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MECHANISM OF ESTROGEN ACTION STUDIES IN THE HUMAN

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SUMMARY

The interaction of both estradiol and estrone with human endometrial tissue obtained during the proliferative phase of the menstrual cycle has been examined by *in vivo* and *in vitro* techniques. Infusions of the tritium-labeled steroids resulted in tissue:plasma concentration gradients for both steroids, although the tissue levels of estrone were generally lower than those of estradiol. Little if any conversion of estrone to estradiol within the tissue was observed. Sucrose density gradient ultracentrifugal analysis of cytoplasmic and nuclear extracts following incubations with [^{3}H]-estradiol revealed the presence of 8S and 4–5S receptors, respectively. While cytoplasmic binding of estrone was observed, transfer to the nucleus, although possible, was not proven. The human estrogen receptor system was found to be more labile than that of other species. Finally, evidence has been obtained which suggests that intranuclear conversion of estradiol to estrone may constitute a "release" mechanism for estradiol turnover in target cells.

INTRODUCTION

EXTENSIVE studies carried out in these laboratories have shown that estrone derived from circulating androstenedione makes an important contribution to total estrogen production in the human[1, 2]. Indeed, it has been found that estrone produced via this extraglandular process can account for total oestrogen production in many anovulatory states, including the postmenopausal woman. Of paramount importance in this process is the fact that the oestrogen so derived is estrone rather than estradiol. It is therefore of importance to elucidate further the mechanism(s) by which estrone exerts oestrogenic activity. It is commonly held that the biological effects of estrone are due to its conversion to estradiol *in vivo*. However, it is difficult to explain our findings in abnormal postmenopausal women on the basis of such a mechanism. For example, when the total production of estrone into plasma exceeds a value of approximately 35 μg/day, oestrogenic effects, as evidenced by uterine bleeding, are always observed. Since it is known that only about 5–10% of circulating estrone is converted to estradiol in plasma, these results would suggest that as little as 3 to 4 μg/day of estradiol, derived from estrone, is effective in causing endometrial hyperplasia and subsequent uterine bleeding. Alternatively, a much higher extent of conversion may occur within the endometrial tissue, and therefore a tissue-localized effect of estradiol derived from estrone might explain these results. Finally, it is possible that estrone possesses inherent activity which may be expressed under certain conditions in the human.

The purpose of the present study was to investigate further the interaction of both estrone and estradiol with human endometrial tissue. The experimental approaches included long-term constant infusions of tritium-labeled estrone or estradiol until a state of equilibrium was achieved, at which time simultaneously obtained endometrial and blood samples were analyzed. These experiments

demonstrated that estrone is concentrated by human endometrial tissue *in vivo*, as was shown previously by Gurpide *et al.*, using *in vitro* superfusion techniques [3]. Furthermore, a very limited conversion of estrone to estradiol within the endometrium was observed. Other experiments have been carried out in which the interaction of both estradiol and estrone with receptors in human endometrium has been studied by sucrose density gradient ultracentrifugation. It has been demonstrated that, as in other species, the cytoplasm of human endometrial cells contains an 8S oestrogen receptor which apparently is transferred to the nucleus, from which a 4–5S form can be extracted by high salt solutions. However, the exact interrelationship of these binding entities is not yet clear because of the relative instability of the human system. Finally, data has been obtained which suggests that intranuclear conversion of estradiol to estrone may constitute a "release" mechanism for estradiol turnover in target cells.

EXPERIMENTAL

Tritium-labeled estrone and estradiol (S.A. 45–100 Ci/mmol) obtained from the New England Nuclear Corporation were purified by celite partition chromatography prior to use as previously described[1]. Routine purity checks were made bi-weekly on aliquots, using thin layer chromatography and the solvent system 10% ether–methylene chloride. Tracers for infusions were prepared in sterile 5% alcohol–saline, and infusions were carried out adjacent to the operating room as previously described[1]. Several blood samples were obtained prior to the time of surgery, at which time a final blood and tissue sample, obtained by curettage, was taken simultaneously. Samples were immediately placed on ice, and the tissue was freed from blood clots and stored frozen until ready for analysis. Tissues were homogenized in 10 ml of methanol to which carbon-14 indicators and nonradioactive carrier steroids had been added, using a Polytron P-10 homogenizer. Following 2 additional extractions with methanol, the methanolic extracts were combined and evaporated to dryness and the residue partitioned between 70% aqueous methanol and *n*-hexane. Methanol was removed from the aqueous phase, and the steroids were extracted 3 times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, evaporated to dryness, and the extract subjected to gradient elution chromatography on celite as previously described[4].

