytosol and particulate fractions were separated by centrifugation. After KCl extraction, the particulate fraction was dissolved by shaking gently in 1 ml of NCS solubilizer (Amersham-Searle). Radioactivity was determined on samples of cytosol, dissolved particulate fraction, and KCl extract of particulate fraction.

Results

Strong binding of 4ME to rat uterine cytosol receptor is demonstrated by the inability to disrupt the complex with organic solvents, as shown in Table I. When estradiol-6,7-*t* is incubated with the cytosol fraction, it can be quantitatively extracted with either benzene or chloroform. Preincubation with unlabeled estradiol does not change the benzene-extractable radioactivity. Incubation of aliquots of the same cytosol with 4ME-6,7-*t* at concentrations of either 1.5×10^{-7} or 9.9×10^{-8} M resulted in a constant high level of nonextractable binding. This binding represents the total concentration of available 4ME-binding sites in the tissue samples, since 4ME has been shown to interact rapidly and nonspecifically with protein sulfhydryl groups (Chin and Warren, 1968). In order to determine the specific binding capacity of the cytosol receptor protein, cytosol samples were incubated with sufficient unlabeled estradiol to saturate this protein, and were then incubated with either of the forementioned levels of 4ME-6,7-*t*. Previous studies (Muldoon and Warren, 1969) have demonstrated that affinity labeling of 4ME to the receptor protein does not occur when the binding sites are presaturated with estradiol. When this is done, benzene-extractable 4ME-6,7-*t* increases by an amount which is considered equal to the minimal value of the saturation level of the receptor. It can be seen from Table I that this amount is constant for the two levels of 4ME chosen and corresponds to about 4 pmoles/uterus.

Confirmation of this finding was sought by fractionating samples of 4ME-6,7-*t* incubated cytosol by sucrose density gradient centrifugation, collecting the fractions corresponding to the central portion of the 8S receptor peak, and attempting

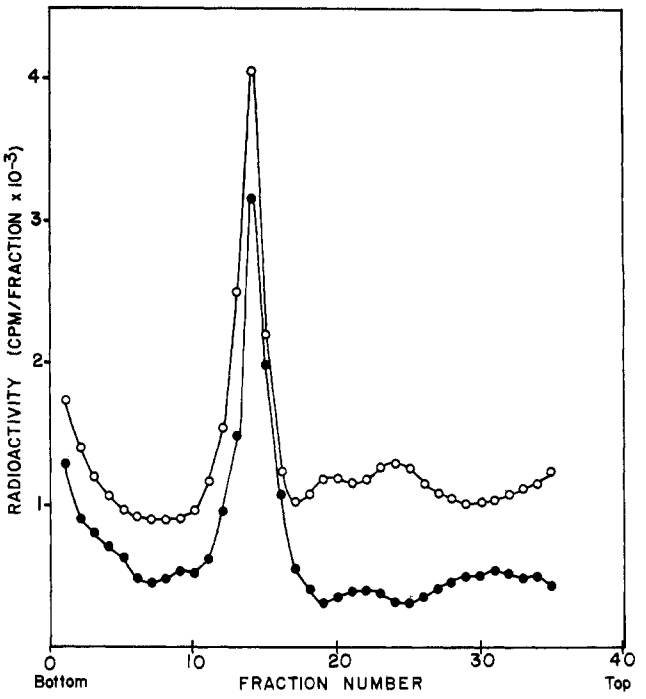

FIGURE 1: Density gradient sedimentation patterns of 0.5-ml aliquots of cytosol incubated at 4° for 30 min with 4ME-*6,7-t* at concentrations of 3×10^{-8} M (closed circles) and 6×10^{-8} M (open circles). Cytosol samples of 200 μl each were layered on a 5–20% linear sucrose gradient in Tris–EDTA. Centrifugation was for 13 hr at 220,000*g*.

