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GENERAL DISCUSSION

Adlercreutz: I would like to stress the importance of having specific antibodies in radioimmunoassay. In Fig. 1 you can see two gas chromatograms from two different samples of pregnancy urine. Both gas chromatograms represent the methylated and silylated estradiol fraction, which has been highly purified and does not contain any ketonic estrogens. All the numbers in the figure represent a different estradiol:

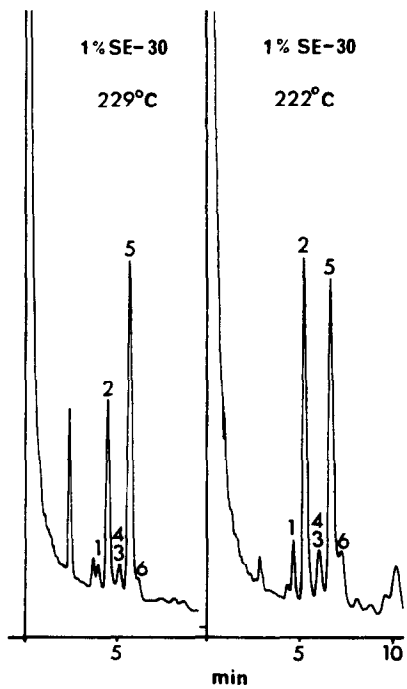

Fig. 1.

No. 1 is an unidentified estradiol with an extra double bond, no. 2 is 11-dehydroestradiol-17α, no. 3 is estradiol-17α and no. 4 (which has almost the same retention time) is another unidentified estradiol with an extra double bond, no. 5 is estradiol-17β and no. 6 is again an unidentified estradiol with an extra double bond. As you can see from these gas chromatograms, estradiol-17β may represent less than half of the total estradiols in pregnancy urine, which means that if this compound has to be determined by radioimmunoassay one may readily obtain a too high value because of interference with these other estradiols, many of them also occurring in plasma.

Then I would like to go to the question of Dr. Grant about other estrogens in plasma than the three "classical" ones. Here you have the values for free and

conjugated estrogens in late pregnancy plasma (mean ± SD of 5 pooled samples):

	Free	Conjugated
Estriol	6·1 ± 2·8	124 ± 17
Estrone	9·9 ± 2·6	80 ± 6
2-methoxyestrone	1·5 ± 1·4	1·3 ± 1·3
Estradiol-17β	15 ± 6	4·5 ± 1·2
Estradiol-17α + unknown estradiols	–	0·5 ± 0·5
11-dehydroestradiol-17α	–	3·5 ± 2·8
16α-hydroxyestrone	2·1 ± 2·2	40 ± 17
16β-hydroxyestrone	–	4·3 ± 3·2
16-oxo-estradiol	2·3 ± 1·9	20 ± 7
16-epiestriol	1·1 ± 1·0	5·4 ± 3·9
17-epiestriol	–	–
15α-hydroxyestrone	+	2·1 ± 2·0

The samples have repeatedly been analysed by combined gas chromatography-mass spectrometry and they were corrected for methodological losses. These results further stress my point that if one is going to make specific radioimmunoassay determinations one has to eliminate the possibility that also other estrogens are not included in the assay. This is a more complete answer to Dr. Grant's question.

Rodbard: I was very interested to see your studies on polyacrylamide gel electrophoresis of estradiol-binding proteins. Until recently, the sucrose gradient centrifugation has been the primary tool employed for the study of this type of steroid-protein interaction. However, gel electrophoresis promises to provide higher resolving power. The major difficulty is that electrophoresis is restricted to use of low ionic strength (e.g. 0·01–0·05), whereas sedimentation can be performed at "physiological" ionic strength (e.g. 0·15).

My question is: does the binding protein, as isolated by polyacrylamide gel electrophoresis, have the same mobility in the presence and absence of estradiol? Also, I wonder if it would be possible to reproduce the basic experiments done with sucrose-gradient centrifugation, using polyacrylamide gel electrophoresis, to follow the conformational change from 4 to a 5S, or from a 4 to an 8S, protein?