Plasma samples were extracted with methylene chloride and the dried extracts were handled in a manner similar to that of the tissue extracts. The fractions containing radioactive metabolites were identified either by counting suitable aliquots or by chemical determination of the added non-radioactive carriers. Estrone and estradiol were further purified by thin layer chromatography in 2 systems, followed by acetylation and rechromatography in a third system. Tissue and plasma concentrations of radioactivity were then calculated from the final tritium:carbon-14 ratios and the amount of carbon-14 indicators added to the original tissue or plasma samples.

Oestrogen binding studies were carried out with endometrium obtained by curettage from women who were scheduled for vaginal hysterectomy immediately prior to surgery. The tissue was placed in ice-cold Krebs–Ringer–Henseleit (KRH) buffer, transported to the cold room, and quickly dissected. Incubations were carried out in the presence of 5×10^{-9} M [^{3}H]-estradiol or [^{3}H]-estrone in the same buffer. Following incubation, tissues were homogenized either in a

Kontes glass or Polytron P-10 homogenizer in 0·01 M tris buffer at pH 7·4 containing 0·0015 M EDTA (tissue : buffer ratio – 1 : 1 or 2). Nuclei were separated by centrifugation at 700 *g*, and the cytosol fraction was prepared from the supernatant by centrifugation at 105,000 *g* for 1 h. The nuclear pellet was extracted by homogenization in 0·01 M tris buffer at pH 8·5 containing 0·4 M KCl and 0·0015 M EDTA, and the suspension was allowed to stand for 1 h at 2°C., followed by centrifugation at 10,000 *g* for 30 min. Samples of cytosol or nuclear extract were layered on 5–20% gradients of sucrose prepared in their respective buffers, and centrigugation was carried out at 157,000 *g* for 17 h. Following centrifugation, the tubes were pierced and fractions collected which were counted directly in Triton x-100 or in standard toluene scintillation mixtures from which the radioactive estrogens were recovered by extraction with sodium hydroxide and their identity determined by thin layer chromatography.

RESULTS

Table 1 summarizes some results obtained following infusions of [^{3}H]-estrone or [^{3}H]-estradiol in human female subjects, where it can be seen that both estrone and estradiol are concentrated by endometrial tissue. Tissue : plasma gradients for estradiol ranged from 2–13 fold when infusions were carried out for at least 4 h. Estrone infusions resulted in tissue : plasma gradients ranging from 2–5 fold. Although estrone appears to be concentrated by human endometrium, it should be noted that the absolute levels of estrone following [^{3}H]-estrone infusion were generally lower than those obtained for estradiol during [^{3}H]-estradiol infusions. Thus, these *in vivo* results are similar to the many *in vitro* observations which indicate that estrone is bound to uterine receptors less avidly than is estradiol.

Table 1. Tissue and plasma concentrations of radioactive oestrogens following infusions of [^{3}H]-estradiol or [^{3}H]-estrone

			d.p.m. × 10^{-3}/100 ml or g			
			Plasma		Endometrium	
Subject	Age	Condition	Estrone	Estradiol	Estrone	Estradiol
E_2						
S.S.	22	normal	41·3	437	197 (4·8)	923 (2·1)
B.D.	26	carcinoma-in-situ	8·2	145	110 (24)	1051 (7·2)
M.W.	77	carcinoma of cervix	3·2	19·7	57 (13·5)	267 (13·6)
E_1						
K.W.	26	normal	43	6·5	115 (2·7)	54 (8·3)
T.J.	26	atypical hyperplasia	150	4·6	368 (2·5)	32 (7)
O.O.	49	carcinoma of cervix	86	3·5	415 (4·8)	192 (54)

E_2 = [^{3}H]-estradiol infused; E_2 = [^{3}H]-estrone infused. Numbers in parentheses indicate tissue : plasma ratios.

