Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 1117–1122. Pergamon Press. Printed in Great Britain

KINETICS OF GLUCOCORTICOID–RECEPTOR COMPLEXES IN RAT THYMUS CELLS

ALLAN MUNCK and ROSEMARY FOLEY
Department of Physiology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

SUMMARY

Using cortisol and dexamethasone, we have studied the kinetic behavior of glucocorticoid receptors in intact rat thymus cells at 37°C. As reported previously, translocation of the cortisol–receptor complexes from "cytoplasmic" to nuclear-bound form takes about 1 min. At 26°C this time is doubled. Consistent with these observations and with the current view that formation of nuclear-bound complex must be preceded by formation of cytoplasmic complex, on addition of hormone to cells at 37°C the initial time-course of formation of cytoplasmic complex precedes that of nuclear complex by about 30 s. After cells have achieved a steady state with [^{3}H]-dexamethasone, however, a "chase" of unlabelled dexamethasone lowers nuclear levels of [^{3}H]-dexamethasone several minutes before cytoplasmic levels, suggesting formation of nuclear complex by direct reaction of hormone with nuclear-bound receptors. Following depletion of cytoplasmic receptor by incubation with a high concentration of dexamethasone and then sudden lowering of steroid concentration by dilution, the time-course of replenishment of cytoplasmic receptors appears to lag significantly behind the time-course of removal of nuclear-bound steroid. The lag is roughly the same magnitude as the time-constant of dissociation of the glucocorticoid used, and is much shorter with cortisol. The pathway of replenishment thus may be different from simple reversal of the pathway of depletion of cytoplasmic receptor, and may depend on the dissociation rate or affinity constant of the steroid.

INTRODUCTION

Much evidence supports the current view [1] that initial formation of steroid hormone–receptor complexes in target cells under physiological conditions takes place via the multi-step process *a*, *b*, *c* shown by solid lines in Fig. 1. Step *a* is the reaction of hormone H with the so-called "cytoplasmic" receptor R to form unactivated cytoplasmic hormone–receptor complex HR. Step *b* is the transformation of HR to give HR′, the "activated" cytoplasmic hormone–receptor complex characterized by having high affinity (compared to HR) for nuclear sites. Step *c* is the reaction of HR′ with nuclear sites to form the nuclear-bound complex HR_N. (The notation HR, HR′, etc. is only symbolic, and does not necessarily represent the true stoichiometry of the reactions and complexes.) Reaction *a* is almost certainly reversible, but as indicated by the dashed arrows in Fig. 1, whether *b* and *c* are reversible is an open question. In rat thymus cells, the system we deal with here, initial formation of glucocorticoid–receptor complexes proceeds by these same steps [2–4], and at 37° *a* is rate-limiting [4].

What happens after steps *a*, *b* and *c* is largely conjectural. Hormone that is bound in the nucleus has been shown to subsequently emerge from the intact cells unchanged [5], but despite much speculation, the fate of the receptor that is bound in the nucleus is not known.

According to a scheme designed to account for dexamethasone binding in hepatoma cells [6], the nuclear-bound receptor reappears in cytoplasmic form by simple reversal of reactions *c*, *b* and *a*. A more general scheme proposed for estrogen–receptor complexes in uterine cells [7] includes in addition a reversible reaction *d* by which hormone dissociates directly from the nuclear-bound complex to leave nuclear-bound receptor R_N, which can then dissociate to form the cytoplasmic receptor R by a reversible reaction *e*. Yet another theoretical possibility is that a nuclear complex reverts to unactivated cytoplasmic complex by a reaction *f*. To account for the apparent dependence on ATP of glucocorticoid receptor levels in rat thymus cells we have suggested [3] that after binding in the nucleus the receptor undergoes a transformation *g* to an inactive state R″ from which the cytoplas-

