Glucocorticoid-Receptor Complexes in Rat Thymus Cells

RAPID KINETIC BEHAVIOR AND A CYCLIC MODEL*

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We have studied the kinetics, on time scales of minutes and seconds, of formation and interconversion of glucocorticoid-receptor complexes in rat thymus cells under physiological conditions. Nonactivated and activated complexes were measured by a minicolumn technique that permits rapid, multiple simultaneous assays. The rate-limiting step in formation of nuclear complexes was activation, which at 37 °C had a half-time of 30–60 s. Activation in cells at 25 °C followed first order kinetics. Nuclear binding at 37 °C was too fast to measure, and probably has a half-time below 10 s.

Earlier findings suggesting that triamcinolone acetonide and dexamethasone give higher steady state ratios of activated to nonactivated complexes than cortisol and corticosterone have been supported by showing that these ratios are concentration-independent, and are unlikely to be due to degradation or dissociation of complexes after cell disruption.

A simple cyclic model of receptor kinetics, in which each glucocorticoid is characterized by its dissociation rate constant, accounts quantitatively for these results and many others. The model is based on the assumption that activation is irreversible, and that energy is required for regenerating functional receptors after each cycle. It yields steady state ratios of activated to nonactivated complexes in agreement with experiment without introducing steroid-specific allosteric influences on activation, and suggests a new mechanism for explaining agonist-antagonist relationships.

Glucocorticoids and other steroid hormones appear to initiate their actions in a cell by interacting with free receptors to form complexes that are rapidly bound to the nucleus. Nuclear binding or "translocation" is thought to be preceded by "activation," a reaction which converts the initially formed nonactivated complexes, HR, to activated complexes, HR', the latter characterized by higher affinity for nuclei and DNA.

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§ The abbreviations and trivial names used are: H, free glucocorticoid; R, free receptor; HR, HR', and HR'' nonactivated, activated, and nuclear-bound glucocorticoid-receptor complexes, respectively; TA, triamcinolone acetonide, 9a-fluoro-11b,16a,17,21-tetrahydroxy-

Firm evidence for the physiological relevance of this reaction, however, has been lacking until recently; indeed, since almost all studies on activation (reviewed by Milgrom, 1981; Grody et al., 1982; Schmidt and Litwack, 1982) have been carried out with cell-free systems, it has often been suggested that activation is an in vitro artifact (cf. Grody et al., 1982).

We have shown that when a glucocorticoid such as dexamethasone is added to rat thymus cells incubated at 37 °C, it initially binds to receptors to form measurable amounts of HR, which are then rapidly converted to HR' (Munck and Foley, 1979, 1980). Furthermore, Markovic and Litwack (1980) have demonstrated that nonactivated glucocorticoid-receptor complexes are present in whole animals, and Schmidt et al. (1980) have found a glucocorticoid-resistant mutant of CEM 7 human leukemia cells that is activation defective. Consequently, the physiological role of activation in glucocorticoid action can now be regarded as established.

HR formed in rat thymus cells have an apparent molecular weight of about 330,000 when analyzed in the presence of molybdate, and are probably oligomers; activation to HR' is accompanied by a decrease to around 100,000, presumably due to dissociation into subunits (Holbrook et al., 1983). Raaka and Samuels (1983) working with GH rat pituitary cells, and Vedeckis (1983) with AT-20 mouse pituitary cells, have observed a similar relation between HR and HR'; these workers and Sherman et al. (1983) have proposed models in which HR is a homologous or heterologous tetramer and HR' a monomer.

In cells at 37 °C, the formation of hormone-receptor complexes and binding of the complexes to nuclei take place rapidly, on time scales of seconds or minutes. Time courses of these initial events of steroid hormone action can provide kinetic parameters important for understanding receptor function, but have seldom been measured because of the technical difficulties they present.

