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GLUCOCORTICOID-RECEPTOR COMPLEXES AND THE EARLIEST STEPS IN THE ACTION OF GLUCOCORTICOIDS ON THYMUS CELLS

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SUMMARY

Cortisol and other glucocorticoids added at physiological concentrations to rat thymus cells *in vitro* at 37°C begin to inhibit glucose transport after about 15 min. This effect corresponds to effects observed *in vivo* and probably is in good part responsible for the catabolic actions of glucocorticoids on lymphoid tissue. From a variety of experiments we have concluded that cortisol initially stimulates synthesis in the nucleus of a specific form of RNA that, after an intermediate step, by 15 min initiates synthesis of a protein that inhibits glucose transport.

The first step in cortisol action is formation of specific cortisol receptor complexes. At 37°C this process is complete by 7 min, by which time the complexes are localized largely in the nucleus. At 3°C, however, most of the complex appears in the supernatant when cells are broken by osmotic shock and nuclei spun down. This non-nuclear bound complex we refer to as "cytoplasmic". On warming the cells to 37°C, [^{3}H]-cortisol bound in the cytoplasmic complex becomes bound in the nucleus within 1 min, probably by transfer of the cytoplasmic complex *in toto* to a nuclear acceptor site.

Isolated cytoplasmic and nuclear receptors bind glucocorticoids specifically, becoming saturated over the physiological range of glucocorticoid concentrations. The isolated cytoplasmic receptor has a half-life at 3°C of about 2 h. Saturating concentrations of cortisol or other glucocorticoids increase this value to more than 20 h. Both α and β sides of the steroid appear to interact with the binding site, which probably consists of a hydrophobic pocket with polar groups that form hydrogen bonds. The principal driving forces for formation of the hormone-receptor complex are probably hydrophobic interactions, the hydrogen bonds conferring specificity to the interactions.

Cortisol-cytoplasmic receptor complexes are transformed by brief warming at 25°C into complexes with high affinity for nuclei. These latter complexes become rapidly associated with isolated nuclei at 3°C. Two alternative functions are proposed for the hormone in this temperature-sensitive transformation: one is that hormone binding displaces the equilibrium of the receptor towards a form with high affinity for nuclei; the other assumes that the cytoplasmic receptor is in a non-equilibrium state, and that hormone binding accelerates its transformation to the equilibrium high-affinity form.

Protein synthesis does not appear to be necessary to replace receptors that are transferred to the nucleus in the presence of hormone. ATP or some related substance, however, does seem to be required. It is proposed that ATP generates the normal cytoplasmic receptor from a precursor. Furthermore, it is suggested that the precursor may be the form in which the receptor leaves the nuclear site, so that in the presence of hormone there is a continuously operating receptor cycle, dependent for energy on ATP.

INITIAL STEPS IN THE ACTIONS OF GLUCOCORTICOIDS ON THYMUS CELLS

IN PREVIOUS studies [cf. 1–10] we have attempted to map the time-course of the initial events in the actions of cortisol on rat thymus cells *in vivo* and *in vitro*. The broad picture that has emerged from the work *in vitro* is outlined in Fig. 1, which summarizes rather schematically the results of a large number of experiments.