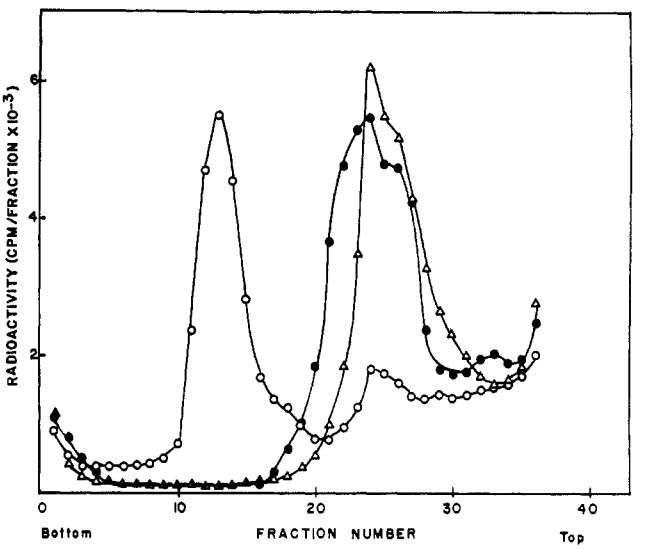

FIGURE 2: Distribution of estradiol-*6,7-t* (1.0×10^{-8} M) in cytosol fractionated by sucrose gradient centrifugation following 30-min preincubation with: ethanol (1% of total volume) (open circles), or with 4ME (1.3×10^{-8} M) in ethanol (triangles). Incubation of preheated cytosol (45°, 30 min) with estradiol-*6,7-t* is also shown (closed circles). Conditions as described in Figure 1.

solvent extraction of the steroid from these fractions. The sucrose density patterns of cytosol after incubation with 4ME-*6,7-t* at concentrations of 3×10^{-8} and 6×10^{-8} M are shown in Figure 1. The greater amount of radioactivity found in the 8S peak at the higher steroid concentration appeared to be the result of nonspecific binding in this region, since the steroid distribution throughout the field was increased to a consistently higher level than at the lower concentration. Complete binding of the 4ME was evidenced by lack of a rise in radioactive content at the top of the gradient. The total radioactivity in the 8S peak at the lower steroid concentration (fractions 12–16, Figure 1; equivalent to 21,640 cpm/0.5 ml of cytosol) closely approximated the cytosol radioactivity which became extractable after preincubation with unlabeled estradiol (Table I). Fractions 13–15 were pooled from concurrently centrifuged samples. Benzene extraction gave the results shown in Table II. In three separate experiments at each level of 4ME, the extractable radioactivity represented less than 1% of the total radioactivity present.

In order to assess specific nuclear uptake of 4ME, it was necessary to demonstrate complete lack of competition

TABLE II: Solvent Extraction of 4ME-*6,7-t* from 8S–Receptor Complex.

Radioactivity (cpm)	4ME-*6,7-t* Incubated	
	3×10^{-8} M (65,110 cpm)	6×10^{-8} M (130,220 cpm)
Recovered[a]	11,576 ± 479	17,916 ± 1954
Aqueous phase	11,361 ± 429	17,726 ± 2161
Organic phase	99 ± 6	157 ± 13

[a] Total radioactivity in three fractions corresponding to maximal 8S binding (fractions 13–15 from Figure 1), pooled from two centrifuge tubes after gradient centrifugation. Extraction performed with benzene. Data are presented as mean of three separate experiments at each level of steroid plus and/or minus standard error.

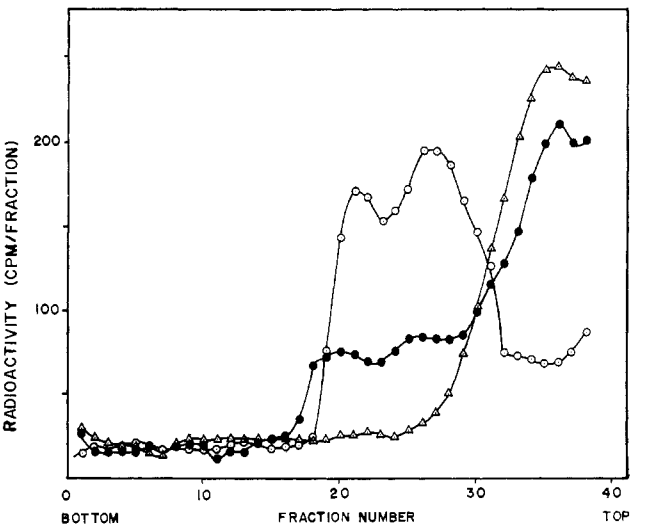

FIGURE 3: Sucrose gradient centrifugation patterns of 0.3 M KCl extracts of particulate fraction after recombination for 5 min at 37° with cytosol treated as indicated. Cytosol was incubated for 30 min at 4° with estradiol-*6,7-t* (2.22×10^{-9} M) following 30-min incubation with: ethanol (1% of total volume) (open circles), 4ME (4.3×10^{-9} M) in ethanol (closed circles), or 4ME (1.72×10^{-8} M) in ethanol (triangles). Centrifugation of KCl extracts was for 13 hr at 220,000*g*. Of total 3-ml extract in each case, 200 μl was analyzed. Total radioactivity in each sample centrifuged was, respectively, 2942, 2761, and 2614 cpm. Details of the procedure are given in the text.