Our earlier studies with thymus cells showed that the overall process of activation and nuclear binding at 37 °C has a half-time of approximately 30 s. Similar rates were obtained with cortisol (Wira and Munck, 1974) and with the powerful synthetic glucocorticoids dexamethasone (Cidlowi and Munck, 1980). Those observations seemed consistent with the fact that under steady state conditions at 37 °C we found no

pregna-1,4-diene-3,20-dione-16,17-acetonide; dexamethasone; 9a-fluoro-16c-methyl-11b,17,21-trihydroxypregna-1,4-diene-3,20-dione; corticosterone, 11b,21-dihydroxyprogesterone-4-ene-3,20-dione; TX, Triton X-100; octylphenoxypolyethoxyethanol; TDM buffer, buffer containing 10 mM sodium Tris, 0.5 mM dithiothreitol, and 10 mM sodium molybdate, pH 7.8 at 3 °C; TES, N-triis(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; KRBg, Krebs-Ringer bicarbonate buffer supplemented with 10 mM glucose; KRBg-HEPES, KRBg prepared with only 0.3% NaHCO3 and supplemented with 25 mM HEPES, pH 7.55 at 25 °C.
major differences in the relative amounts of total nuclear (HR') and total cytosolic (HR + HR') complexes generated by these two steroids, although differences have been observed after extraction of nuclei (Cidlowski and Munck, 1978, 1980) or preparation of nuclei with Triton X-100 (Svec and Harrison, 1979). Under steady state conditions, however, both dexamethasone and TA, another synthetic glucocorticoid, formed three to four times more HR' (in proportion to total cytosolic complexes) than cortisol or corticosterone (Munck and Foley, 1980). These preliminary results, since confirmed by Miyabe and Harrison (1983) with AtT-20 cells, were recognized to be subject to technical limitations that we discuss below, and that are minimized by the new methods used in the present work.

In the course of our studies on physicochemical characterization of HR and HR' in rat thymus cells, we developed a minicolumn procedure by which these complexes can be separated within minutes (Holbrook et al., 1983). This method, coupled with the use of molybdate as a stabilizing agent (Sando et al., 1979; Sherman et al., 1982), largely overcomes the difficulties due to spontaneous activation, dissociation, and degradation of complexes in the full column procedures used earlier, and can be conveniently applied to many samples simultaneously. Here we have employed the minicolumns, along with previously developed techniques for preparing nuclei and cytosols rapidly from thymus cell suspensions (Wira and Munck, 1970, 1974; Munck and Wira, 1975), to follow the kinetic interplay of HR, HR', and HR'.

Our results, which include substantiation of the relationship mentioned above of HR' to HR for different steroids, can be interpreted quantitatively in terms of a simple cyclic (irreversible) model of steroid hormone-receptor complexes. This model requires few assumptions, and accounts for many results besides those reported here, including some that have been taken to support reversible or "equilibrium" models.

**EXPERIMENTAL PROCEDURES**

**Materials**

[6,7-3H]TA (37 Ci/mmol), [6,7-3H]dexamethasone (50 Ci/mmol), [1,2-3H]cortisone (50 Ci/mmol), and [1,2-3H]corticotestosterone (40 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Unlabeled steroids were from Steraloids, Wilton, NH. Triton X-100 was from Sigma, St. Louis, MO. Materials used for columns were obtained from sources given previously (Holbrook et al., 1983).

**Methods**

**General Procedures**—These have been described in detail elsewhere (Holbrook et al., 1983; Munck, 1988; Munck and Wira, 1975), and specific methods for each experiment are given in figure and table legends. Briefly, thymus cells from adrenalectomized rats were prepared in suspensions with cymcitrits of 0.3–0.4 m of packed cells/ml of suspension, and incubated in KRBg or KRBg-HEPES with labeled steroids, which were purchased from New England Nuclear, Boston, MA. Unlabeled steroids were from Steraloids, Wilton, NH. Triton X-100 was from Sigma, St. Louis, MO. Materials used for columns were obtained from sources given previously (Holbrook et al., 1983).

**Cytosolic Complexes**—These complexes were obtained by adding aliquots of cell suspension to 1.5-ml Eppendorf centrifuge tubes at 0 °C containing 5 volumes (occasionally 10 volumes for special purposes) of 1.5 mM MgCl₂ with dextran-coated charcoal (Munck and Wira, 1975) and 20 mM sodium molybdate (Holbrook et al., 1983), then mixing vigorously to lyse the cells rapidly and adsorb free steroid. Molybdate was omitted when the cytosols were used to study cell-free activation. Low speed cytosols, obtained by centrifugation 7–15 min later at 11,000 × g for 2.5 min at 3 °C, were then placed on minicolumns or treated in other ways as required. To measure total cytosolic complexes, an aliquot of the cytosol was assayed directly for radioactivity.

