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GENERAL DISCUSSION

De Moor. When you talk about pathological pregnancies did you take into account the fact that starvation has a definite influence on the half-life of DHEA sulphate in serum and on the excretion of DHEA in the urine?

Cohen. All the normal patients or pathological cases during pregnancy took the test between 8 and 9 o'clock in the morning. They stayed in bed in the hospital and had lunch at 12 o'clock.

De Moor. But what had they eaten the day before?

Cohen. They were admitted in hospital on the night before the test, so they had the same diet.

De Moor. We examined the half life of DHA sulphate in a patient who had vomited for several days. This made a big difference, this prolonged the half life.

Cohen. Our patients who took the test did not show any signs of vomitting due to pregnancy.

Arai. I would like to ask Dr. Cohen a question. When you infused dehydroepiandrosterone sulphate into the maternal compartment, what percent of the infused steroid would go into the fetal placental units? The reason I ask this question is because you pointed out the half life changes when the fetus is abnormal.

Cohen. We have not studied the percentage of DHA that passes from the mother to the placenta but other authors have given 40% as the percentage.

Arai. I wonder how you can tell the function of the fetal placental units by infusing dehydroepiandrosterone sulphate into the maternal compartments only.

Cohen. 40% of the infused DHAS enters the placenta and is metabolized at the placental level in normal pregnancy. So, in normal cases, DHAS half-life is 3–4 h. If DHAS is not normaly metabolized into estrogens (lack of sulphatase or aromatase for example) the DHAS half-life will be greater.

Macnaughton. I have a question for Dr. Salvatori. I am just a little puzzled, but he seemed to be doing planned deliveries very early in pregnancy if I read his slide correctly. Presumably there was some reason for the planned delivery. I wondered if the reason for the planned delivery could be something wrong with the fetus. This might have accounted for some of the values he got rather than anything else. Could he answer why he was doing planned deliveries so early in pregnancy?

Benassi. The cases of planned premature delivery refer to pregnancies complicated by severe maternal disorders (diabetes-ecclampsia-placenta previa- and so on). When possible, the L/S ratio analysis in amniotic fluid was effected to assess fetal lung maturity, or to stimulate it by means of corticoid administration.

Macnaughton. Yes, but if you had given dexamethazone that might affect the fetal adrenal and cause low oestriol levels and perhaps low levels of some of the other steroids you were measuring.

Benassi. No S.G.A. cases were found.

Macnaughton. There might be, from what you say, something wrong with the fetus which might account for the low levels of the 16-hydroxylated steroid.

Grumbach. The question Dr. Benassi is whether the control group you did planned interruption of pregnancy on was really a control group in that the pregnancy was pathological and this could have affected the synthesis and secretion of the 3β steroids that you mentioned.

Benassi. These steroids are estriol precursors in the fetus and our data show that they have different trends according to whether labour occurred or not. Estriol too, which is not only a product of fetal metabolism, but needs placental aromatisation as well, shows a similar pattern.

Macnaughton. Yes, but I think if you have some disease like that causing a small fetus then you probably have deficient placental function as well.

Grumbach. The question is, and really it is more a comment that Dr. Macnaughton is making is the problem of having a control group where you may have, in a situation of toxemia of pregnancy, or a diabetes that the fetal adrenal function as well as the placental function may possibly be affected particularly in some of these patients.

Benassi. Those pathologies had arisen shortly before or might hardly affect fetal and placental functions like 16-hydroxylation or aromatisation.

Macnaughton. 16-hydroxylation takes place in the pathway of oestriol from the fetal precursor in the fetal liver.

Benassi. In the fetus only.

Macnaughton. Well, I think it is still doubtful.

Tamaoki. May I proceed to the second question. Do you have any direct evidence on the incorporation of the progesterone which was bound to the binding protein, to uterine nuclei, *in vitro*? Judging from the dissociation constant of the binding protein–steroid complex in comparison with the value of the receptor, the progesterone could be transfered from the binding protein to the receptor at the cell membrane of the uterus, if the complex of progesterone with the binding protein is incubated with free uterine cytosol receptor.