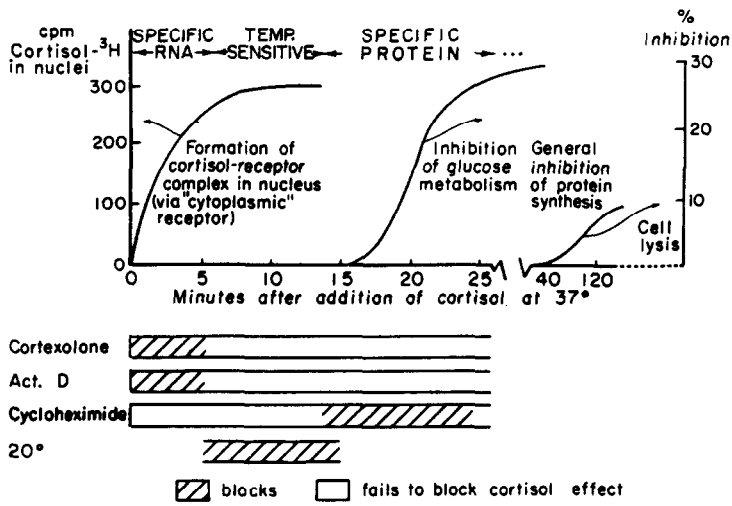

Fig. 1. Time course in rat thymus cell suspensions at 37°C of cortisol-receptor complex formation, cortisol-induced inhibition of glucose transport and inhibition of protein synthesis. Cross-hatched segments of the horizontal bars in the lower part of the figure indicate roughly the periods (on the time scale above) during which emergence of the cortisol effect on glucose metabolism can be blocked by treatment with cortexolone (which displaces cortisol from the glucocorticoid receptors), actinomycin-D, and cycloheximide, and delayed by lowering temperature. Open bars indicate periods during which these treatments have no effect. At the top of the figure is given the sequence of steps by which it is hypothesized that the cortisol-receptor complex leads to synthesis of a specific protein that inhibits glucose transport.

Our reference point for most of these studies has been the inhibition of glucose uptake, an early effect of cortisol *in vivo*, and the most rapid metabolic effect so far demonstrated *in vitro*. It begins to appear *in vitro*, as shown in Fig. 1, between 15 and 20 min after addition of cortisol to a suspension of rat thymus cells at 37°C. It is not present by 10 min. A number of results have shown that the step in glucose metabolism on which the cortisol effect is exerted is probably glucose transport [cf. 8]. The effect on glucose is specific for steroids with glucocorticoid activity such as corticosterone, cortisol and dexamethasone, relative activities of which correspond well with activities *in vivo*. Cortisone is inactive, however, in agreement with evidence that cortisone *in vivo* owes its activity to conversion to cortisol, a conversion that does not take place to any significant degree in thymus cell suspensions. Cortisol activity is displayed over a range of concentrations that coincides approximately with physiological range of concentrations of free cortisol in plasma, roughly 10^{-8} to 3×10^{-6}M. Much evidence [8, 9] supports our hypothesis that the inhibition of glucose uptake is an essential step in the thymolytic actions of glucocorticoids, the first signs of which manifest themselves after about one hour as inhibition of protein synthesis (Fig. 1).

The earliest interaction of cortisol with thymus cells consists in noncovalent, reversible binding. Immediately upon addition to cells at 37°C, cortisol becomes bound both specifically and nonspecifically [2, 4]. We shall not consider further the nonspecific associations, which, although they account for most of the bound steroid, and undoubtedly are responsible for the multitude of nonspecific metabolic

effects that cortisol and other steroids give rise to at high concentrations, are probably of little importance at physiological concentrations[11].

Specific binding was initially characterized by the fact that cortisol bound in this fashion dissociates relatively slowly from the cells, with a time constant of 3 min at 37°C. Nonspecifically bound steroid dissociates much more rapidly [2, 4]. By a number of criteria, specific binding represents binding to glucocorticoid "receptors", the molecular entities through which glucocorticoid effects are initiated. Thus, this form of binding becomes saturated over the physiological range of concentrations, and is roughly proportional to glucocorticoid activity, dexamethasone, cortisol, and corticosterone showing decreasing affinity and cortisone almost none. An exception is cortexolone, which has no glucocorticoid activity of its own but competes for binding with cortisol. In doing so it blocks the activity of cortisol, acting as an antiglucocorticoid[4].

By disrupting cells with 1·5 mM $MgCl_2$, a procedure that breaks the cell membranes but leaves nuclei relatively intact, we find that the receptors to which cortisol is bound after an incubation at 37°C are localized largely in the nucleus. Following incubation at 3°C, however, the cortisol-receptor complex is not associated with the nucleus but can be identified in the supernatant after nuclei are sedimented by centrifugation. For convenience we refer to this complex as "cytoplasmic", although we do not know whether in the intact cell it is in the cytoplasm or the nucleus or both. Upon warming the cells to 37°C the complex immediately becomes bound to the nucleus. A similar two-step transfer to the nucleus has previously been demonstrated with estrogens and other steroid hormones[12]. Radioactive cortisol initially bound at 3°C to the cytoplasmic receptor is by this process transferred to the nucleus even in the presence of excess unlabelled cortisol.