**Nuclear Complexes**—Nuclear pellets for assaying HR' were prepared by modifications of earlier procedures (Munck and Wira, 1975). Unless otherwise indicated in the figure or table legends, 20-µl aliquots of a cell suspension were added to 1 ml of 0.8% (v/v) bovine serum albumin, 0.1 M MgCl₂ at 0 °C in 1.5-ml Eppendorf centrifuge tubes, mixed vigorously, and centrifuged at 11,000 × g as above. After aspiration of the supernatant, the tips of the tubes were cut off above the pellets and placed in 4 ml of scintillation fluid with vigorous mixing for assay of radioactivity.

**Minicolumn Procedure**—This method was applied to measure HR, HR', and mero-receptor complexes (Sherman et al., 1978) exactly as described by Holbrook et al. (1983). Three small columns are connected in series, and 0.1 ml of cytosol is passed rapidly through all three with TDM buffer. The top column, of DNA-cellulose, retains HR'; the middle column, of DEAE-cellulose, retains HR; and the lower column, of hydroxyapatite, retains mero-receptor and any other complexes not retained by the first two columns. The three column beds are then placed in scintillation vials for counting radioactivity. Although the mero-receptor complex was detected in all experiments reported here, the amounts found were generally small and varied little, so for most results these values are omitted. The correction for nonsaturable binding, assayed with cytosol from incubations with excess unlabeled steroid, was usually negligible and always less than 10% of total binding to each column. A source of error noted by Milgron (1981) in the use of DNA-cellulose and other polyanions for assay of HR' arises from spontaneous inactivation of HR' on contact with the polyanions. This error is effectively eliminated in the minicolumn assay by the brevity of the exposure of cytosol to DNA-cellulose (less than 5 min), and by the presence of 20 mM molybdate, which blocks activation.

**Comparison of the Efficiency of DNA-cellulose with Nuclei for Assay of HR'**—The efficiency with which the DNA-cellulose and DEAE-cellulose columns retain HR' and HR, respectively, is above 90% as determined by repeated passage of a cytosol through the same type of column (Holbrook et al., 1983). Since it has been found by some workers (Milgron, 1981) that HR' binds to nuclear pellets more efficiently than to DNA-cellulose, we have compared binding of HR' to nuclei with binding to the DNA-cellulose columns used in the minicolumn procedure. A cytosol containing HR' was obtained by incubating thymus cells with 26 nM [3H]TA at 37 °C for 30 min. Aliquots (0.1 and 0.3 ml, respectively) were used for parallel minicolumn and nuclear binding assays. The nuclear binding assay was performed essentially as described by Cidlowski and Munck (1978), except that the nuclear pellets were about 2.5 times larger to ensure excess binding capacity. After correction for nonsaturable binding (less than 7%) the radioactivity retained was 9600 cpm/ml of cytosol with DNA-cellulose and 5200 cpm/ml of cytosol with nuclei, showing that by our methods the efficiency of binding to DNA-cellulose is greater than to nuclei. The HR' remaining unbound in the cytosol after exposure to nuclei (4900 cpm/ml of cytosol by DNA-cellulose assay) did not represent a separate population of complexes with low affinity for nuclei, since 3100 cpm/ml of cytosol were bound when exposed to fresh nuclear pellets.

**Radioactivity Measurement**—Radioactivity was measured with a Packard 3390 liquid scintillation counter, using 4 ml of Hydrolfluor scintillation fluid (National Diagnostics) in plastic minivials at about 44% efficiency for tritium. All minicolumn samples were corrected for quench by dividing the counts/min by 0.8 as determined in preliminary experiments.