Jensen: The use of polyacrylamide gel electrophoresis in the case of estrogen receptors is subject to some technical difficulties which gave us trouble for a long time. One cannot put the crude cytosol on polyacrylamide gel. The receptor complex just doesn't move into the gel, probably because there is aggregation and association with other proteins in the cytosol, just as the 8S protein comes in the excluded volume on a Sephadex G-200 column where an 8S protein should be included. Apparently the 8S complex exists in some kind of aggregated state except in sucrose where it seems to be smaller. It has been our experience that one has to purify these receptors about 1000-fold before you can put them on acrylamide gel; then it seems to work all right. We have not tried the uncomplexed receptors on acrylamide gel; we usually purify the receptor protein with the radioactive hormone bound to it, both as a marker and to stabilize it. We have partially purified the calcium-stabilized cytosol receptor unit without estradiol, and then shown that you can add the steroid later and obtain the 4S complex. We know that on sucrose density gradients the protein without the hormone sediments at the same rate that it does with the hormone, but we don't know that for

acrylamide gel electrophoresis. Finally, I should mention that the 5S complex you ordinarily get from the nucleus by salt extraction will reaggregate to something bigger (8 or 9S) when you remove the salt. But after you purify this nuclear complex, first by ammonium sulfate precipitation and then G-200 filtration in the presence of salt, it somehow becomes altered so that now it is stable in low salt where it sediments at 5S. Now you can put it on acrylamide gel at low ionic strength, which you couldn't do with the original salt-containing nuclear extract.

Kellie: I'd like to address to Dr. Jensen, the question of the temperature-dependent conversion of the 4S unit in the cytosol to the 5S unit, which I think you describe as a test-tube experiment. I am aware that you have shown this to be so, and that it has also been independently confirmed by Dr. Gorski. But both Dr. Gorski and yourself have done this by means of sucrose gradient centrifugation, a procedure which appears to us to lack precision. We prefer to make measurements not by sucrose gradient centrifugation, but by using a Scatchard plot to measure the molar concentration of cytosol receptors. We took uterine tissue from immature rats, divided into halves, and incubated the uterine tissue with and without estradiol. We expected that under these circumstances, in the presence of estradiol there would be a diminution in the concentration of cytosol receptor, but we have not found this to be so. Whether this means that the cytosol receptor 4S is being converted to 5S and is being replaced, I don't know, but under these *in vitro* conditions, with intact tissue, with the fine structure of the cell retained we haven't found the decrease in cytosol receptor that we expected.

Jensen: Was the temperature 37°C?

Kellie: Yes.

Jensen: I can only comment that both we and Gorski have observed this depletion of the cytosol receptor both *in vivo* and *in vitro*.

Siiteri: I might add that in looking at the human system by the same technique, we find that at 25 or 37°C, we get relatively little receptor in the nucleus. Most of it breaks down to the 4S, which still binds very adequately, but it does not move in, to the nucleus.

Kellie: I think the essential difference between Dr. Jensen's and your type of experiment is that you concentrate on recognizable macromolecules as revealed by sucrose gradient centrifugation, whereas we do not consider what size the molecule is, but merely its capacity to bind estradiol.

O'Malley: Is there a decrease in cytoplasmic androgen binding proteins during this two-step transfer?

Liao: Since the nuclear 5α-dihydrotestosterone binding protein originates in cytoplasm as we demonstrated earlier, the cytoplasmic androgen binding protein must lose the same amount which enters nuclei. This loss is apparently compensated in a matter of a few hours either by resynthesis or by a supply from a storage site. I might add that it is very difficult to measure the exact amount of the cytosol receptor. The work done in the laboratories of Dr. Baulieu, Dr. Mainwaring and others showed that their 8–10S materials dissappear rapidly after the castration of rats. We found, however, that this was not the case for β-protein within 40 h after castration if a small amount of 5α-dihydrotestosterone was added at the time of homogenation. Since we also know that the androgen can stabilize the binding protein *in vitro*, the loss of the detectable binding protein after castration may be due to the decreased amounts of the receptor being protected by the endogenous

androgen at the time of homogenation and isolation. It is also very interesting to point out that there is a large amount of 5α-dihydrotestosterone-binding protein in the cytoplasmic particulate fractions such as microsomes. It is a very large proportion of total cytoplasmic binding protein (10–30%). It may play a functional role there, but it is also possible that it is a major source of supply for the cytosol receptor protein.

O'Malley: That's interesting. I believe Jungblut feels there's a substantial amount of estrogen-binding protein on the microsomal fraction in uterine tissues. Is that correct?

Jensen: Yes, Dr. Jungblut has found that when he homogenizes in sucrose he gets much less (only about 20%) of the binding capacity in the cytosol than he does when he homogenises in a hypotonic medium. We do not agree with this observation. We find in homogenates, whether it be in 0·25, 0·32 or 2·2 M sucrose, that there is as much, and in some cases more, cytosol binding capacity. I don't know the reason for this discrepancy.

O'Malley: You mean you don't find any binding protein on microsomes.