Westphal. Let me first say that we have not done these experiments. However, if I were to comment on that point I would say that so far we do not know of a single definite case where a transporting serum protein enters cellular structures. Over the years, there has been a great deal of discussion of the question of whether we might have what we call a directed transport, that means that the carrier protein–steroid complex becomes attached in a specific manner to the target site, and would thus contribute to the specificity of the hormonal effect. So far we do not have a single example for such mechanism. At the same time we know, and I come back to the PBG in your question, that the dissociation rate of the PBG–progesterone complex (and that is also true for other steroid complexes with PBG) is the highest that we have found among the steroid complexes with serum proteins. This would mean that, although the affinity is very high, the dissociation is very readily accomplished so that in fact we can assume that the free unbound hormone enters the cytoplasm. Such a mechanism would be in perfect harmony with the physico-chemical parameters that we know today. Would that answer your question?

Tamaoki. Yes. Thank you Dr Westphal.

Crastes de Paulet. My first question is: you have shown by circular dichroïsm that progesterone induces a conformational change in the protein when binding. Could it be possible that such a modification in conformation introduces a modification in the accessibility of the tryptophan residue to hydroxynitrobenzobromide?

Westphal. My answer would be if I understand you correctly: yes. It is a possibility and all we have done so far leaves open the question of conformational change versus a direct interaction at the binding site. Unfortunately, we have no means of determining this at present without additional chemical studies. But at the present time we would not be able to distinguish between the two possibilities. Did I understand your question correctly?

Crastes de Paulet. Yes, so it could be possible that the tryptophan residue was beside or close to the binding site.

Grumbach. How did you block your tryptophan Dr Westphal?

Westphal. We blocked the tryptophan residue with hydroxynitrobenzylbromide, also called Koshland's reagent, which is rather specific for tryptophan. So we have a good reason to known that the tryptophan is modified by this substitution.

Crastes de Paulet. My second question is on this point. This protein is a glyco-protein. What about the possibility that glucides have something to do with the affinity or the conformation of the molecule?

Westphal. Yes this is a good question which has also bothered us considerably. With a protein that has 70% carbohydrate and only 30% polypeptide, one would assume that much could happen with the carbohydrate portion of the molecule. Our experience so far with glycoproteins that bind steroids with high affinity has shown that whatever was done with the carbohydrate moiety did not affect the binding. However, I would say at the same time that we have never removed all carbohydrate from the binding protein. Most work has been done on sialic acid which has been removed from α_1-acid glycoprotein (AAG), CBG or transcortin, and also from PBG. In all cases the binding affinity was essentially unaffected by the removal of sialic acid. However, what the effect would be if we removed more of the carbohydrate we do not know. Carl Schmidt in Boston has determined the amino acid sequence of α_1-acid glycoprotein and has cleaved the molecule with cyanogen bromide into three peptides. We have prepared these peptides and tested their binding affinity for progesterone, the steroid that binds best to AAG. We have determined the binding to the AAG fragments in 4 M NaCl, a solvent that increases the binding affinity several-fold. We found that one peptide showed binding to progesterone indicating that apparently enough of the conformational structure was intact to provide binding affinity although we have to assume that the conformation of the split product is considerably changed from that of the native AAG. These and other results on AAG have now been published (Kute, T. and Westphal, U. (1976) *Biochim. biophys. Acta* **420** 195–213).

Grumbach. It is interesting that in terms of the glycoproteins, disyalisation doesn't seem to affect binding to, for example, the cell but it does affect its disposal rate in the peripheral circulation. Do you have any information of that?

Westphal. I can only agree with you on the important difference that binding to the hepatocytes is very much affected by the presence or absence of sialic acid. Work in Dr. DeMoor's and other laboratories has shown this extensively. The removal of sialic acid inactivates *in vivo* for all practical purposes because the desialated glycoprotein is immediately taken up by the hepatocytes and metabolized by the liver and excreted, whereas in the native, sialic acid-containing molecule this constituent provides a protecting effect for the protein.