**RESULTS AND DISCUSSION**

**Time Course of Formation of HR, HR', and HR'**—Fig. 1 shows that, when TA is added to cells at 37 °C, rapid formation of HR is followed by increases in HR' and HR' after a delay of 0.5–1 min. The initial sequence HR → HR' → HR', which is in accord with all our results, is assumed throughout this discussion on the basis of the evidence cited above. The fact that values of HR' even at the earliest times are slightly above zero in this experiment does not indicate that HR' is formed before HR'; this is not a consistent observation, and may be due to imprecise compensation for nonsaturable binding, which is relatively larger and more variable for HR' than for the other complexes.) Levels of all
Glucocorticoid-Receptor Kinetics and a Cyclic Model

Fig. 1. Time course of formation by TA of HR, HR', and HR'n in rat thymus cells at 37 °C. [3H]TA at 10 times final concentration in KRBg-HEPES at 37 °C was added at time 0 to a thymus cell suspension in KRBg-HEPES at 37 °C, to give a final concentration of about 15 nM. At the indicated times, aliquots of cell suspension were removed for assay of HR, HR', and HR'n as described under "Methods." Nonsaturable binding, determined from a parallel incubation with 1 μM TA, gave corrections (independent of time) of 1280 cpm for HR'n and of less than 2% for HR and HR'.○, HR; □, HR'; ■, HR'n.

The three complexes reach a steady state by 30 min. The results for HR and HR' in Fig. 1 agree with earlier results (Munck and Foley, 1979, 1980) obtained by full column methods with dexamethasone, but yield much more information. The earlier studies were severely limited because each time point required a separate experiment.

We have previously seen a delay at 37 °C in the appearance of HR'n compared to total cytosolic complexes (Munck and Foley, 1976). With the results in Fig. 1, we can ascribe most of this delay to the time required for generation of HR', the presumed intermediate between HR and HR'n, rather than to the time required for binding of HR' to the nucleus; while formation of HR' clearly lags behind HR, no obvious delay is detectable in the formation of HR'n from HR'.

Temperature Jump Experiments—Activation can be separated kinetically from the formation of HR by means of a "temperature jump" experiment such as used previously to measure the combined rate of activation and nuclear binding (Wira and Munck, 1974). In that procedure, a cell suspension which had been incubated for several hours at 3 °C with labeled glucocorticoid to form HR was warmed rapidly to a given temperature by dilution with 10 volumes of warmed buffer, and then cooled rapidly by a further 50-fold dilution with cold 1.5 mM MgCl₂, which lysed the cells and yielded nuclei.

For present purposes, we required both nuclei (to assay HR'n) and cytosols (to assay HR and HR'), so that large dilutions had to be avoided. The procedure described in Fig. 2 was a compromise designed to match exactly the heating schedule for the separate aliquots of cell suspension used for HR'n and for minicolumn analysis. Since warming was not instantaneous, the time courses in Fig. 2 display initial lags of 10-20 s. Despite these limitations, the time courses for HR'n are in good agreement with those measured previously with cortisol (Wira and Munck, 1974), reaching maximum levels after about 1 min at 37 °C and 2 min at 25 °C. The initial values of HR' and HR'n, both above zero, result from slow activation and nuclear binding during the incubation at 0 °C.

Fig. 2. Temperature jump experiments with TA at: a, 37 °C; b, 25 °C; c, 15 °C. Suspensions of rat thymus cells were incubated in KRBg-HEPES at 0 °C for 2.5-3.5 h with 25-56 nM [3H]TA to form HR. The experiment for each temperature was with a different suspension. For each time point, 40-μl aliquots of the suspension were added to two 1.5-ml Eppendorf centrifuge tubes warmed to the appropriate temperature in a water bath. For the 0-s time points, the aliquots were added to tubes at 0 °C. At the indicated times, 400 μl of 1.5 mM MgCl₂ at 0 °C was added to one tube, and 400 μl of 1.5 mM MgCl₂ with dextran-coated charcoal and 20 mM molybdate (Holbrook et al., 1983) was added to the other. The tubes were mixed vigorously and immediately placed in an ice bath. To remove as much nonsaturably bound steroid from the nuclei as possible, an
Our principal goal with the temperature jump experiments was to establish limits on the rate of nuclear binding. A measurable rate of nuclear binding would appear as a delay in the time course of HR' compared to HR. No such delay is discernible at any temperature. We conclude that nuclear binding must be faster than activation. How much faster is a question we take up later.