Jensen: We don't find less binding protein in the high-speed cytosol of a sucrose homogenate than we do in the cytosol of a Tris hypotonic homogenate, nor do we observe much in the microsomal fraction. You always find a little bit, because no separation is really complete.

O'Malley: Gary Rosenfeld, who is presently a fellow in our lab also did some of this with progesterone binding in oviduct. We actually find a significant amount on the microsomes. It doesn't seem related to the preparation and most of it is solubilized but there seems to be a substantial (~25%) receptor still associated with microsomes.

Jensen: The hormone-receptor complex, at least with estrogens, will bind non-specifically to all sorts of things, such as ground glass, as Gorski showed.

O'Malley: But under these 0° conditions, though, you cannot bind the receptor onto non-target microsomes. It seems to be not a product of homogenization.

Liao: The microsome DHT-binding protein we extracted from microsomes will end up in the nuclei also. It seems to be similar at least to the β-protein of cytosol.

Grant: I would like to ask you if you're quite sure that this DHT-binding protein in the prostate isn't in any way related to the reductase. There's something rather odd about the way in which DHT binds to some of these proteins in the prostate in relation to the zinc concentration. The binding to the reductase and to certain other proteins is dramatically influenced by exceedingly low concentrations of zinc: we've found the order of 10^{-8}, 10^{-9}M zinc having an effect. Now if you study these things out of the cell and in different zinc concentrations, you might influence dramatically the results you get. Is it not possible you have DHT bound to the enzyme?

Liao: As far as reductase is concerned, I think there are a number of indications that the binding protein is not reductase itself. For example, there is no correlation between the amount of binding protein inside or outside the nucleus and the enzyme activity. In addition, if the binding protein is the reductase which binds the product tightly, one might expect some inhibition by a large amount of DHT, which we couldn't see, even when we put in a really large amount, a saturated amount, of DHT. We, of course, would like to see whether the binding protein is a known enzyme or an enzyme regulator. As to the second question you have on zinc, we actually found that zinc will precipitate the 5α-dihydrotestosterone

binding protein. However, the retention of the binding protein by nuclei or microsomes is clearly not due to the simple precipitation of the protein by the metal.

Morfin: Dr Liao, our recent work led us to consider that the microsomal 5α-reducing enzyme is one of the binding possibilities for either testosterone or 5α dihydrotestosterone. We have performed experiments (*Endocrinology* **89**, (1971) 465) where dogs were either castrated or treated with estrogens. In both cases we observed variations of the prostatic fine structure and C_{19}-steroid metabolism. There was after treatment a dramatic disappearance of rough endoplasmic reticulum and shift from a 17β-hydroxy to a 17-keto pathway in testosterone metabolism. We tentatively explained these results by considering a block of the 5α-reduction by estrogens, more actively bound than androgens, which induced cellular alterations similar to those obtained after castration.

Munck: I have a question for either Dr. Liao or O'Malley. If you give the ribosomal-associated receptor a chance to bind either to ribosomes or to nuclei, which does it choose? Have you tried this?

O'Malley: No.

Siiteri: There's been a lot of discussion about cytoplasmic receptors and formation of dihydrotestosterone in the cytoplasm. I'd like to mention again Dr. J. Wilson's view concerning dihydrotestosterone receptors and their localization in the nucleus, the point being that the cytoplasm is a very active site of metabolism of not only testosterone, but also of dihydrotestosterone, so that there is a large spectrum of metabolites formed in the cytoplasm at all times. This differs markedly from the situation in the nucleus, in which the 5α-reductase is the only major enzyme. Therefore, in Dr. Wilson's view at least, the nucleus is the important site of formation of dihydrotestosterone and also of its binding. Perhaps I could ask Dr. Grant a question concerning his differences in uptake and metabolism between hyperplastic and normal prostatic tissue. I believe you indicated that these preparations are compared on a wet tissue weight basis. Is it possible that the morphological characteristics of these tissues are sufficiently different to account for your results? Could the fibrous tissue in the hyperplastic material prevent maximal uptake and also be responsible for a relative lack of metabolizing enzyme?