The time course at 37°C of specific binding of cortisol to thymus cells and transfer to the nucleus is shown by the first curve on the left in Fig. 1. By 5–10 min the process has reached a steady state. Between the formation of nuclear cortisol-receptor complexes and inhibition of glucose metabolism there is thus a 5–10 min time lag. We have so far identified at least three steps leading from one of these events to the other[6–8, 10].

First there is an irreversible step, characterized by the fact that removal of cortisol from the receptors after 5 min—by displacement with cortexolone, as indicated in Fig. 1, or by washing—does not prevent a cortisol effect on glucose transport or protein synthesis from appearing later. During this same period there is sensitivity to actinomycin-D, presumably due to a requirement for RNA synthesis. Actinomycin-D, just like cortexolone, prevents cortisol effects from appearing if it is added together with cortisol at 0 min but not if it is added 5 min later. (Actinomycin-D does not affect binding.) Next there is a temperature-sensitive step, which is almost completely blocked at 20°C. And finally, coinciding in time with the inhibition of glucose transport, there is a cycloheximide-sensitive step, presumably involving protein synthesis.

Our working hypothesis regarding these observations (see top, Fig. 1) is that the hormone-receptor complex, on reaching the nucleus initiates synthesis of a specific form of RNA that, after translocation to the cytoplasm (perhaps via a temperature-sensitive step or steps) leads to synthesis of a specific protein. The protein in turn, directly or indirectly, rapidly inhibits glucose transport [7, 8].

In the following sections we shall deal in somewhat speculative fashion with certain properties of the glucocorticoid receptors in isolation and in intact cells.

PROPERTIES AND NATURE OF GLUCOCORTICOID-RECEPTOR COMPLEXES

Glucocorticoid receptors, with or without hormone attached, can be isolated from both the nucleus and the cytosol from disrupted cells[5, 10, 13]. The isolated molecules, which are probably in part protein since they are inactivated by proteolytic enzymes, have affinities for various steroids similar to those of intact cells. In particular, they have higher affinity for dexamethasone than for cortisol, a characteristic that distinguishes them clearly from corticosteroid-binding globulin (CBG).

At 37°C cortisol dissociates from the isolated cortisol-receptor complexes with a time constant very close to the 3-min time constant for dissociation from intact cells, indicating that the cell is freely permeable to cortisol. If similar conditions apply *in vivo*, there is no need for a transport system—a function occasionally suggested for receptors—to carry cortisol into the cell[10].

From preliminary results it appears that the differences in binding constants of various steroids such as cortexolone, cortisol and dexamethasone are determined largely by their dissociation rates. As far as we can tell, the association rates of these steroids are very similar. The implication of these findings is that the groups that distinguish these steroids, particularly the 11β-hydroxyl and 9α-fluoro, do not come into play until the steroid has entered the steroid binding site of the receptor. It may be supposed, therefore, that the complementary group on the receptor with which the 11β-hydroxyl group reacts (presumably through hydrogen bonding) lies deep inside the site.

In considering the nature of steroid hormone-receptor interactions one is immediately struck by the lack of any relation between the number of hydrogen bonds a steroid hormone can form (as judged by the number of polar groups it possesses) and the affinity it has for its receptor. For example, dihydrotestosterone and progesterone have much higher equilibrium constants for binding to their respective receptors than does cortisol for binding to glucocorticoid receptors: but cortisol has far more polar groups than the other hormones. It is clear, therefore, that although the specificity of a steroid for its receptor must depend to a large extent on polar groups, a major contribution to the free energy of binding must come from hydrophobic interactions of the non-polar steroid nucleus with non-polar regions of the receptor.