**Steady State Levels of HR' and HR Formed by Various Glucocorticoids**—Preliminary results obtained by DNA-cellulose chromatography with salt gradient elution of cytosols from cells incubated at 37 °C for 30 min with various steroids, showed that the proportion of HR' formed was several times higher with TA and dexamethasone than with cortisol, corticosterone, and cortexolone (Munck and Foley, 1980). The findings were interpreted with caution because the chromatographic procedures used were lengthy and performed without molybdate, so that degradation, dissociation, and spontaneous activation of complexes after cell disruption could have influenced the outcome. We have since obtained the same results by these procedures using buffer that included molybdate, as have Miyabe and Harrison (1983) with AtT-20 cells. Although molybdate blocks spontaneous activation, dissociation and degradation, it will have limited effectiveness. With these reservations, which are dealt with in experiments described below, the results were important in revealing that cytosolic complexes of glucocorticoid receptors with different steroids behaved differently in the cell.

Precedents for an influence of steroid structure on more than just the equilibrium binding constant already existed from work on biological activity of steroids. In early studies (Munck and Brinck-Johnsen, 1968) we noticed that when compared to cortisol, certain synthetic glucocorticoids had relative biological activities that exceeded their relative affinities for receptors. We inferred that glucocorticoids such as dexamethasone had greater "intrinsic activity" or "efficacy" than cortisol. In the same studies, cortexolone behaved as a glucocorticoid antagonist, i.e. had lower efficacy. From observations on enzyme induction in hepatoma cells with a large group of steroids, Samuels and Tomkins (1970) proposed that the glucocorticoid receptor is an allosteric protein with two states, as in the model formulated by Rubin and Changeux (1966). According to this model, steroids would behave as inducers or anti-inducers in relation to their ability to form complexes with one state or the other. Rousseau et al. (1973) postulated that the state of the receptor that led to enzyme induction, i.e. the one with highest affinity for inducers, formed complexes capable of activation and binding to the nucleus, whereas the other state formed complexes incapable of activation.

To establish whether our preliminary results (Munck and Foley, 1980) reflected the levels of complexes formed by various steroids in the cell, we used minicolumns to determine what happens to HR and HR' in cytosols after the cells are broken. The results in Fig. 3 show that, with all four glucocorticoids tested, the relative distribution of HR and HR' in these molybdate-containing cytosols remains fairly constant from 11 min up to at least 1 h after cell lysis. Dissociation of cortisol and corticosterone, probably accompanied by degradation of the receptor, was quite rapid, but the rates for HR and HR' did not differ sufficiently to alter their proportions. As we have noted before (Holbrook et al., 1983), the slow increase for the TA curve of HR in Fig. 3c, which strictly speaking should be labeled "DEAE-cellulose binding" rather than "HR," does not indicate reversal of activation since the complexes that accumulate in this fraction cannot be reactivated and are probably proteolytic degradation products of HR'. The standard minicolumn assay for HR and HR' in cells uses only the first (11-min) time point.

Several conclusions can be drawn from Fig. 3. (i) Analysis by the rapid minicolumn technique reveals very different values of [HR']/[HR] for different steroids, ranging from about 4 for TA to 0.3 for corticosterone. (ii) Those differences are present from the earliest measurements and therefore seem to have been established in the intact cells; they cannot be accounted for by any differences in rates of dissociation or degradation of HR and HR' detectable in the cytosols after 11 min from the time cells are broken.

Although not entirely comparable to our preliminary results, because there we measured the equivalent of HR' (labeled Complex Ib) but not the direct equivalent of HR, the quantitative relationships in Fig. 3 agree well with those results since the two synthetic glucocorticoids, TA and dexamethasone, exhibit much higher levels of HR' relative to HR than the natural glucocorticoids. This relation has remained consistent throughout our experiments, despite quite wide fluctuations from one cell preparation to another in [HR']/[HR] for the same steroid. For example with TA, steady state values at 37 °C of [HR']/[HR] have varied from below 2 up to 5, and with cortisol from 0.5 to 1.5.

Fig. 4 compares the formation in the cell of HR' and HR with TA and cortisol after various times of incubation at 37 °C. These time courses do not include the initial 5-min period detailed in Fig. 1, but with both steroids the drop from an early high value of HR is evident. As shown most clearly in the lower parts of Fig. 4, the values of [HR']/[HR] are nearly constant by 15 min. In other experiments, we have found that dexamethasone and corticosterone similarly reach constant values by 15 min.