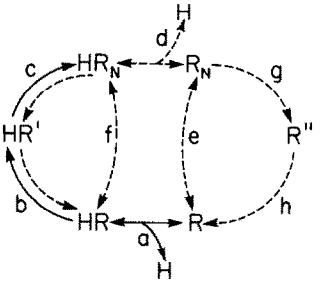

Fig. 1. Established pathways (solid arrows) and hypothetical pathways (dashed arrows) between H (steroid hormone), R (cytoplasmic receptor), HR (unactivated cytoplasmic complex), HR′ (activated cytoplasmic complex), HR_N (nuclear-bound complex), R_N (nuclear-bound receptor), and R″ (inactive receptor). See text for explanation.

mic receptor is regenerated by an ATP-dependent reaction *h*. Since the ATP-related phenomena can be observed in the absence of protein synthesis [3,8], reaction *h* does not include synthesis of new receptor. It should be emphasized that although it is very likely that at least some of the hypothetical reactions indicated with dashed lines in Fig. 1 take place in the intact cell, there is little evidence for any particular one of them.

Additional hypothetical reactions and states should no doubt be included in Fig. 1 to take into account the likelihood that *de novo* synthesis of receptors is important in long-term maintenance of receptor levels [9–11], that receptors may be associated with microsomes [12], that the nuclear-bound complex consists of several distinct subfractions [13], etc. Reactions such as $H + R' \rightleftharpoons HR'$ should also be added [3]. For the short-term kinetic results that have been published so far and for those we discuss below, however, the scheme in Fig. 1 as it stands is already sufficiently complex.

Our earlier studies [2–5, 8] have shown that cortisol binds rapidly to cytoplasmic and nuclear glucocorticoid receptors in rat thymus cells in suspension at 37°, reaching a steady state by about 10 min. Cortisol dissociates from receptors in intact cells as well as from extracted receptor complexes with a rate constant of 0.3–0.4 min^{-1}. Dexamethasone (9α-fluoro-16α-methyl-11β,17,21-trihydroxypregn-1,4-diene-3,20-dione) has 4–5 times greater affinity for glucocorticoid receptors than cortisol, a difference largely due to a lower dissociation rate constant.

To gain a more detailed understanding of the dynamic interrelationships of various forms of receptor and hormone–receptor complexes in the intact, normal cell at 37°, we have begun to carry out experiments of the kind described below. Most of these are "pulse" experiments, designed to reveal the time-course of transition from one state to another following a sudden change in temperature, concentration, metabolic condition, etc. Generally we test both cortisol and dexamethasone, since the different kinetic properties of these steroids often provide instructive contrasts.

METHODS AND MATERIALS

The following procedures have been described in earlier publications: preparation and incubation with steroids of suspensions in Krebs–Ringer bicarbonate buffer of thymus cells from adrenalectomized rats [4,14]; measurement of nuclear hormone–receptor complex (HR_N in Fig. 1) from the radioactivity in a "nuclear" pellet obtained by centrifugation following 50- or 200-fold dilution of a 20 μl aliquot of cell suspension into aqueous 1.5 mM $MgCl_2$ at 0–3° [14,15]; measurement of "cytoplasmic" hormone–receptor complex from the radioactivity in the supernatant obtained by centrifugation following 6-fold dilution of 20 μl aliquot of cell suspension into 100 μl Dextran-coated charcoal suspension at 0–3° [15]. This latter procedure measures unactivated and activated complexes together (HR and HR′ in Fig. 1), but we are not sure that it measures both with the same efficiency.

Activated complexes in cells incubated with radioactive hormone are measured by incubating cytosol from those cells with isolated nuclei at 3°. The cytosol is prepared as the supernatant from a 6-fold dilution of the cell in 1.5 mM $MgCl_2$, 0–3°, centrifuged 4 min at about 15,000 ***g***. The nuclear pellet is obtained by adding 50 μl of unexposed cell suspension to 10 ml 1.5 mM $MgCl_2$ and then centrifuging at about 1500 ***g*** for 6 min and decanting, keeping all components at 0–3°. A 25 μl aliquot of the cytosol is added to the nuclear pellet (which is in a 15-ml conical polyethylene or polypropylene centrifuge tube), mixed vigorously for a few seconds and left for 10–15 min. The mixture is then resuspended by adding 10 ml 1.5 mM $MgCl_2$, the nuclear pellet sedimented again by centrifugation and the amount of activated complex determined from the radioactivity associated with the pellet.