The results of 2 experiments in which human proliferative phase endometrial tissue was incubated with [^{3}H]-estradiol at 2°C for 2 h in the presence of 5×10^{-9} M ^{3}H-estradiol are shown in Fig. 1, where it can be seen that both cytoplasmic and nuclear binding of estradiol were demonstrated by density gradient ultracentrifugation. In contrast to earlier reports on human oestrogen receptors [5, 6], the cytoplasmic binding component observed under these conditions has an

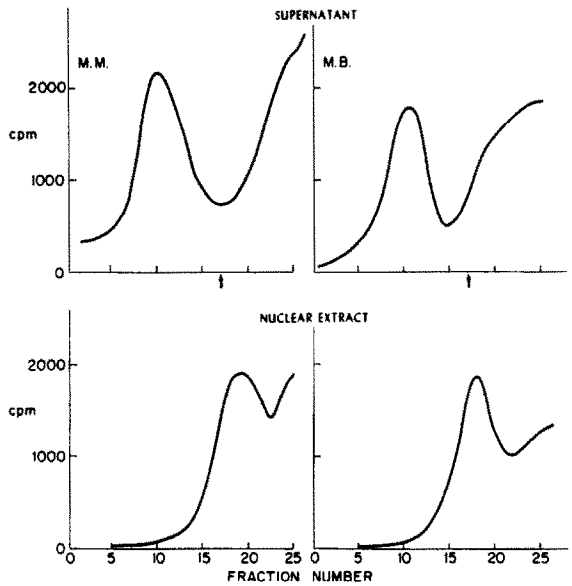

Fig. 1. Sucrose density gradient ultracentrifugal patterns of cytosol and nuclear extracts of human endometrium (proliferative) following incubation in the presence of 5×10^{-9} M [^{3}H]-estradiol for 2 h at 0°C. The tissue samples were homogenized in Tris buffer (M.B. – 0·5 g in 1 ml; M.M. – 0·36 g in 0·5 ml), and 0·3 ml aliquots of high speed supernatant and nuclear extract (total volume equal to volume of homogenate) were analyzed. The arrows indicate the position of bovine serum albumin.

approximate sedimentation coefficient of 8S, similar to that which has been observed in other species[5, 7, 8]. Also, the nuclear binding moiety extractable by high salt solutions has a sedimentation coefficient of 4–5S, as has been observed for the rat[9] and other species. The amount of bound radioactivity present in the nuclear extract was approximately equal to that present in the cytoplasm in both experiments. This is perhaps surprising in view of other reports in which very little transfer of estradiol into the nucleus occurred when similar *in vitro* incubations of rat uteri were carried out at 0°C. Figure 2 illustrates the results obtained when a similar experiment was carried out at 25°C. Under these conditions, no 8S but a 4–5S binding component was detected in the cytosol even after only 15 min of incubation. Nevertheless, transfer of [^{3}H]-estradiol in a bound form to the nucleus was demonstrable despite the apparent absence of the 8S cytosol receptor, as shown in the lower portion of Fig. 2. When human endometrial tissue was incubated with [^{3}H]-estradiol at 37°C for periods of time greater than 30–45 min, no binding could be demonstrated in the cytosol although limited transfer to the nuclei was evident. Brief incubations at 37°C (5–10 min) resulted in minimal 8S and 4–5S binding in cytosol fractions and of 4–5S binding in the nuclear extracts.

These data suggest that the general characteristics of the human receptor system are similar to those of other species, but the cytoplasmic receptor of the human appears to be relatively unstable. To rule out the possibility that this apparent temperature sensitivity was not due to artifacts of homogenization, experiments with broken cell preparations have also been carried out. Figure 3 illustrates the results obtained when a whole homogenate of human endometrium

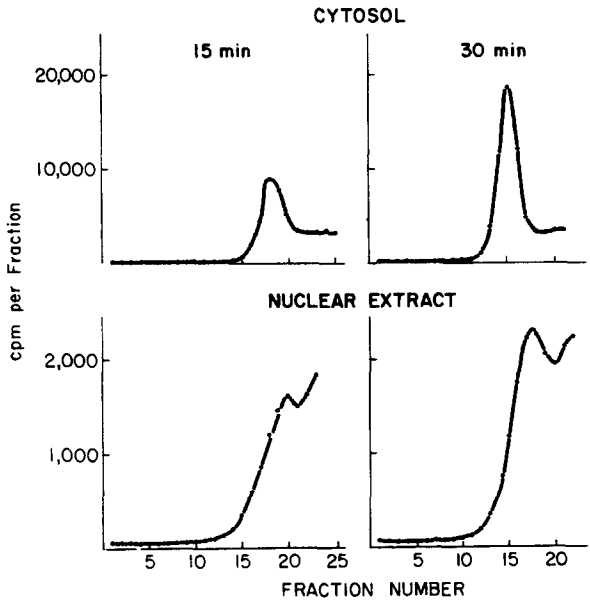

Fig. 2. Sucrose density gradient ultracentrifugal patterns of cytosol and nuclear extract following incubation of 2 g of human endometrium (proliferative) in the presence of 5×10^{-9} M [^{3}H]-estradiol at 25°C.