An important question is whether [HR']/[HR] depends on steroid concentration. It is a well established fact (cf. Svec and Harrison, 1979; Bloom et al., 1980), which we have verified with thymus cells, that the ratio of nuclear to total cytosolic complexes is independent of the concentration of steroid. The data in Table I show that the same conclusion applies to steady state values of [HR']/[HR], since over wide concentration ranges of TA and cortisol the ratios change insignificantly.

Taken together, the results of this section demonstrate that different glucocorticoids generate different steady state values of [HR']/[HR] in the cell. If one makes the reasonable assumption that the magnitude of the biological effect of a steroid is proportional to the concentration of HR', and that HR' is proportional to HR', then the relative values of [HR']/[HR] for TA, dexamethasone, cortisol, and corticosterone vary in a way that would account roughly for the differences mentioned above between the biological efficacies of these glucocorticoids.

Our results do not find a ready explanation in the allosteric model of Samuels and Tomkins (1970) as elaborated by Rousseau et al. (1973). That model is based on equilibrium between two states, only one of which can be activated. In all, three complexes are postulated: the one that can undergo activation (corresponding to HR), its activated form (corresponding to HR'), and the one that cannot be activated. We find no...
FIG. 3. Changes with time after cell lysis of HR and HR' from thymus cells incubated with various glucocorticoids at 37 °C. Suspensions of rat thymus cells in KRBg were incubated for 30 min at 37 °C with: a, 65 nM [3H]cortisol; b, 39 nM [3H]corticosterone; c, 10 nM [3H]TA; d, 15 nM [3H]dexamethasone. Incubations a and b were with the same cell suspension, as were c and d. Parallel incubation with 1 μM TA were run to determine nonsaturable binding. At time 0, cells were broken to obtain cytosols as described under “Methods.” The cytosols were left at 0 °C. After the indicated times, aliquots of cytosol were removed and placed on minicolumns for analysis. The first time point in each case was at 11 min. All values were corrected for nonsaturable binding, which was negligible for TA and dexamethasone, about 25% of each value for cortisol, and 10% for corticosterone.

FIG. 4. Comparison of formation of HR and HR' in thymus cells by TA and cortisol at 37 °C. A suspension of thymus cells in KRBg was incubated at 37 °C with a, 23 nM [3H]TA, and b, 80 nM [3H]cortisol. Parallel incubations with 1 μM TA were run to determine nonsaturable binding. At the times indicated after addition of steroid to cells, aliquots were taken and cytosols prepared as described under “Methods” for minicolumn analysis of HR, HR', and mero-receptor complex. The results, corrected for nonsaturable binding (negligible for [3H]TA and about 20% of each value for [3H]cortisol), are given in counts/min in a and b, and as percentage of total receptor-bound counts/min at each time point in c and d. O, HR; ●, HR'; x, mero-receptor complex.
In Table I we compare nuclear and cytosolic binding with cortisol and TA, using nuclei obtained by lysing cells in our standard medium (no TX) and in a medium with Triton X-100 (TX). The main conclusions from these results are: (i) nuclear to cytosolic ratios measured by our methods (no TX) are not significantly higher with TA than with cortisol. (ii) Triton X-100 reduces by 45-75% the nuclear-bound cortisol, but has no significant effect on nuclear-bound TA, so it reduces the nuclear to cytosolic ratio for cortisol but not for TA. The variation in Table II in nuclear to cytosolic ratios (no TX) from one experiment to another is typical of our experience over the years with different groups of rats and from season to season.

We have not investigated further how Triton X-100 reduces nuclear-bound cortisol. It may do so by dissociating the relatively weakly bound cortisol from the receptor, by extracting the whole complex or removing it from the outer nuclear membrane. Nuclear-bound cortisol complexes are known to be more susceptible than TA complexes to extraction with KCl solutions and DNase (Cidlowski and Munck, 1978, 1980). We have avoided using Triton X-100 or other extractants routinely for preparing nuclei, since the fraction of receptor-bound steroid that is extracted would then appear in neither the nuclear nor the cytosolic assay.

**Comparison between Rates of Activation in Cells and in a Cell-free System**—With the minicolumn procedure, we have compared the rates of disappearance of HR in cells and in a cell-free system after warming to 25 °C. These rates provide the most direct comparison of rates of activation between two such systems, since in the cells HR is converted to HR'. Because activation is strongly influenced by ions, we used approximately the intracellular concentrations of NaCl and KCl in the cell-free system.