A relative measure of total cytoplasmic receptor (i.e. R + HR + HR′) is obtained with the cytosol prepared as described above. The cytosol is brought to pH 7 with Tris (10 mM), EDTA (25 mM) and dithiothreitol (5 mM) and incubated for 2 h at 3° with [^{3}H]-dexamethasone or [^{3}H]-cortisol to label the free receptors. The level of bound hormone is then determined by the Dextran-coated charcoal procedure using 50 μl cytosol and 100 μl suspension.

$(S)_e$ is the concentration of free radioactive steroid (in M) with which cells are initially incubated. When used in the denominator it is a constant that serves to normalize the values of bound steroid. (Sc) is the radioactive steroid (mol per liter of packed cells) associated with cells or cell fractions at various stages of an incubation [4, 14]. All suspensions were generally incubated at a concentration of 0.30 to 0.36 ml packed cells per ml suspension.

Dextran-coated charcoal is prepared by mixing 0.1 g Norit-A charcoal (Fisher) and 0.01 g Dextran type 60C (Sigma) in 10 ml 1.5 mM $MgCl_2$, allowing the suspension to settle at 3° for 24 h, aspirating off the supernatant and bringing the vol. back to 10 ml with 1.5 mM $MgCl_2$.

[1,2-^{3}H]-cortisol (S.A. 40 Ci/mmol) and [1,2,4-^{3}H]-dexamethasone (S.A. 22.6 Ci/mmol) were obtained from New England Nuclear, and used without further purification. Cortisol, dexamethasone and dithiothreitol were obtained from Calbiochem.

RESULTS AND DISCUSSION

Rate of cytoplasmic-nuclear translocation at 37, 26 and 15°C

To measure these rates we first incubated thymus cells with [^{3}H]-cortisol at 3° for about 2 h to form cytoplasmic, but not nuclear, hormone–receptor com-

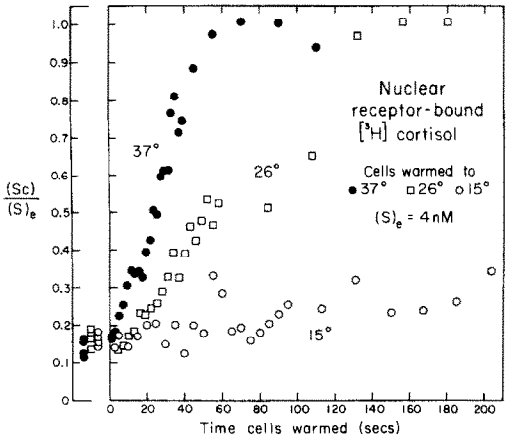

Fig. 2. Cytoplasmic-nuclear translocation of cortisol–receptor complexes at 37°, 26°, and 15°C. [From 4.] For each temperature a thymus cell suspension was incubated with 4 nM [^{3}H]-cortisol at 3°C. Between 80 and 140 min after the start of the incubation successive 20-μl aliquots of the suspension were removed and brought instantaneously to the appropriate temperature by adding 200 μl warm buffer. After the indicated number of s the cells were simultaneously cooled and disrupted by addition of 10 ml of 1.5 mM $MgCl_2$ at 3°C. The resulting suspension was then centrifuged to yield a nuclear pellet. Warming times for successive aliquots were chosen in random order. Points to the left of 0 s were obtained with unwarmed aliquots. (Sc)/(S)e = 1 corresponds to about 2400 d.p.m. per nuclear pellet. Further experimental details are given in [4].

plex. The cells were then abruptly warmed to initiate the cytoplasmic-nuclear transfer and further formation of cytoplasmic complex stopped. After a given number of seconds the cells were cooled and broken by hypotonic shock, and the amount of nuclear complex determined from the radioactivity in the nuclear pellet.