From the structural requirements for glucocorticoid binding to thymus cell receptors[4], as well as from arguments such as that above regarding the location of the receptor group complementary to the 11β-hydroxyl, we have come to the conclusion that probably both the α and the β sides of the steroid interact with the receptor, rather than just the β side as proposed by Sarett on the basis of structure-activity relationships[14]. We thus picture the steroid binding site as a hydrophobic pocket, with polar groups strategically placed so as to form hydrogen bonds with the polar groups of the steroid. A similar picture was arrived at some years ago by Engel[15] from general considerations such as that the hydrophobic regions of proteins are usually inside the molecule.

Hydrophobic interactions have been extensively investigated in connection with stabilization of tertiary structures of proteins. From such studies, and from

earlier studies on solubilities of hydrocarbons in water, it has been concluded that the stability of hydrophobic bonds in aqueous media is due principally to the increase in entropy (resulting from decreased ordering of water structure) that accompanies formation of such bonds [cf. 16]. As far as they go, our measurements on binding of cortisol to thymus receptors are consistent with an entropy-driven reaction, but for firm conclusions we need more precise data than we have at present.

Hydrogen bonds can confer specificity to such hydrophobic reactions without necessarily contributing net negative free energy, since if a steroid is to fit closely into a site the water molecules normally associated with polar groups on the steroid and in the site must first be removed. This process requires input of free energy, tending to drive the reaction backwards. The increase in free energy can be compensated for by release of free energy if each polar group then forms a hydrogen bond with a complementary polar group. If no complementary group is encountered, however—i.e. if hydrogen bonding groups on the steroid and in the site are not matched—then the reaction is held back.

It is likely that the 21-hydroxyl group of cortisol does not penetrate deeply into the glucocorticoid site, since glucocorticoid activity is known to be relatively insensitive to changes at this position. Furthermore, we have recently found that in thymus cell suspensions cortisol-21-hemisuccinate competes with cortisol for specific binding and exhibits considerable glucocorticoid activity, without being hydrolysed (Munck and Brinck-Johnsen, unpublished). It is difficult to see how a large hydrophilic group such as the succinate could be accommodated inside a close-fitting site. The cortisol binding site on CBG is probably quite different from the receptor binding site. Not only are there differences in steroid specificity, as already noted, but, despite the fact that the binding constants are almost identical, the rate constants for association and dissociation of cortisol at 37°C appear to be much greater with CBG than with the receptors[17]. The free energies of activation for binding to CBG are therefore lower, suggesting that cortisol does not burrow as deeply into CBG as into the receptor.

STABILIZATION OF GLUCOCORTICOID RECEPTORS

A serious difficulty we have had in making equilibrium and kinetic binding measurements, and until recently in purifying receptors, is that the isolated thymus receptor is very unstable. At 3°C it has a half-life of about 2 h, which is increased in the presence of EDTA to about 7 h. A remarkable degree of stabilization is achieved by having a steroid bound specifically to the receptor. Saturating concentrations of cortisol, for example, even without EDTA, increase the half-more to more than 20 h. Preliminary analysis indicates that with less than saturating concentrations, receptors become inactivated at a significant rate only during the periods when they are free. Triamcinolone acetonide, as noted by Kirkpatrick and Rosen (personal communication), has very high affinity for glucocorticoid receptors. This steroid can stabilize individual receptors by the fact that at 3°C, once it is bound it practically does not dissociate. Stabilization by steroid binding, which is of course closely akin to substrate stabilization, should considerably facilitate purification of the receptors. It may also provide us with significant clues to the nature of the transformations the receptor undergoes when it forms a complex with the hormone.

TEMPERATURE-SENSITIVE TRANSFORMATION AND NUCLEAR BINDING OF THE CORTISOL-RECEPTOR COMPLEX

As already mentioned, with intact thymus cells at 3°C cortisol becomes bound to the cytoplasmic receptor. When the cells are warmed, cortisol, probably still bound to the receptor, rapidly becomes associated with the nucleus. From the studies of others[18, 19] we assume that the nuclear acceptor sites with which the cortisol-receptor complex becomes associated are part of the chromatin fraction of the nucleus.