Fig. 5 shows that the rates of disappearance of HR in the two systems are indistinguishable. Within experimental error, both sets of points for HR fit a first order decay curve. To some extent these results have been foreshadowed by those of Atger and Milgrom (1976), who showed that in a cell-free system, thermal activation of glucocorticoid-receptor complexes follows first order kinetics. Although the cell-free conditions used for the results in Fig. 5 are not unusual, many factors influence cell-free activation (Milgrom, 1981; Schmidt and Litwack, 1982), so it is possible that under other conditions the coincidence of rates seen in Fig. 5 would not have been observed.

**Cyclic Model of Receptor Kinetics**—Two types of model have been proposed to account for the behavior of steroid hormone receptors in cells: reversible or "equilibrium" models, such as that of Samuels and Tomkins (1970) discussed above, and irreversible, energy-dependent, or "cyclic" models (Munck et al., 1972). Conclusive experimental support for one or the other is still lacking. The most recent version of the equilibrium model has been put forward by Raaka and Samuels (1983) to account for their observations on glucocorticoid receptors in GH1 cells. They suggest that some of their results,
Glucocorticoid-Receptor Kinetics and a Cyclic Model

TABLE II

Comparison of nuclear receptor-bound complexes assayed with and without Triton X-100 with cytosolic complexes formed in thymus cells by TA and cortisol at 37 °C

Data are from four experiments, each run with both steroids simultaneously. Rat thymus cell suspensions were incubated in KRBg for 60 min at 37 °C with

| Experiment | Steroid | Nuclear Receptor-bound | Cytosolic | Nuclear/Cytosolic |
|------------|---------|------------------------|-----------|------------------|
|            |         | No TX | TX | No TX | TX |
| 1          | Cortisol | 1561 ± 166 | 931 ± 59 | 1.68 |
|            | TA      | 2395 ± 82 | 1309 ± 127 | 1.83 |
| 2          | Cortisol | 1573 ± 90 | 853 ± 68 | 1.07 |
|            | TA      | 3491 ± 43 | 4825 ± 255 | 1.50 |
| 3          | Cortisol | 1283 ± 114 | 513 ± 19 | 1.10 |
|            | TA      | 2110 ± 61 | 3080 ± 392 | 1.50 |
| 4          | Cortisol | 783 ± 76 | 202 ± 57 | 0.87 |
|            | TA      | 2041 ± 308 | 1763 ± 58 | 1.28 |

in particular, those indicating that the amount of nuclear complex at 37 °C for various hormone concentrations is proportional to the total amount of complex formed, would be difficult to explain with an irreversible model.

We originally proposed a cyclic model (Munck et al., 1972) for glucocorticoid-receptor complexes to account for the observed requirement for metabolic energy to maintain the binding capacity of receptors in thymus cells (Munck and Brinck-Johnsen, 1968). Cyclic phosphorylation and dephosphorylation of the receptors were suggested as a possible mechanism. Reutilization of receptor protein was indicated by observations that, even in the presence of cycloheximide, receptor levels were maintained and could manifest reversible energy dependence (Munck et al., 1972; Bell and Munck, 1973). Evidence for such a cyclic model, with glucocorticoid and other steroid hormone receptors, has come in recent years from a number of investigations (e.g., Schmidt and Litwack, 1982; Grody et al., 1982) and others, many inspired by the elegant studies of Pratt and colleagues on the role of phosphorylation in the binding activity of glucocorticoid-receptors (Wheeler et al., 1981; Housey and Pratt, 1983; Housley et al., 1983).

To our knowledge, no attempt has ever been made to test against experiment a completely defined model of steroid hormone receptor kinetics in the cell. That is precisely the purpose with the cyclic model shown in Fig. 6. It includes only those forms of the receptor that are known to be present in the cell, and that can be measured independently. These forms are linked through reactions which all have some experimental basis.