Naftolin. Firstly, I think that your cautious use of the term "transport protein" is very valid. There clearly are evidences that so called "transport proteins" exist within cells and that they are manufactured in cells before entering the blood. I would like to know what other roles these "transport proteins" might be playing. Secondly, I would like someone to discuss the relationships of enzymes and receptors. For example, must an enzyme handle the steroid before it is converted and is then bound to receptor? Does the receptor hold the steroid for the enzyme?

Westphal. Let me begin with a comment on your first point, transport proteins versus other designations of these steroid-binding serum proteins. Your question is very pertinent; we have emphasized for years that transport is probably not the important function for the simple reason that all these steroid hormones at normal physiological levels are completely soluble in water. They do not need any protein to help dissolve them. This is sometimes not recognized in the literature when it is said that there is a dissolving effect of the proteins for the steroid hormones. Progesterone which is the least soluble of them because of its hydrophobic nature is still soluble in water to an amount of about 12 micrograms per millilitre; this is at 4°, at 37°C it would be even higher. Under most conditions, therefore, the organism would not need the protein for transport, on the contrary, it would be much easier for the steroid to get around and into the cells if there were not such a large protein attached to it. This attachment of the protein, however, is for very good reasons. For one, we know that the complex is much less affected by all kinds of reactions. That means the protein protects the steroid hormone from chemical attack, such as oxidation for instance, or from enzymatic attack. In other words it binds the steroid hormone and keeps it away from the enzyme.

This brings me to your second question, the relationship of the binding protein to the enzyme. You have just seen from Dr. Crastes de Paulet's presentation that the binding affinity of the enzyme 17β-dehydrogenase was roughly about $10^6\ M^{-1}$ judging from the K_M value. This is a binding affinity about two orders of magnitude lower than that of most receptor proteins and also of the serum proteins. To be sure we can only consider this in a general way not knowing any other specific physical or chemical influences. In physico-chemical terms it would mean there is a competition between one protein that binds with an affinity which we call 1, namely the enzyme protein, and the affinity of the receptor protein which binds with an affinity equal to 100. Now obviously the receptor protein would have a great advantage if it comes to competition for the steroid hormone. This actually is a question I had meant to ask Dr. Crastes de Paulet. It is, I think, similar to your question: what is the relationship of these two phenomena, let us say, in a steroid target cell where enzymes are also present. Is it the excess of steroid that would then have a chance to be metabolized by the enzyme or is there any other effect that would come into play here and would perhaps direct in some form the fate of the steroid hormone. So may I pass the question to Dr. Crastes de Paulet.

Crastes de Paulet. Enzymes are in a "difficult situation" in competition with receptors, I agree with that, but perhaps also as shown by many authors the enzyme has a function in regulating the "form" of the hormone. As you know there are differences in affinity of estradiol and estrone for the receptor and of some androgens for their receptors, and the enzymes can, by their concentration, "modulate" the action of the hormone on the receptor. So enzymes can play an important "modulating" role in the action of hormones.

Naftolin. If I understand it rightly, you are saying that within the cell an enzyme can modify a steroid and change it into one which has more or less affinity for the binding receptor, or may even bind to a different receptor. If so, there is yet another control point within the cell. Is that correct?

Crastes de Paulet. The enzymatic transformation of steroids, for instance from one of high affinity for the receptor into another having a lower affinity is perhaps the most important. But now a practical question arises: What is the concentration of the enzymes in comparison with that of the receptors, this would be exactly what we need to know. Let us say that the enzyme concentration is 100–1000 times higher than the receptor concentration; that would be an interesting situation since with more enzyme of lower affinity than receptors, the "affinity gap" could be compensated.

Westphal. If the enzyme concentration would be 1000 times as high as the receptor protein concentration then our example would mean that the enzyme has 10 times the binding advantage, compared to the receptor. That would mean a considerable influence of the enzyme and

this actually makes much more sense because it is our experience that the enzymes have this influence on metabolism. If there is an affinity disadvantage for the enzyme it would never get to the steroid, except if the concentration is much higher, then we have obviously an advantage for the enzyme and things can function as they do.