As can be seen from Fig. 2, which comes from earlier work [4], at 37°C formation of nuclear-bound complex begins within seconds of warming and reaches completion by about 1 min. At 26° and 15°C the rates are progressively slower.

In preliminary experiments we have compared the rates of nuclear translocation at 37° of cytoplasmic complexes formed with dexamethasone and cortisol, and have found no significant difference between them. As far as it goes, this observation is consistent with the fact that we find no significant difference in the ratio of nuclear to cytoplasmic complex formed by dexamethasone and cortisol under steady-state conditions.

Time-course of formation of cytoplasmic and nuclear complexes on addition of [^{3}H]-dexamethasone to thymus cells at 37°C

The time-course of formation of cytoplasmic and nuclear complexes shown in Fig. 3 are entirely in accord with the sequence of steps *a*, *b*, *c* in Fig. 1, which predicts that cytoplasmic complex HR should be formed first and then give rise to nuclear complex HR_N. Cytoplasmic complex begins to form immediately on addition of [^{3}H]-dexamethasone, and is followed after a slight delay by appearance of nuclear complex. The delay is of the magnitude expected from the rate of cytoplasmic-nuclear translocation shown in Fig. 2. Cortisol gives almost the same initial time-course. These results are similar to those found with estrogen receptors in the uterus at 37°C [7], and are consistent with time courses measured at lower temperatures in a variety of systems including thymus cells [4].

Time-course of decay of cytoplasmic and nuclear complexes with [^{3}H]-dexamethasone following chase with unlabelled dexamethasone

The cold chase in Fig. 3, added after 28 min (by which time steady-state levels have been reached) gives somewhat surprising results. In contrast to what happens in the formation of complexes, there is now first a drop in level of nuclear complex, followed after a delay of about 5 minutes by a drop in level of cytoplasmic complex. We have repeated these observations many times with similar results, and have also found that a somewhat slower decay of cytoplasmic than nuclear complex follows removal of steroid by washing (Fig. 4), or by sudden lowering of ATP levels.

At least two questions are raised by these results. One is, why do the cytoplasmic levels not begin to drop immediately after the unlabelled hormone is added, as would be expected if the cytoplasmic levels are maintained through reaction *a* alone? A possible answer, among several, is that the lower rate of for-

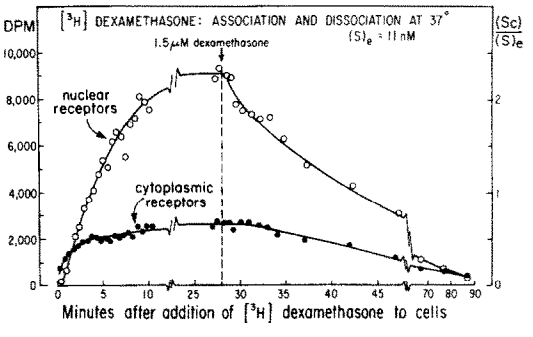

Fig. 3. Time-course of formation, and of decay following a cold chase, of cytoplasmic and nuclear [^{3}H]-dexamethasone complexes in thymus cells at 37°C. To a thymus cell suspension at 37°C, [^{3}H]-dexamethasone was added at 0 min to give a concentration of free steroid of 11 nM. At intervals, 20-μl aliquots were removed to determine alternately cytoplasmic and nuclear-bound [^{3}H]-dexamethasone. At 28 min dexamethasone was added to give a free concentration of 1.5 μM, and sampling continued. A separate cell suspension to which 1 μM dexamethasone had been added initially was incubated simultaneously with [^{3}H]-dexamethasone and assayed to provide a correction for nonsaturable binding; these values were subtracted from the values for each aliquot above. The differences are plotted in the figure, each point corresponding to one aliquot.