The temperature-sensitive transfer to the nucleus can be reproduced by adding the isolated cortisol-receptor complex to isolated nuclei and warming to 25°C. The amount of nuclear-bound cortisol rises to a maximum by about 10 min; after that it decreases, probably due to inactivation of the receptor. Just as in the intact cell, the transfer process is not influenced by a large excess of free, unlabelled cortisol, demonstrating that in the course of transfer to the nucleus, receptor-bound cortisol is not released.

Warming is necessary only in order to produce a transformation of the cortisol-receptor complex, the transfer to the nucleus being a separate step, as shown already with the estrogen-receptor complex[20]. Thus, prewarming to 25°C of the isolated cortisol-receptor complex transforms the complex in such a way that it subsequently can become bound to nuclei at 3°C. Under our conditions, the rate-limiting step in the overall process is the transformation of the complex: transfer of the transformed complex to the nucleus is extremely rapid, being completed in less than 1 min even at 3°C.

We may symbolize formation of the cortisol-receptor complex by $H + R \rightleftharpoons HR$ (where H stands for hormone, and R for receptor; for simplicity we suppose there is one H per receptor, but the discussion below does not depend on this assumption), and the presumably allosteric temperature-sensitive transformation by $HR \rightleftharpoons HR'$ (where HR' has high affinity for the nuclear acceptor whereas HR has low affinity). Writing out the full set of possible reactions and equilibrium constants we get:

$$\begin{aligned} H + R &\rightleftharpoons HR & K_1 &= (HR)/(H)(R) \\ HR &\rightleftharpoons HR' & K_2 &= (HR')/(HR) \\ R &\rightleftharpoons R' & K_3 &= (R')/(R) \\ H + R' &\rightleftharpoons HR' & K_4 &= (HR')/(H)(R'). \end{aligned}$$

A simple way of accounting for the experimental observations described above is to assume that what the hormone does is alter the equilibrium between R and R' so as to favor the form with high affinity for the nucleus, i.e. $K_2 \gg K_3$. It is worth noting an immediate consequence of this assumption. The equilibrium constants are related by the equation $K_4/K_1 = K_2/K_3$. Therefore if we assume $K_2/K_3 \gg 1$, it follows that $K_4 \gg K_1$. In other words, if hormone binding alters the equilibrium between R and R' by a factor of K_2/K_3, then the equilibrium constant (K_4) for binding of the hormone to the transformed receptor must be increased over that (K_1) for binding to the untransformed receptor by the same factor. Although we do not have definitive data we would expect that if this mechanism is valid, K_2 would have to be increased over K_3 by a factor of 5 at least; this would lead to a substantial increase in affinity of the hormone for the transformed receptor, one that we should eventually be able to measure.

An alternative way of accounting for the observations is to assume that the hormone, without altering the equilibrium between R and R′, alters their rates of interconversion, i.e. H accelerates the temperature-sensitive transformation. Since on this assumption K_2 and K_3 are equal, so are K_1 and K_4. Another consequence, perhaps more easily testable, that distinguishes this assumption from the previous one is that once HR′ is formed, if the complex is made to dissociate then the free receptor should remain in the form R′ rather than reverting to R, even when warmed.

In the absence of hormone the predominant form of the receptor in the intact cell is shown by experiment to be R. If the second mechanism is valid, this form is not at equilibrium. Such a non-equilibrium state could arise if R were being generated continuously from a precursor, a possibility considered in the next section.

The two mechanisms discussed here are by no means the only ones that can be devised. But they are perhaps the simplest and the most clearly distinguishable, the first being an equilibrium mechanism and the second a kinetic mechanism. They are not mutually exclusive, so a combination of the two is quite conceivable.

ENERGY DEPENDENCE OF CORTISOL BINDING: A HORMONE-RECEPTOR CYCLE?