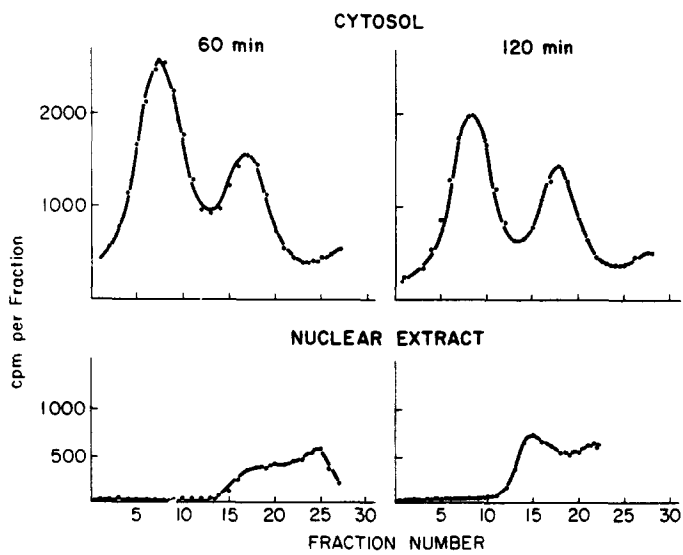

Fig. 3. Binding of [^{3}H]-estradiol to cytosplasmic and nuclear macromolecules following incubation at 0°C. of a whole tissue homogenate of human endometrium (proliferative) prepared in KRH buffer containing 5×10^{-9} M [^{3}H]-estradiol.

was incubated with 5×10^{-9} M [^{3}H]-estradiol at 0°C. It can be seen that both 8S and 4–5S cytoplasmic binding components were readily demonstrated at both 60 and 120 min of incubation. However, the relative amount of the lighter component increased with time, suggesting that the 8S receptor is converted to the 4–5S even at 0°C. Transfer of bound E2-[^{3}H] into the nuclear fraction occurred in the

homogenate at 0°C, although not as efficiently as in the whole tissue incubations. However, when similar whole homogenate incubations or recombinations of cytosol previously incubated with [^{3}H]-estradiol and nuclei were carried out at room temperature or at 37°C, very little binding in either the cytoplasm or nuclear fractions could be demonstrated. Again, these data suggest that the human oestrogen receptor system is peculiarly temperature sensitive when compared with that of other species.

The next experiments were undertaken to compare binding of estradiol and estrone when incubated with human endometrial tissue. Figure 4 illustrates the results obtained when equal portions of tissue obtained from the same patient were simultaneously incubated for 2 h at 0°C in the presence of 5×10^{-9} M concentrations of each tritium-labeled steroid. [^{3}H]-estradiol binding to cytoplasmic 8S and 4–5S components was again observed, and a nuclear binding peak was obtained whose sedimentation coefficient was in the range of 4–5S. On the other hand, estrone binding could only be demonstrated in the smaller cytosol binding component (4–5S), and no clear-cut binding peak was evident in the nuclear extract. More recent experiments with estrone have indicated that an 8S peak is occasionally observed accompanying the major 4–5S binding component in the cytosol, but as yet nuclear binding of estrone has not been clearly demonstrated. However, as is evident from Fig. 4, it has been difficult to rule out the possibility of a small extent of binding of estrone in the nuclear extract due to the presence of large amounts of free radioactivity in such gradients even when several preliminary washes of the nuclear pellet with Tris buffer were carried out. The nature of the oestrogen in both the cytoplasmic and nuclear peaks was investigated by thin layer chromatography in several experiments, and no evidence was found for interconversion of estrone or estradiol under these conditions.