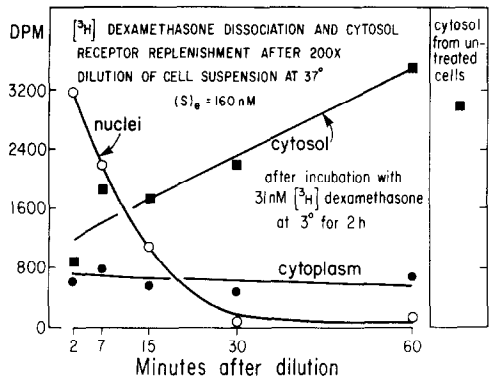

Fig. 4. Time-course at 37°C of decay of nuclear (open circles) and cytoplasmic (solid circles) [^{3}H]-dexamethasone–receptor complexes, and of replenishment of cytoplasmic receptors (solid squares), following 200-fold dilution of a cell suspension initially brought to a steady state at 37°C with [^{3}H]-dexamethasone. A suspension of thymus cells was incubated initially at 37°C with 160 nM [^{3}H]-dexamethasone for 30–40 min. A set of aliquots was diluted 200-fold with buffer at 37°C, and cooled rapidly to 0° after 2, 7, 15 min, etc. All subsequent procedures were at 0–3°C. The diluted suspensions were centrifuged 6 min at about 1500 *g* to sediment the cells, the supernatants decanted and the cells resuspended in buffer to their original vol. From these suspensions, 20-μl aliquots were taken in duplicate for determining nuclear and cytoplasmic complexes. The values are plotted as d.p.m. per 20 μl aliquot, and are corrected for non-saturable binding determined from a similarly treated batch of cells incubated initially with [^{3}H]-dexamethasone plus 1 μM dexamethasone. Cytosol preparations for determining total cytoplasmic receptor were obtained from the same suspensions. They were incubated with 31 nM [^{3}H]-dexamethasone for 2 h at 3°C, and then assayed in triplicate for bound hormone using Dextran-coated charcoal. To determine total receptor in untreated cells (value plotted on right), a cytosol preparation was also obtained from a similarly treated batch of cells that was incubated initially without hormone. An aliquot of this latter cytosol, incubated with [^{3}H]-dexamethasone and 1 μM dexamethasone, gave the correction for non-saturable cytosol binding (848 d.p.m.) that has been subtracted from all the cytosol points (solid squares). Each point is the mean value from two or three assays.

mation of HR is compensated for temporarily by replenishment from nuclear complexes, through reversal of reactions *c* and *b* or through a separate path such as *f*. This mechanism is in accord with the fact that there is a drop in nuclear complex, but does not explain what causes the nuclear complex to dissociate in the first place.

The second question is, why do the levels of nuclear complex drop in the absence of any measurable change in cytoplasmic levels? One reasonable possibility is that once nuclear complexes have been formed through the paths *a*, *b*, *c*, hormone dissociates from HR_N and associates directly with nuclear-bound receptor R_N through reaction *d*. The unlabelled hormone would then stop formation of labelled nuclear complex by blocking both reaction *d* and reaction *a*. In the so-called "nuclear exchange" assay [16], nuclear complexes are formed by reaction of hormone with isolated nuclei, apparently by direct reaction with nuclear-bound receptors R_N. Thus there is some evidence that a reaction such as *d* can take place under certain cell-free conditions.

In similar experiments with cortisol the overall time course is much more rapid, and it is difficult to discern significant differences between the rates of decay of the cytoplasmic and nuclear complexes.

Levels of activated (HR′) and unactivated (HR) receptor complexes under steady-state conditions

A missing element in the experiments of Fig. 3, as well as in the experiments described below, is the time-course of the level of activated complex HR′. So far we have measured HR′ only under steady-state conditions with dexamethasone. Our preliminary results indicate that of the total cytoplasmic receptor complex in intact cells at 37°C, some 30–50% is in the activated form HR′, the rest being in the unactivated form HR.