Pasqualini. I think that at the present, to know the transformation of the steroid hormone-receptor complex is an important problem as was to establish the transformation of the hormone itself many years ago. Concerning this transformation of the hormone–receptor complex, in studies on mineralocorticosteroid-receptors in the fetal kidney of guinea pig. When we incubated [^{3}H]-aldosterone and [^{3}H]-tetrahydroaldosterone separately, in the cytosol fraction there was a significant quantity of [^{3}H]-tetrahydroaldosterone binding from the incubated aldosterone and there was very little if any binding of [^{3}H]-tetrahydroaldosterone when this is incubated with [^{3}H]-tetrahydroaldosterone alone. (*J. steroid Biochem.* (1972) **3** 543–556.) This shows the possibility of the aldosterone bound to the receptor being metabolized to the tetrahydro derivative and perhaps it constituted a form of the inactivation of the complex hormone–receptor.

Thorburn. I would like to raise another point. We have recently postulated that progesterone is secreted packaged in granular form by the corpus luteum. So far we have studied the sheep and cow. We proposed that progesterone is packaged in the Golgi apparatus bound to a steroid binding protein and is then exocytosed. In the case of the guinea pig, we would like to think that the progesterone binding globulin was a transport protein which is carrying progesterone out of the cells of the guinea pig placenta. It is possible that progesterone and other steroids are compartmentalized in the cell and are not in ready access to some of the enzymes that have a chance of metabolizing them. In support of this view, we have shown, by electron microscopy, exocytosis of granules from the corpus luteum of the sheep and the cow and you can actually see these granules being exocytozed. If we administer colchicine to sheep *in vivo*, we can block secretion of progesterone and circulating progesterone levels decrease. Progesterone accumulates within the corpus luteum and the material normally seen in the granules accumulates in the Golgi apparatus.

Westphal. May I ask, concerning the sheep and cow, do they have a progesterone binding globulin? Is it CBG that takes over the binding? We have looked for a specific progesterone-binding globulin in several species and have never found one except in the pregnant guinea pig. You know, of course, that the corticosteroid-binding globulin is the one that takes over the function of progesterone binding in most other species including man. I wonder since we have never looked for a PBG in sheep or in cows do you know if progesterone is bound to CBG?

Thorburn. In the sheep and cow there is relatively little CBG but it is the main transport protein for progesterone and cortisol in these species. Leymarie and Gueriguian have isolated from the cow corpus luteum a specific binding protein for progesterone which was not CBG. This work which was done in 1969 has really not been followed up since then. In the cow, the progesterone binding protein of the corpus luteum is presumably not secreted into the general circulation. This is probably true of most species in which the intra-cellular carrier protein is not exocytosed and may be broken down by the enzymes of the cell surface. However, it is possible that the guinea pig and other hystricomorphs are unique in that they secrete the carrier protein (i.e. progesterone binding protein) together with progesterone into the general circulation.

Grumbach. I think the analogy is quite interesting in terms of the enzyme binding protein. The Sertoli cell in the rat has a very small amount of sex steroid binding protein in the periphery. Whereas in the human from the studies which have been done so far at the NIH, the sex steroid binding globulin in serum, and also the so called androgen binding protein in the Sertoli cell, seems to be if not the same, very very closely related. So this may be quite comparable to the situation you are describing in the sheep.

Thorburn. We have some preliminary evidence for a similar mechanism in the adrenal. I shall describe briefly how we handle the corpus luteum. Firstly, we homogenize the corpus luteum, then spin it down at 500 ***g*** and remove the debris. Then the supernatant is centrifuged at 10,000 ***g*** and the pellet is placed on a sucrose gradient. We get a marked enrichment of the progesterone/protein ratio in some fractions (density Ω 25% W/W sucrose) and there is an enrichment of the granules in the same fractions. However, this work is still in preliminary stages. We have some preliminary data on the cow adrenal to suggest a similar distribution for cortisol.

Grumbach. So you think that steroids are just glorified catecholamines.

Thorburn. Yes.