In view of the apparent inability of estrone to be transferred in a bound form to the nucleus, a possible role of estrone in oestrogen action was considered from another point of view. Previous studies by other workers have demonstrated

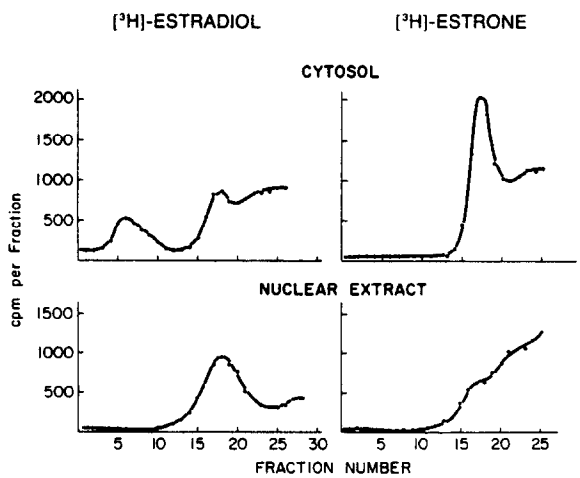

Fig. 4. Sucrose density gradient patterns of cytoplasm and nuclear extracts following incubation of human endometrium (proliferative) samples in the presence of 5×10^{-9} M [^{3}H]-estradiol or [^{3}H]-estrone for 2 h at 0°C.

the interconversion of estrone and estradiol in human uterine tissue with the conversion of estradiol to estrone being predominant under a variety of experimental conditions[10, 11]. Additionally, other workers have demonstrated that estrone is more weakly bound to uterine receptors in a variety of species, and the present data suggests that a similar relationship obtains in human endometrium. Therefore, it was attractive to consider the possibility that the formation of estrone from estradiol in human endometrium may provide a mechanism by which estradiol is released following its interaction with nucleus. In order to test this hypothesis, the subcellular distribution of 17β-estradiol dehydrogenase in human endometrium was determined. The results shown in Fig. 5 indicate that the enzyme is present in all of the subcellular fractions investigated. The highest activity was found in the microsomal and mitochondrial fractions, but significant and approximately equal amounts of activity were present in both the supernatant and nuclear fractions. Furthermore, most of the nuclear activity was

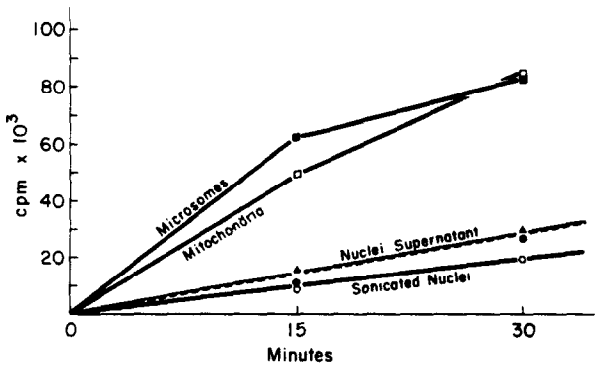

Fig. 5. Subcellular distribution of estradiol 17β-dehydrogenase in human endometrium. 2 g of tissue (proliferative) were homogenized in 4 ml of KRH buffer, and subcellular particulate fractions and high speed supernatant were prepared by differential centrifugation. The 800 *g* nuclear pellet was resuspended and purified nuclei were obtained by centrifugation through a solution of 2·2 M sucrose. One half of the nuclear pellet was subjected to ultrasonication in KRH buffer, followed by centrifugation at 800 *g* for 30 min at 0°C. Aliquots of each fraction equivalent to 1/8 of total tissue were incubated at 37°C with 20 mμ mol of [^{14}C]-estradiol and 20 μmol of TPN in a volume of 1·0 ml. Data presented are c.p.m. isolated in estrone, corrected for procedural losses.

released by sonication. Next, the experiment shown in Fig. 6 was carried out in which human endometrial tissue was incubated at 0°C for 2 h in the presence of 5×10^{-9} M [^{3}H]-estradiol, and cytoplasmic and nuclear fractions were prepared as previously described. The nuclear pellet was subjected to further purification by passage through a discontinuous 2·2 M sucrose barrier. The resulting pellet, which contained in excess of 95% whole nuclei with relatively minor amounts of cytoplasmic tags, was washed once with cold KRH buffer and then resuspended in the same buffer containing 0·2 mM TPN. The suspension was incubated with shaking at 37°C, aliquots were removed at various time intervals, and the nuclei and supernatant buffer separated by centrifugation at 800 *g* for 15 min at 0°C. Aliquots of the nuclei and supernatant were extracted with chloroform methanol (2 : 1), and, following the usual work-up, the extracts were analyzed by thin layer chromatography. Initially, the bulk of the radioactivity within the nuclei consisted