An observation we made several years ago[2, 4] is that at 37°C there is a correlation between levels of ATP in thymus cells and the magnitude of specific cortisol binding. As shown in Table 1, where conditions 1 give the standard values for specific binding and ATP levels, in the absence of O_2 and substrate (condition 2), both ATP levels and specific binding are low. Either O_2 alone (condition 3) or glucose alone (condition 4) can reestablish approximately normal values for these parameters, showing that either oxidative or glycolytic processes can serve as sources for whatever ATP is necessary. Finally, conditions 5 show that these effects are reversible, since introduction of O_2 after 20 min incubation

Table 1. Correlation between magnitude of specific binding of cortisol at 37°C to thymus cells, and cellular levels of ATP. Specific binding is given in relative units, the maximum value for which is about 0·3 under the conditions of these experiments. ATP levels are in μmol per ml of cells. Cortisol concentration is about 10^{-7} M. From [4]

Incubation conditions	Incubation time (min)	ATP levels	Specific binding
1. O_2, glucose	20	2·3	0·30
2. N_2, no glucose	20	0·12	0·04
	80	0·06	0·00
3. O_2, no glucose	20	2·3	0·30
	80	2·2	0·21
4. N_2, glucose	20	1·5	0·30
5. N_2, no glucose	20	0·15	0·05
O_2 after 21 min	26	2·1	0·12
	38	2·0	0·24
	64	2·1	0·26

without O_2 or substrate, rapidly restores ATP levels and somewhat more slowly restores specific binding. These results suggest that ATP or some related substance is necessary for normal formation of cortisol-receptor complexes, perhaps by supplying energy. Cortisol almost certainly enters the cells freely under all the conditions described, so the deficiency caused by absence of ATP presumably has to do with the receptor itself—with its availability, or its ability to bind cortisol at 37°C.

Binding to the cytoplasmic receptor, R, isolated from metabolically intact cells, can proceed in absence of ATP. ATP therefore probably enters at a stage prior to R. A recent experiment suggests that in cells deprived of ATP there may exist another form of the receptor, perhaps a precursor of R. Cells incubated with cortisol under condition 2 (Table 1) for about 90 min at 3°C (rather than at 37°C) form substantial amounts of a cytoplasmic cortisol-receptor complex. When these cells are warmed to 37°C, however, only very slight transfer to the nucleus occurs. Addition of oxygen reestablishes normal nuclear binding.

We thus appear to be dealing with a form of the receptor that in the cell is unable to bind cortisol at 37°C, and that even with cortisol bound is not transferred to the nucleus. To distinguish this hypothetical form from the R and R′ forms introduced previously, we designate it as R″. Our working hypothesis is that ATP converts R″ to R, the normal cytoplasmic receptor.

If R is indeed derived from R″, the question that immediately arises is, where does R″ come from? Our present tentative idea is that R″ may be the form in which the receptor is released from the nucleus, or in other words, that R″ may be part of a receptor cycle.

Figure 2 is a highly speculative synthesis of these ideas and experimental observations. The left half of the scheme is fairly solidly established, at least in outline. It includes the reactions and transformations discussed in the previous

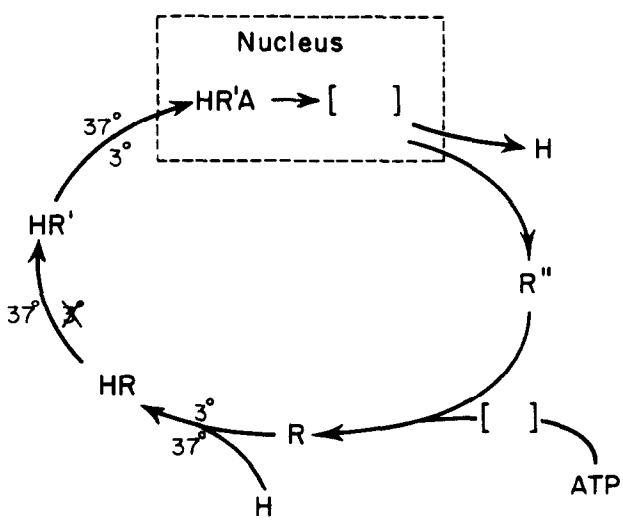

Fig. 2. Hypothetical glucocorticoid receptor cycle. H stands for hormone, R, R′ and R″ for different forms of the receptor, and A for nuclear acceptor site. Temperatures give the conditions under which the various reactions can proceed. The reactions do not go at temperatures that are crossed out. See text for details.