TABLE III: Lack of Particulate Fraction Uptake of Cytosol 8S-Bound 4ME-*6,7-t*.[a]

	Reisolated Particulate Fraction (cpm)		0.3 M KCl Extract (cpm)	
4ME-*6,7-t* Incubated	Expt 1	Expt 2	Expt 1	Expt 2
4.3×10^{-9} M (164,500 cpm)	1540	1110	107	79
8.6×10^{-9} M (329,000 cpm)	1650	2106	77	73
1.72×10^{-8} M (658,000 cpm)	2218	1742	80	62

[a] 4ME-*6,7-t* (25.1 Ci/mmole) was incubated with 0.8-ml samples of cytosol in the amounts indicated. Recombinations with 0.8-ml portions of resuspended particulate fractions were performed for 5 min at 37° in duplicate. Final particulate extraction was effected with 3 ml of 0.3 M KCl.

TABLE IV: Distribution of 4ME-*6,7-t* in Uterine Fractions Following *in Vivo* Administration.[a]

Group	Cytosol (cpm)	Solubilized Particulate Fraction (cpm)	0.3 M KCl Extract
I	582,640	826,665	6420
II	620,400	1,360,500	4875

[a] Groups of six animals were injected intraluminally with 4ME-*6,7-t* (25.1 Ci/mmole, 0.1 μg/animal). Group I was sacrificed 2 hr after injection; group II, 6 hr after injection. Fractions of the uterine homogenates were prepared and analyzed for radioactivity.

between 4ME and estradiol for specific cytosol binding sites. The experiments described above, in conjunction with previous data (Muldoon and Warren, 1969), clearly show that pretreatment with estradiol blocks subsequent binding of 4ME to the cytosol receptor. The data in Figure 2 are the results of experiments designed to examine the ability of estradiol to interact with 8S receptor which has been saturated with 4ME. Preincubation of cytosol with the mercury steroid (1.3×10^{-8} M) completely blocks uptake of labeled estradiol by the 8S protein. Most of the estradiol becomes associated with a 4–5S peak. The pattern of estradiol distribution is similar to that observed when the cytosol receptor binding capacity is destroyed by preheating to 45° for 30 min (Figure 2; *cf.* also, Jensen *et al.*, 1968).

Subcellular distribution of estradiol between cytosol and particulate fractions was studied in the presence and absence of 4ME. After centrifugation of uterine homogenates at 105,000*g*, the supernatant cytosol fraction was decanted (3.2 ml). The sedimented particulate fraction was rinsed with buffer and recentrifuged. The remaining sediment was resuspended by gentle homogenization in 3.2 ml of cold buffer. Recombinations were performed for 5 min at 37° using 0.8 ml of cytosol and 0.8 ml of resuspended particulate fraction. After incubation, the combined fraction was chilled in an ice bath and recentrifuged for 90 min at 105,000*g*. The sediment was extracted into a total of 3 ml of 0.3 M KCl in Tris–EDTA buffer. When cytosol was incubated with estradiol-*6,7-t* (2.22×10^{-9} M) and recombined with the particulate fraction, 65% of the radioactivity was found associated with the particulate fraction (107,250 cpm). Extraction of this fraction with 0.3 M KCl released 37% of the radioactivity (39,540 cpm). The sucrose density centrifugation analysis of the KCl extract (Figure 3) showed binding in the 4S and 5S regions. Preincubation of cytosol with 8S-saturating levels of 4ME (1.72×10^{-8} M) did not inhibit uptake of estradiol into the particulate fraction or into the KCl extract thereof; binding to specific receptors, however, was eliminated. Preincubation with a nonsaturating amount of 4ME (4.3×10^{-9} M) again did not decrease either particulate uptake or the amount extractable, but binding in the 4S and 5S regions was observed at a lower level, in approximate relation to the concentration of cytosol binding sites not occupied by 4ME.

Attempts to effect direct particulate uptake and specific binding of 4ME from the cytosol fraction are summarized in Table III. Levels of 4ME-*6,7-t* both above and below the calculated 8S receptor binding capacity were incubated with cytosol fractions in the same manner as described above for estradiol. Presence of 8S binding was demonstrated by sucrose gradient centrifugation of one sample. In contrast to the results obtained with estradiol, very little uptake by the particulate fraction was found. The amount associated with the particulate fraction was not proportional to the amount added to the cytosol and was well within the experimental range of error of the procedure used to separate cytosol from the particulate fraction after recombination. The very low amounts of radioactivity extracted by KCl are a further indication of lack of nuclear receptor association.

When 4ME-*6,7-t* was injected intraluminally into immature rats, the uptake of radioactivity in the cytosol fraction was essentially the same 2 hr after injection and 6 hr after injection (Table IV). Large amounts of radioactivity were found associated with the particulate fractions, but the KCl extraction removed only a small percentage of this radioactivity, indicating that most, if not all, of this steroid was bound to species other than specific nuclear receptors. Specific 8S binding was readily demonstrable by sucrose gradient fractionation of the cytosol fractions, but no specific binding was observed upon similar treatment of the KCl extracts.