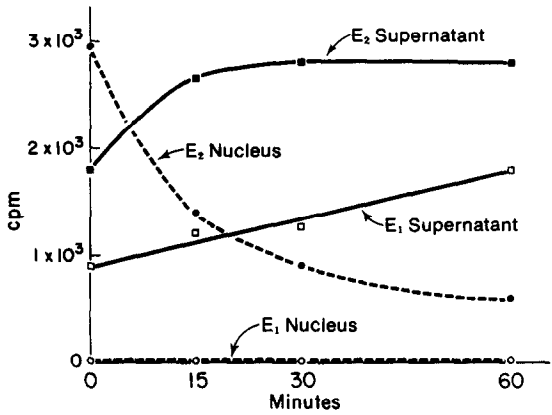

Fig. 6. Release of bound radioactivity from isolated human endometrial nuclei, following exposure of tissue to 5×10^{-9} M [^{3}H]-estradiol. See text for details.

of estradiol with essentially no estrone detectable. However, even at this initial time, estrone was present in the supernatant buffer. During the first 15 min of incubation, the amount of radioactive estradiol in the nuclei decreased rapidly, resulting in the appearance of both estradiol and estrone in the supernatant. After this time, the rate of decrease of intranuclear estradiol was slower, whereas the amount found in the supernatant remained essentially constant. The fall in intranuclear estradiol was accompanied by a steady rise in the amount of estrone in the supernatant. In other similar experiments small amounts of estrone have occasionally been found in the nuclei, but the relative increase of estrone in the supernatant at the expense of intranuclear estradiol has been a consistent finding. On the other hand, when similar experiments were carried out with uteri obtained from immature rats exposed to [^{3}H]-estradiol either *in vivo* or *in vitro*, only a very slow release of radioactivity has been observed with little estrone formation.

DISCUSSION

The results of the *in vivo* studies shown in Table 1 indicate that both estradiol and estrone are concentrated by human endometrium. It is possible that the tissue concentration of estrone observed when [^{3}H]-estrone was infused arose from the conversion within the tissue of estradiol, derived from infused [^{3}H]-estrone in the peripheral circulation, back to estrone in the tissue. However, this is unlikely if one considers the results obtained with [^{3}H]-estradiol infusions, where in every instance the tissue concentration of estradiol was much greater than that of estrone. That the tissue:plasma concentration gradients of estrone resulting from [^{3}H]-estradiol infusion (mean, 13·1) were considerably greater than when [^{3}H]-estrone was infused (mean, 3·3) demonstrates extensive conversion of estradiol to estrone within the tissue. On the other hand, with one exception (subject 0·0.), the tissue concentration of estradiol found during [^{3}H]-estrone infusion could be explained by uptake of [^{3}H]-estradiol from the circulation (cf. [^{3}H]-estradiol infusions). Therefore, it is unlikely that the *in vivo* conversion of estrone to estradiol within the target tissue can account for the estrogenic stimulus that has been observed under conditions in which estrone is the principal estrogenic hormone in the circulation. However, further experiments utilizing

tracers of both oestrogens simultaneously are needed to determine accurately the dynamics of estrone-estradiol interconversion in blood and uterine tissues.

The results of the experiments in which binding of estradiol and estrone by human endometrial tissue was studied by density gradient ultracentrifugation are of interest in several respects. First, the present data indicates that the 8S cytoplasmic receptor of human endometrium is less stable than uterine receptors described in other species, which may account for the failure of others to observe this binding component[5, 6]. While this may be due to inherent species differences in the binding macromolecules, a more likely explanation is rapid proteolytic degradation of the human receptors as a result of tissue damage incurred when endometrium is obtained by curettage. On the other hand, the physiological state of the individual from which the tissue is obtained is also of importance since rarely has it been possible to demonstrate any binding of estradiol in endometrium obtained from patients during the secretory phase of the menstrual cycle. Using other techniques, Evans and Hahnel[12] have also shown a similar marked difference in uptake and binding of estradiol between proliferative and secretory human endometrium. Whether this difference is due to the absence of receptors during the secretory phase or the presence of large amounts of bound endogenous estradiol which prevents binding of [^{3}H]-estradiol is not clear. Nuclear binding of estradiol (4–5S) was readily demonstrable even when incubations were carried out at 0°C. The work of others has shown that in the rat, the cytoplasmic-nuclear translocation step is temperature dependent[13, 14]. Whether the human receptor system differs in this regard is difficult to ascertain because of the loss in binding capacity when incubations are done at elevated temperatures. More recent experiments have shown that the nuclear binding protein has a sedimentation constant of 3·6–4·1S when nuclear extracts containing an internal standard of ^{14}C-albumin are analyzed at higher centrifugal forces. This contrasts with results (unpublished) obtained with immature rat uteri in which the major nuclear binding component sediments somewhat faster than albumin (5·0–5·3S), as shown by Jensen *et al.*[14].