Solomon. I wonder if Dr Westphal would like to comment on the possibility of CBG being inside cells. It is a bit of a loaded question because at one point Dr Giannopoulus was trying to get rid of the CGB. He thought it was in the cell and couldn't wash it out, there was always some residual amount left which looked like CBG.

Westphal. We have not worked on this specific question ourselves, but I have been interested in the work that was done by Dr. Rosenthal in Dr. Sandberg's laboratory in Buffalo (Rosenthal, H. E., Paul, M. A. and Sandberg, A. A.: *J. steroid Biochem.* (1974) **5** 219–225). The authors reported the presence of CBG, or protein antigenically related to CBG, in liver, uterus, and kidney of the guinea pig. We have to be careful, of course, in interpreting the presence of CBG in the liver since we know that CBG is produced in the organ as was shown early in our laboratory for rat liver, and has also been reported by Guidollet and Louisot in France. As to your question, I am sorry that I cannot give you more information than refer to these reports in the literature.

Solomon. The reason I ask this question is to address myself to Dr Thorburn. I am wondering whether those granules you talked about have not CBG enclosed within them. Would it be too difficult to try and eliminate the others.

Thorburn. That is a very interesting point because in the sheep fetus there is a marked increase in the plasma concentration of CBG in the 7–10 days before delivery. It is the only time in the sheep's life that there are significant levels of CBG in the plasma. There is no increase in plasma estradiol levels to account for it. Since at this time the fetal adrenal is maximally stimulated, I wondered whether CBG was being secreted with cortisol by the fetal adrenal. The fetal adrenal grows considerably during this time.

However, I should mention in relation to the other question that we would like to isolate the progesterone binding protein from the cow corpus luteum and to raise antibodies to this protein. We then hope to use the immunoperoxidase method for localizing the protein in the granules. We would hope to show that these binding proteins are in the granules. I believe that Dubois and Corteel in Nouzilly have some preliminary evidence in the pig corpus luteum, using the immunoperoxidase technique with a progesterone antibody, indicating that progesterone is inside the granules.

Grumbach. Thank you very much. I think that it is very fitting that Dr Naftolin who opened up the discussion should make the last comment.

Naftolin. I wonder, we have managed to get next to the cell, we have managed to go into the cell, and we talked about what we did in the nucleus but it is still a mystery

about what happens to this steroid. Do you think that any of what you are telling as has got to do with what happens to the steroid. Do you think that these proteins could be the way that the steroid is packaged up and got out of the cell system without being thrown back into the machinery because we know that does not happen.

Thorburn. Yes, I think this is where we came into the argument. As I see it, the problem is to get progesterone out of the cell without it being metabolized or without it getting caught on a cytosol receptor. I consider the synthesis and secretion of progesterone is a highly organized sequence of events.

In the past we were probably very naive to think that progesterone could simply diffuse out of the cell because it is lipid soluble. The concentration of progesterone within the sheep corpus luteum is something like 25 micrograms per gramme whereas the concentration of progesterone in the ovarian vein plasma is something like one microgramme per mille. With a substance which is as lipid soluble as progesterone you should get diffusion equilibrium and since you don't, it would indicate that a lot of the progesterone is packaged or bound in some way and, furthermore, that the secretion of this steroid hormone is active.

McEwen. I wanted to respond to Dr. Solomon about the existence of CBG inside cells. Dr. deKloet and I have evidence in the rat pituitary for a resident population of CBG-like proteins. Dr. deKloet, now in Utrecht, has evidence that CBG may actually reside inside of or tightly attached to pituitary cells. This CBG seems to function in the pituitary as a competitor with the receptors for circulating corticosterone. A steroid like dexamethasone, which does not bind to CBG, is therefore much more potent in binding to receptors and in suppressing ACTH secretion.

Solomon. It is really very much like what we found. Dexamethasone doesn't bind to CBG and doesn't stay in the cell the way cortisol does in rabbit nuclei at 28 days gestation. It was this inability to get it out which started us worrying about the possibility of CBG and we have never followed this through the way we should have and definite identification of CGB by criteria which are well known, but the experience is much the same as Dr McEwen just had.