Rate of replenishment at 37°C of cytoplasmic receptor following removal of steroid after depletion by incubation with saturating concentrations of [^{3}H]-dexamethasone or [^{3}H]-cortisol: comparison with the rate of dissociation of hormone from nuclear complexes

From Fig. 1 it is clear that if in the intact cell under physiological conditions nuclear-bound hormone leaves the nucleus exclusively by reversal of paths *c*, *b* and *a* or by path *f*, then a decrease in nuclear-bound hormone must lead to a stoichiometrically equivalent increase in the total amount of bound and free receptor in the cytoplasm, i.e. R + HR + HR′.

In Fig. 4 we show the results of an experiment designed to test this conclusion. Cells were first incubated with a high concentration of [^{3}H]-dexamethasone at 37°C to deplete free cytoplasmic receptor. The concentration of steroid was then lowered by dilution at 37°C. At various times afterwards the levels of nuclear complex (open circles) and cytoplasmic complex (solid circles) were determined, and the level of total cytoplasmic receptor (solid squares) measured by incubating cytosol with [^{3}H]-dexamethasone at 3°C. The level of total cytoplasmic receptor in untreated cells (i.e. cells incubated without steroid) is shown on the right.

Compared to the level in the untreated cells, at the 2-min point there is very little cytoplasmic receptor above that accounted for by cytoplasmic complex. This shows that virtually complete depletion has taken place. After 60 min dilution there is complete replenishment (the difference between the levels after 60 min and the untreated cells is within experimental error). Similar results have been found with dexamethasone and glucocorticoid receptors in hepatoma cells, and have been interpreted to support a scheme consisting of a reversible set of reactions *a*, *b*, *c* [6].

When, however, the full time-course of replenishment of total cytoplasmic receptor is compared to

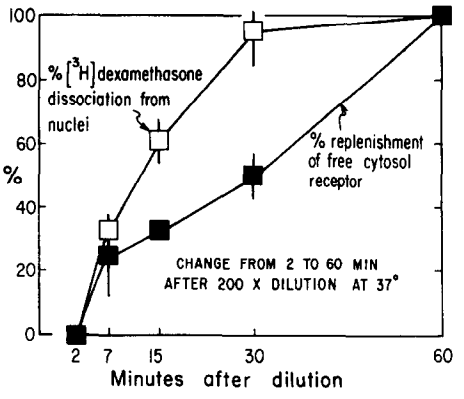

Fig. 5. Comparison of the time-courses of simultaneous decay of nuclear receptor-bound [^{3}H]-dexamethasone (open squares), and of replenishment of total cytoplasmic receptor (solid squares), following removal of [^{3}H]-dexamethasone by dilution. For each of three experiments such as that illustrated in Fig. 4, the 2-min and 60-min values for nuclear complex and total cytoplasmic receptor (cytosol) were taken as 0% and 100% respectively, and the remaining points scaled accordingly. The points are the average of the means and the vertical lines the range of the means from the three separate experiments.

the time-course of decrease of nuclear complex, there is a significant discrepancy that is particularly noticeable at 30 min. By 30 min the disappearance of nuclear complex is almost complete, but the replenishment of cytoplasmic receptor is not.

We have repeated this kind of experiment several times, with the same results. The combined results of three such experiments are given in Fig. 5. They illustrate clearly the degree to which the time-course of cytoplasmic replenishment lags behind the time-course of disappearance of dexamethasone from the nuclei.

There is no way of explaining this result with the model in Fig. 1 except by means of paths such as *d*, *e* or *d*, *g*, *h*, which allow for the possibility of H leaving the nucleus without the receptor that was part of the nuclear complex reappearing simultaneously in the cytoplasm. We have no proof, of course, that the receptors that replenish the cytoplasm are indeed derived from the nuclear complexes, and paths that require synthesis of new receptor or that tap a store of "pro-receptor" could also explain our results.