To date, our studies of estrone binding in human endometrium are inconclusive. That estrone can form cytoplasmic complexes has been established, but translocation into the nucleus has not been demonstrated. However, it cannot be concluded that estrone is ineffective in the translocation of the cytoplasmic receptor to the nucleus. Our experience with human endometrium suggests that the cytoplasmic-estrone complex may be transferred to the nucleus, but the lower affinity of estrone for the nuclear binding component, especially in high salt solutions, results in dissociation of the complex during centrifugation (Fig. 4).

The results of the experiments shown in Figs. 5 and 6 are of interest in relation to the problem of estradiol turnover in target tissues. The dissociation constant for the interaction of estradiol and uterine receptors has repeatedly been estimated to be about 10^{-10} M. Conversion of estradiol to estrone, whose association constant is considerably less than that of estradiol, would therefore facilitate the release of the hormone from the nucleus. The fact that most of the nuclear 17β-estradiol dehydrogenase activity was released by sonication suggests that the enzyme may be present in nuclear membranes. If the dehydrogenase is localized in the nuclear membrane, a mechanism can be envisioned in which free estradiol in equilibrium with the nuclear receptor would tend to be removed from

the nucleus by conversion to estrone. It should be pointed out that such a hypothesis is in accord with the findings of Gurpide and Welch, who showed that estrone and estradiol are equally well taken up by human endometrial tissues during superfusion, but that estradiol is completely converted to estrone, which then leaves the cell[3].

In summary, the essential features of a cytoplasmic 8S estradiol receptor which is transferred to the nucleus, from which a slower sedimenting form (4–5S) can be recovered, suggest that human endometrium has the same characteristics as oestrogen target tissues of other species. The results of this study further suggest that estrone may have an important role(s) in the mechanism of oestrogen action in the human, other than serving as a precursor of estradiol. The infusion studies demonstrated that estrone itself is concentrated by human endometrial tissue, albeit at lower levels when compared with estradiol. Therefore, human endometrium must be considered a target organ for both estrone and estradiol if the criterion of the establishment of a significant tissue:plasma concentration gradient is used. While the binding studies indicated difference in behavior between estradiol and estrone, these may be explained by the lower affinity of estrone for oestrogen receptors. Therefore, the possibility remains that estrone may mimic the action of estradiol, particularly if present at high levels in the relative absence of estradiol.

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DISCUSSION

Grant: Dr. Siiteri, did you try washing off cytoplasmic contaminants from your nuclei with Triton X-100 or some detergent. It is terribly difficult, as you probably know, to clean up nuclei going through these sucrose density gradients, even through very dense ones.

Siiteri: Yes, we are doing this at present. After conferring with Dr. Wilson, who has been facing similar problems, I'm not very optimistic about ever getting nuclei to the state of purity where one can exclude a small contaminant. The only

thing I can say here is that the levels of activity are present in the microsomal fraction as compared with the nuclei, are not terribly different, so that we would have to have a very large contamination to account for the activity we see in the nuclei. If there were 50 times more activity outside the nuclei, then the possibility of contamination would be much greater. We don't have that big disparity, so at the moment I tend to think that there is in fact 17β-dehydrogenase activity in the nuclear associated perhaps with the nuclear membranes.

Munck: What's your guess on the reason for the apparently excessive activity of very low amounts of estradiol? Have you tried a bioassay with injected amounts of estradiol that would give blood levels of the order of what you find, to see if in fact for some reason uteri under these conditions are more sensitive than normal?

Siiteri: I'm not familiar with any experiments designed specifically to test this particular point. Perhaps Dr. Jensen knows of some. We are carrying out such comparisons in the rabbit at the present time. Trying to determine whether or not one can dissociate the two at some fixed end point. Perhaps Dr. Tuohimaa would like to comment here.

Touhimaa: I have made some preliminary experiments with 10 pg of estrone and estradiol injected into the lumen of ovarectomized rat uteri. Estradiol increase RNA synthesis by 36% and estrone by 30% at 4 h after administration.

Munck: Did you analyse your estrone at the end of this experiment?

Tuohimaa: No.

O'Malley: What was the state of maturity of those animals?

Tuohimaa: Adults.