Grant: I think you're absolutely correct: the cell type is of great importance. I didn't mention that we are endeavoring to get separated cell types. We think we can do this but we do not know that this is going to help us. We are influenced by what Dr. Frank says at the Imperial Cancer Research Fund. He thinks that the growth of alveolar cell fragments in organ culture does not take place unless these cell fragments are associated with stromal cells. The problem is that you want to try and get separated cells to see how they are going to behave; you can do this, but you may alter the situation so drastically from what it's like in the solid tissue that you're not going to find any really useful information. May I in turn make another comment? We've had a good deal of emphasis on the formation of dihydrotestosterone. Somebody yesterday mentioned the fact that we have an effect of testosterone on muscle, possibly without the formation of dihydrotestosterone. I would like to point out that Dr. Jose Minguell in Santiago, Chile has shown, that testosterone has got quite a marked effect on protein and RNA synthesis in the rat bone marrow cell. When he came to work with us, we wanted him to put up the rat bone marrow cells in the superfusion system, but to our disappointment we found that the bone marrow cell will not metabolize testos-

terone at all. We get over 90% of the testosterone back unchanged. When Dr. Minguell gave the rats dihydrotestosterone, it had absolutely no effect on either protein synthesis or RNA synthesis in the bone marrow cell. This is another case in which we don't need metabolism to get an androgenic steroid effect.

Exley: May I ask Dr. Jensen if he knows anything about the turnover at the receptor sites and synthesis of the receptor sites in the cytosol? If no estradiol is available, do they regress?

Jensen: Not a lot is known about this. Jack Gorski has one paper where I believe he presents some evidence on the half-life of the cytosol receptor *in vivo*. As far as the stimulation for receptor synthesis goes, the only evidence we have is that after the depletion by giving estradiol, we see a restoration which is cycloheximide-sensitive. In reply to your question about whether any estradiol is there or not, this is somewhat curious; the immature rat has a higher concentration of receptor sites than does the mature rat, even after correcting for endogenous estrogen, but whether this reflects something left over from the natal environment, I don't know. If one ovariectomizes a mature rat, for the first five or six days the receptor content of the cytosol will increase to nearly double the original level per mg of tissue weight. It still doesn't come up to that of the immature rat, however, but it does increase markedly. We think this is due to the removal of endogenous estradiol which is occupying the binding sites and which does not readily exchange, especially in the cold. After this first 5–6 day period, there is a gradual decrease in total receptor content over the next seven weeks, suggesting there is something in the ovarian secretions that seems to be necessary to maintain the receptor level in the adult rat uterus. May I ask Dr. Siiteri a question: in regard to your studies of human uterus, in some of your slides you showed only an 8S sedimentation peak, in other case you seem to have both 8 and 4S. Are these under different conditions, or are these different individuals? Because this is what we see in human breast cancer; we never see 4 without 8, but we often see 8 without 4. We see both in some cancer specimens. I wonder whether you have any correlation between the two different forms.

Siiteri: I don't believe we have enough information at the present time to make any firm conclusion. I have the impression at least that the problem is one of stability of the 8S form. If one is not extremely careful, one can generate the 4S from the 8S in our whole tissue incubations upon homogenization. Clearly, if we do the incubation at room temperature or 37°C, we can lose the 8S completely. At the present time, we can't make a physiological distinction between presence of 8 or 4 or both.

Grant: I'm intrigued by this temperature-sensitivity, and I'd like to hear what Dr. Jensen has to say about the fact that we do have these receptors in man and in women in their bodies at 37°C. Now, are these receptors protected by other proteins, how are they protected by the environment from this temperature *instability*.

Jensen: In answer to this question, all I can say is that nature is wonderful. If we excise rate uterus from its environment at 37 or 38°C and keep it at 38°C for half an hour on a moist filter paper or in a buffer solution, it markedly loses receptor content, so there must be some mechanism of stabilization in the whole animal which is lost when the tissue is taken out of its natural environment.

Kellie: May I extend Dr. Jensen's comments on Dr. Exley's question with regard to the concentration of uterine receptor sites. We have independently confirmed

that whether expressed in terms of mg wet tissue, mg dry tissue DNA some of the highest concentrations that are encountered, are found in the uterus of a newborn rat, and that as the animal grows to maturity, the concentration drops quite substantially. As the rat goes through the 4-day oestrus cycle, the concentration of estrogen receptor site cycles in exactly the same manner, changing by a factor of 10-fold; the highest concentration of tissue estrogen receptors coincides with the highest concentration of blood estradiol. One suspects that in some way the synthesis of the estradiol receptors may be associated with the presence of estrogen.

O'Malley: In relation to your comment, didn't Clark and Gorski find that receptor concentration is low in the newborn rat and increased during maturation at about 10 days.

Kellie: Yes, that is true. It reaches its maximum at 10 days, and then it falls until the rat matures, and then it begins to cycle.

Jensen: In your cycle, do you ever reach the original level of the immature rat?

Kellie: No, the highest levels that you ever find are in the 10-day old rat. They are substantially higher than at any point in the cycle.