Whatever causes the delay in replenishment of cytoplasmic receptor in cells exposed to dexamethasone, one might expect that the same phenomenon would be observed in cells exposed to cortisol. It is not, however. Cortisol-exposed cells exhibit a much smaller delay (Fig. 6), one that is hardly significant.

Taken at face value, the results in Figs. 5 and 6 lead to the conclusion that the rate of replenishment of cytoplasmic receptor depends on the steroid that causes the depletion. As a concrete example of what this could mean, we can suppose that the path *d*, *e* in Fig. 1 is the main route of replenishment from HR_N through R_N to R. It must then be postulated that the rate of the reaction *e* by which R_N is converted to R depends on the particular hormone, H, by which HR_N is initially formed, even though H does not participate in reaction *e*.

Alternatively, if it is supposed that several replenishment paths are available, such as *d*, *e* and *c*, *b*, *a*, the influence of the hormone may be to determine which path predominates. Thus the difference between the delays obtained with cortisol and dexamethasone could be accounted for if replenishment in cortisol-treated cells were mainly by path *c*, *b*, *a* and in dexamethasone-treated cells by path *d*, *e*.

Although we have no reason to doubt the validity of these results, we hesitate to accept the conclusions they lead us to in the absence of further evidence, and are conscious of the many artifacts that can distort the outcome of experiments of the kind we are dealing with. One point that emerges clearly from Figs. 5 and 6 is that the delay in replenishment, greater for dexamethasone than for cortisol, is roughly proportional to the time-constant of dissociation of the steroid and will therefore, as noted above, be proportional to the affinity of the steroid for the receptor. It would not be remarkable, in fact, if any influence the steroid had on replenishment were related to its affinity.

GENERAL CONCLUSIONS

Our results lead us tentatively to the conclusion that the kinetic behavior of glucocorticoid receptors in intact thymus cells at 37°C cannot be adequately accounted for by the set of reactions *a*, *b*, *c* in Fig. 1, whether these are assumed to be fully reversible or are supplemented by a separate reaction such as *f*.

Several observations, furthermore, suggest that, at least with dexamethasone, reaction *d* plays a significant role in both the formation and the dissociation of nuclear complex.

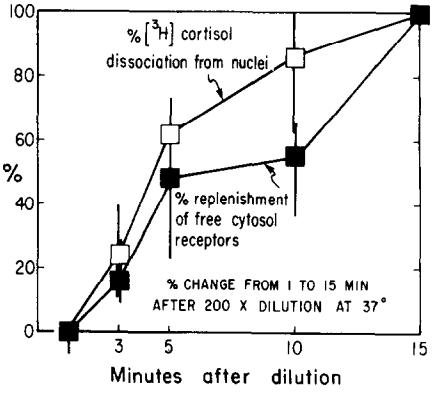

Fig. 6. Comparison of the time-courses of disappearance of nuclear receptor-bound [^{3}H]-cortisol (open squares) and of replenishment of total cytoplasmic receptor (solid squares). The data are derived from three experiments similar to that illustrated in Fig. 4, but using [^{3}H]-cortisol and different time-points. The form of plotting the data is the same as in Fig. 5.

Finally, our evidence appears to indicate that the two glucocorticoids, cortisol and dexamethasone, generally regarded as identical except for their different affinities, can induce qualitatively different kinetic behavior in glucocorticoid receptors. This observation can perhaps account for the differences that have been noted between the intrinsic activities or efficacies of cortisol and fluorinated synthetic glucocorticoids such as dexamethasone [14, 17].

Acknowledgements—The work described was supported by research grant AM03535 from the National Institute of Arthritis and Metabolic Diseases and CA17323 from the National Cancer Institute.