Crabbé: Dr. Siiteri, may I take issue with the suggestion you made that estrone would have significant estrogenic activity. We know that in females suffering from congenital adreno-cortical hyperplasia, for instance or from Cushing's disease, one usually sees signs of lack of estrogenic activity. In such circumstances, however, one has an overproduction of androstenedione which would probably give rise to larger than normal amounts of estrone. Why is it then, that at the periphery one doesn't see any convincing signs of estrogenic activity?

Siiteri: I would agree that this is certainly possible, I would also point out the question of when one sees androgen versus estrogenic hormonal effects is a very difficult one to answer, particularly if one considers the variety of target organs. We've studied men with gynecomastia, in whom the development of the abnormality was brought about by a great variety of stimuli. The only common factor which we were able to find in some 15 patients, is that there was a decreased ratio of testosterone to estrogen. There appears to be a balance point, at least for as far as the male breast is concerned, which determines whether estrogenic hormones can express themselves or not. So what I'm suggesting is that the conversion of androstenedione to testosterone in females with CAH provides a sufficient amount of testosterone to suppress any estrogenic effect of estrone derived from the same precursor. It's not a very satisfactory answer, but I think this perhaps relates to the problem.

Exley: Regarding biological activities, we are told that diethyl-stilbestrol is captured. Does this go into the nucleus? If so, what enzyme is around to change this and remove it from the nucleus?

Siiteri: I have no experiments with diethyl-stilbestrol; Dr. Jensen has shown, I believe, nuclear uptake of stilbestrol.

Jensen: We have only shown the 4–5S transformation with stilbestrol. With

hexestrol we have shown in the whole animal that it goes into the uterine nucleus without chemical transformation. How it gets out, we don't know.

Siiteri: I hope I haven't indicated that there is an obligatory mechanism for removal of estradiol.

Wira: In the context of trying to relate estradiol and estrone binding to metabolic activity I might mention some results which Dr. Baulieu and I have recently found. Very simply, we have developed an *in vitro* system consisting of intact uterine segments from immature rats which responds to 1 nM estradiol, but not 1 nM estrone, by synthesizing an induced protein (IP). This IP, present initially after 1 h of *in vitro* incubation, appears to be the same as that synthesized *in vivo* following estradiol administration (*C.R. Acad. Sci. Paris* **273** (1971) 218). When actinomycin D (20 μg/ml) or α-amanitin (0·02 μg/ml) are added simultaneously with estradiol *in vitro*, IP is suppressed. These findings led us to look for direct evidence of messenger RNA synthesis. Only recently have we succeeded in identifying a 15 S (and sometime an 8 S) messenger-like RNA(s). These values are at present only approximate estimates. These results have been observed in both *in vivo* and *in vitro* experiments 15–30 min after estradiol treatment (*C.R. Acad. Sci. Paris*, in press). While the appearance of these messenger-like RNAs coincides with the actinomycin D and α-amanitin results, which suggest that the early action of estradiol on induced protein synthesis may be mediated through mRNA synthesis, further studies must be undertaken to determine the exact nature(s) of these RNA(s) and their inter-relationship to IP.

Siiteri: Have you done the experiments with graded dosages, or have you looked for metabolism of estrone? Can you get any response with estrone?

Wira: No, at present we have not looked at higher concentrations. One limitation to our studies is that induced protein, as well as IP m-like RNA, have been observed by looking at $^{3}H/^{14}C$ ratios. Estradiol-exposed tissues are incubated with [^{3}H]-leucine or uridine, while control tissues are incubated with ^{14}C samples. At the end of the incubation, ^{3}H and ^{14}C tissues are pooled and extracted. Following electrophoresis we look at the ratio in the various bands, such differences are essentially qualitative.

Siiteri: I would just like to make one final comment, if I may. At the outset I indicated that the in studies we have performed have been done in individuals who are making only estrone; there essentially is no estradiol present. Certainly I think the possibility exists that in the relative absence of estradiol, estrone can substitute for estradiol and perhaps exert the same effects as estradiol.

Summerville: As you said, Dr. Siiteri, the interconversion of estradiol-17β to estrone favours the formation of the latter but this is then followed by the formation of numerous estrone derivatives. I should like to ask whether compounds of the 2-methoxy series or the 6, 11 or 18-hydroxy estrones have been studied in the present context and whether you think that their formation might be a factor in the apparent anomaly which we are discussing.

Siiteri: We have looked very carefully for other metabolites in our infusion studies, and if they occur, they must be in very much smaller quantities than the estrone-estradiol pair.