sections. The right half of the scheme contains our working hypotheses regarding R″, the role of ATP, and the cyclic nature of the process. So far there is no evidence whatsoever on whether or how the receptor comes off the nuclear acceptor site. We have included a second, hypothetical, nuclear state, because it seems reasonable to suppose that removal of the receptor from the nuclear site is accompanied by a nuclear-induced transformation of HR′, perhaps coupled to the effect HR′ produces on the nucleus. The hormone, H, is assumed to leave the cycle in the same form in which it enters the cycle. This assumption is in accordance with our observation that little if any metabolism of cortisol takes place during a long incubation with thymus cells, even though the hormone is continuously associating with and dissociating from the specific sites.

Once it is assumed that the receptor goes through a cycle, the role of ATP is seen in a new light. Any cyclic process requires free energy, regardless of the details of the cycle. In principle the hormone, H, could supply the energy by being metabolized; but if H remains unchanged, as appears to be the case, the energy must be sought elsewhere. ATP might well be the source. By regenerating R from R″, ATP could raise the receptor to a sufficiently high free energy level so that it would pass without further addition of free energy through the various transformations leading to the nuclear complex. Even without a cycle ATP could of course be acting in this way. The hormone, in such a scheme, would be expected to operate through the second, kinetic, mechanism proposed in the previous section rather than through the equilibrium mechanism.

Through what metabolic processes ATP acts we do not know. Aside from their intrinsic interest such processes, particularly if coupled with a cycle, could be of practical importance in purifying receptors. We have been testing, so far with inconclusive results, for the possibility that ATP might promote phosphorylation of the receptor, perhaps with the intervention of cyclic AMP.

An obvious alternative to a cyclic process is one in which receptors in a hormone-stimulated cell are continuously being synthesized, and are destroyed after use. As a test of this alternative we have measured specific binding of cortisol by thymus cells at 37°C over a period of one hour in the presence of 10^{-4} M cycloheximide, which we know will reduce protein synthesis by more than 90 per cent. The concentration of cortisol employed was such that about half the receptors were occupied. From the rates of association of specifically bound cortisol we could calculate that if every receptor with which cortisol became bound to the nucleus was destroyed, then in the absence of synthesis all receptors in the cell would be used up in about 10 min. In fact, there was practically no change in specific binding over the whole hour, showing that synthesis and destruction of receptors probably do not occur at rates comparable to the turnover of specifically-bound hormone.

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DISCUSSION

Martini: I have one small question about your Fig. 2. Are you sure that if the steroid you use is cortisol, what comes out of the nucleus is still cortisol? Or could it be cortisone, as was suggested this morning for oestradiol and oestrone?

Munck: If I hadn't already thought about this question, I would have said yes, it's cortisol that comes off; but we haven't done quite the right experiment yet. What we have done is removed the supernatant from cells incubated with [^{3}H]-cortisol, removed the non-specific fraction from the cells, and then extracted and analyzed the specific fraction left on the cells. That turns out to be cortisol – purer cortisol than in the supernatant. But we've never done the direct experiment to test that question, which is to first allow the specific fraction to dissociate at 37°C, and then analyze it. We find no indication of any cortisone being produced even after a one-hour incubation, but that still does not answer the question. [Note in proof: Dr. Brinck-Johnsen and I have now done the right experiment, and the answer is quite unambiguous. The radioactivity that dissociates under physiological conditions at 37° from cells that have been incubated with [^{3}H]-cortisol, is still 100% [^{3}H]-cortisol (with a possible error of 5%). So we find no evidence that metabolic transformation of the hormone precedes or accompanies its release from the nucleus.]