Discussion

Specific uptake and retention of estradiol by cell-free preparations of rat uterine tissue has been shown, by extensive investigations in the laboratories of Jensen and Gorski, to involve binding of the hormone to an 8S cytosol protein, followed by temperature-dependent appearance of a 5S steroid–protein complex in the particulate fraction. In the present studies, the interrelationship between the cytosol and particulate receptors was studied by taking advantage of the formation of a specific and nondissociable complex between the 8S cytosol receptor and a synthetic estrogen.

The work of Chin and Warren (1968) established that 4ME reacts rapidly with sulfhydryl compounds to form stable mercaptide linkages. Disruption of the sulfhydryl integrity of the 8S cytosol receptor results in loss of estrogen binding capability of this protein (Jensen *et al.*, 1967). The nature of the

reaction between 4ME and the receptor thus appears to involve specific mercaptide formation. Since 4ME cannot displace estradiol from the 8S protein, even though the latter steroid interacts in a reversible fashion, a mechanism of affinity labeling, requiring binding site-localized concentration of steroid prior to binding (Wofsy *et al.*, 1962), is confirmed for the formation of 4ME–8S complex.

The minimal binding capacity of the 8S receptor for 4ME has been calculated, from the solvent extraction of whole cytosol, as 4 pmoles/pair of uterine horns. Assuming a true affinity-labeling mechanism, whereby the steroidal portion of the molecule directs its interaction with the receptor, the binding capacity for estradiol should also be 4 pmoles/uterus. This is somewhat higher than the value of 2.1 reported for immature rats (Jensen *et al.*, 1968), and agrees more closely with a total binding capacity of 3.13 pmoles determined by Notides (1970) in castrate adult rats. Sucrose gradient fractionation of 4ME-treated cytosol (Figure 1) substantiates the level of binding when the amount of 4ME added does not greatly exceed the capacity of the 8S protein.

The molecular basis for specific transfer of estradiol from the cytosol into the nuclear fraction has not been elucidated. Formation of the nuclear complex results in a proportional decrease in binding capacity of the 8S cytosol receptor (Jensen *et al.*, 1968; Gorski *et al.*, 1968), but is not accompanied by a fall in the total intracellular concentration of binding sites (DeHertogh *et al.*, 1971). These findings suggest either that the 8S–steroid complex directly enters the nucleus in a process which involves degradation to a 5S–steroid complex without dissociation of steroid, or that estradiol dissociates from the 8S protein (in a manner such that the 8S receptor does not retain its affinity for estrogen) and is complexed to a different protein species (5S) in the nucleus. If the latter were the true mechanism, it would be expected that an irreversible association with 8S receptor, such as that observed with 4ME, could not lead to nuclear binding. Transfer of the entire complex to the nucleus should be possible, but the data presented show that this does not occur with 4ME. It is possible that the mercurial interaction with the cytosol receptor physically prevents either the degradation of the 8S protein to a 4S or 5S component, or the uptake of the complex by the nuclei. It should be noted that subsaturation levels of cytosol 4ME do not prevent specific transfer of estradiol into particulate fraction peaks in the 4S and 5S regions. The 4S component appears to be a subunit of the transferred 8S protein (Jensen *et al.*, 1969) and has been observed by other workers (Musliner *et al.*, 1970).

Since estradiol rapidly accumulates in the nuclear fraction both *in vitro* and *in vivo*, it has not been possible to assess the direct physiological significance of the cytosol binding system. A working hypothesis of "induced derepression" has been proposed (Jensen *et al.*, 1969) to correlate estrogenic activity with specific receptor binding systems. By analogy, the estrogen-like activity of 4ME (Muldoon and Warren, 1969) would appear to result from its specific cytosol interaction, since it does not subsequently enter the nucleus. The activity of glucose-6-P dehydrogenase and the accumulation of tissue glycogen, measured at various time intervals after administration of 4ME, increased to levels which were not so high as those induced by the same amount of estradiol, and these levels were maintained for a much-prolonged period of time. It is suggested that this anomalous activity pattern reflects irreversible cytosol binding with concomitant elicitation of biological activity at a reduced level. It cannot be ascertained at this time whether this activity is a general result of estrogen treatment or whether it occurs with 4ME by a compensatory mechanism in the absence of nuclear binding; nevertheless, since *in vivo* experiments also show no specific nuclear uptake, the cytosol receptor interaction may be related to the activity of this synthetic steroidal estrogen by serving a function other than transfer of steroid into the nucleus. An alternate mechanism of genetic activation might be proposed, involving an effect of the steroid–protein complex on some cytosol component which can then effect intranuclear changes. Investigation of this possibility is presently being undertaken in this laboratory.

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