REFERENCES

1. Jensen E. V., Mohla S., Gorell T., Tanaka S. and DeSombre E. R.: *J. steroid Biochem.* **3** (1972) 445–458.
2. Munck A. and Wira C.: *Adv. Biosci.* **7** (1971) 301–330.
3. Munck A., Wira C., Young D. A., Mosher K. M., Hallahan C. and Bell P. A.: *J. steroid Biochem.* **3** (1972) 567–578.
4. Wira C. and Munck A.: *J. biol. Chem.* **249** (1974) 5328–5336.
5. Munck A. and Brinck-Johnsen T.: *J. steroid Biochem.* **5** (1974) 203–205.
6. Rousseau G. G., Baxter J. D., Higgins S. J. and Tomkins G. M.: *J. mol. Biol.* **79** (1973) 539–554.
7. Williams D. and Gorski J.: *Proc. natn. Acad. Sci., U.S.A.* **69** (1972) 3464–3468.
8. Bell P. A. and Munck A.: *Biochem. J.* **136** (1973) 97–107.
9. Jensen E. V., Suzuki T., Numata M., Smith S. and DeSombre E. R.: *Steroids* **13** (1969) 417–427.
10. Gorski J., Sarff M. and Clark J.: *Adv. Biosc.* **7** (1971) 5–20.
11. Cidlowski J. A. and Muldoon T. G.: *Endocrinology* **95** (1974) 1621–1629.
12. Liao S., Liang T. and Tymoczo J. L.: *J. steroid Biochem.* **3** (1972) 401–418.
13. Middlebrook J. L., Wong M. D., Ishii D. N. and Aronow L.: *Biochemistry* **14** (1975) 180–186.
14. Munck A. and Brinck-Johnsen T.: *J. biol. Chem.* **243** (1968) 5556–5565.
15. Munck A. and Wira C.: In: *Methods in Enzymology 36, Hormone Action, Part A, Steroid Hormones* (Edited by B. W. O'Malley and J. G. Hardman). Academic Press, New York (1975) pp. 255–264.
16. Anderson J., Clark J. H. and Peck E. J. Jr.: *Biochem. J.* **126** (1972) 561–567.
17. Munck A. and Leung K.: In: *Receptors and Mechanism of Action of Steroid Hormones* (Edited by J. R. Pasqualini). Marcel Dekker, Inc., New York (1976) (in press).

DISCUSSION

O'Malley. How about explaining replenishment in relation to steroid binding affinity for the receptor.

Munck. Basically that is what we are beginning to do with cortisol and dexamethasone. Their affinities, which largely reflect their dissociation rates, differ by a factor of about 5. They, in turn, seem to be closely related to the rates of replenishment.

Jungblut. Did you do any inhibitor experiments puromycin, actinomycin etc. etc.

Munck. No, that's next on the programme.

Siiteri. Have you done any experiments in which you have used other than hypotonic buffers for preparing the cytosol and the nuclei?

Munck. No.

Siiteri. The reason I ask is because we've been working recently with guinea pig uteri and have found that if we use a standard sucrose–calcium medium for disrupting the tissue, as a good biochemist would who is interested in preserving subcellular structure, we find very little cytoplasmic binding and we find to our great amazement, that it is almost impossible to extract nuclear receptors with KCl solutions. This suggests to me that perhaps what we are calling cytoplasmic receptors may in fact be artifacts.

Munck. Well, I am certainly in sympathy with your last point. It could be that the hypertonic shock does in fact loosen up nuclear receptors that don't have any business being in the cytosol.

Jungblut. Were the guinea pigs primed with estradiol or with other steroids, Dr. Siiteri?

Siiteri. These are *in vivo* experiments in which we have injected the labelled hormone and then looked at different ways of getting back receptors.

Birmingham. What kind of curve do you get with corticosterone?

Munck. We have not tried it, because corticosterone is very messy. It gives a lot of nonspecific binding, and that becomes especially troublesome in replenishment experiments where we start with almost saturating concentrations of steroids.