Jensen: You have in your list of equations the complex HR going to HR′, and then you have R going to R′. You mentioned that the presence of the hormone may change the position of equilibrium. Do you have any evidence that R goes to R′ at all when the hormone is not there?

Munck: No, we don't have any direct evidence. But I think that as a matter of principle at least a theoretical equilibrium must exist between R and R′. Whether the equilibrium is way over to the left side (in other words, that R for all practical

purposes is the only form) or whether it's slightly over, is another question. The only point I can make is that we do find that nuclei from thymuses from adrenalectomized rats (i.e. from cells that have not been exposed recently to cortisol) do have extractable glucocorticoid receptors. So something is in there. That's one reason we included that second, hypothetical, nuclear stage.

Jensen: You don't know that it is the same receptor that you extract from the nuclei of cortisol-treated animals, do you? Because you don't characterize it by any parameter, such as an S value.

Munck: That's right. The curious thing is that the intact nuclei will not bind cortisol. But we can extract a binding protein from them, suggesting that there is a receptor in there that is unable to bind cortisol. It just might be a stage in the process of getting out.

Pasqualini: Is it your conclusion, then, that there is no equilibrium between 11-keto and 11-hydroxy functions, and both the steroids are absolutely unmetabolized? Do you think the receptor is formed through the 11β-hydroxy function?

Munck: Yes, we find no evidence for conversion of cortisol to cortisone or vice-versa. We believe the complex is highly specific for the 11β-hydroxyl group. We get binding with cortisol, corticosterone, dexamethasone, prednisolone, 9α-fluoroprednisolone and triamcinolone acetonide, but not with cortisone or 11-epicortisol (which has the 11-hydroxyl in the α position). We do get binding with cortexolone (Reichsteins Substance S) which is identical to cortisol and cortisone except that it lacks a group at position 11. Cortexolone has no glucocorticoid activity, but functions in our system as a very effective antiglucocorticoid. The fact that cortexolone binds indicates to us that cortisone and 11-epicortisol fail to bind not just because they lack an 11α-hydroxyl, but because the 11-keto and 11α-hydroxyl groups interfere with binding by bulging out on the α side. These and other arguments have convinced us that glucocorticoid-receptor complexes are formed through both α- and β-side interactions, with the 11β-hydroxyl group perhaps leading the way into a hydrophobic pocket. We do not think that simple β-side interactions, such as were postulated many years ago from structure-activity relationships *in vivo*, can account for our data.

Rosner: Can you remove R (Fig. 2) from the cytoplasm and get cortisol to bind to it? I got the impression that it was turning over very fast, and I'm therefore surprised that you can isolate the receptor if you take away glucose and oxygen, since it seems to go away so fast.

Munck: Well, I think what we're doing is always isolating the receptor in the form R. In that form it's ready to bind cortisol without any need for ATP, if this hypothesis is correct. We extract R from cells that have been cooled, in which the receptors are not turning over fast. R″ we usually would not see at all.

O'Malley: I'm not sure whether thermodynamically I understand what you mean by the fact that the affinity constant of the receptor after binding the steroid is different from that of the uncomplexed receptor. You mentioned something about an allosteric change in the receptor which gives it a new affinity constant.

Munck: What it amounts to is that we are able to measure two separate affinity constants. Normally, if you have a steroid binding to a protein and transforming it instantly, you don't have a chance of catching it before the transformation in the protein has taken place. But it just so happens that here we require a high temperature (25 or 37°) in order for the next transformation to take place. So we've got two separate affinity constants: one is for the untransformed receptor

R, to which the hormone initially binds; the other is for the receptor after it has been transformed to R′ by warming (with cortisol bound to it).

Rosner: Is there any evidence that there's only one binding site per mole of receptor, or could there be more? If there's more than one, it very easily takes care of Dr. O'Malley's question, through a simple allosteric effect affecting the second and third and X number of binding sites.

Munck: We don't know how many sites there are on the glucocorticoid receptors.