The simple formulation of the initial reversible reaction, H + R ⇔ HR, appears adequate for representing kinetic data (Yeakley et al., 1980). The next reaction, HR → HR', embodies the postulate that activation is irreversible (e.g., Housley et al., 1983). There is no direct evidence concerning reversibility of activation in intact cells. With cell-free systems, reversibility has been inferred from experiments in which pH and ionic strength were manipulated (e.g., Milgrom, 1981). We do not find this evidence compelling as applied to intact cells, particularly since the experiments were done under conditions that are now known to lead to rapid degradation of receptors (Sherman et al., 1982). In agreement with Milgrom (1981) and with our results in Fig. 5, activation is taken to be a first order reaction. Neither the mathematical analysis of the model given in the Appendix nor the predictions of the model in Fig. 8 depend on the detailed molecular mechanism of the activation reaction. They are compatible, for example, with dissociation of a tetramer, HR, through a mechanism favored by several groups (see above), and with any other mechanisms that are consistent with the empirical observation that the reaction appears to be first order.

For reasons similar to those detailed by Raaka and Samuels (1983) and Miyabe and Harrison (1983), we assume that nuclear binding, HR' ⇔ HR'n, is reversible and rapid, so that HR' and HR'n can be regarded as similar complexes in two different locations. Biological activity is presumably generated by a small subpopulation of HR'n (Payvar et al., 1981; Pfahl, 1982), that may be more tightly and specifically bound than the general population.

Finally, we assume that the receptor is reutilized, and that it is degraded and synthesized at rates that are negligible compared to those of the kinetic phenomena studied. These assumptions are based on our own results with cycloheximide mentioned above, on the studies of Housey et al. (1983), and particularly on the experiments of Raaka and Samuels (1983) with receptors synthesized with heavy amino acids. The unliganded receptor R must therefore be regenerated from HR'

FIG. 6. Cyclic model of receptor kinetics. Hormone, H, is assumed to react reversibly with free receptor, R, to form a nonactivated complex, HR, that through an irreversible reaction produces the activated complex HR'. HR' rapidly equilibrates with the nuclear-bound complex, HR'n. HR' and HR'n both regenerate R through irreversible reactions.
and HR'n. These processes are shown in Fig. 6 by dashed arrows. They really constitute only one reaction, because of the similar properties assumed for HR' and HR'n. The dashes emphasize that the mechanism and location of this reaction are unknown, and that it may consist of several steps. It corresponds to the ATP-dependent reaction in the original cyclic model (Munck et al., 1972). Although the reaction may be complex, we assume that the rate-limiting step involves dissociation of H and represent it kinetically by the first order rate constant \( k_{11} \), which is the rate constant for dissociation of H from HR.

Our choice of a single rate constant, \( k_{11} \), for these reactions is probably the most radical assumption in the model. We justify it on several grounds. First, dissociation rates at 37 °C of cortisol from extracted nuclear complexes (Wira and Munck, 1970) and of cortisol and TA from complexes formed under cell-free conditions (Bell and Munck, 1973) are similar to the dissociation rates from whole cells. Second, the rates of regeneration of free receptor, R, after removal of hormone from cells incubated at 37 °C with saturating concentrations of cortisol and dexamethasone, appear to be of comparable magnitude to the dissociation rates of the hormones from the nuclei and from the whole cells (Munck and Foley, 1976). Third, use of \( k_{11} \) instead of an arbitrary constant for the regeneration reactions simplifies the model, and makes it more testable by limiting the number of constants that can be adjusted to fit new data.

Numerical values of rate constants used to calculate the kinetic and steady state behavior of the model with TA and cortisol are given in Fig. 8. All the rate constants except \( k_1 \) are first order, with units of min⁻¹; \( k_1 \) appears only in the product \( k_1[H] \), which has the same units. Earlier studies (Munck et al., 1972; Bell and Munck, 1973) indicated that differences in affinity between steroids such as TA, dexamethasone, and cortisol were due mainly to differences in their rates of dissociation. Therefore the only constant used in the model to characterize a particular steroid is \( k_{11} \). For TA, \( k_{11} \) was determined from results on dissociation of TA from receptors in thymus cells at 37 °C (Bell and Munck, 1973); for cortisol, \( k_{11} \) was determined from analogous results (Munck and Brinck-Johnsen, 1967, 1968). The appropriateness of these values will be illustrated in Fig. 8. All other constants are common to both steroids. The value for the association rate constant, \( k_3 \), is rounded off from that calculated for binding of cortisol to receptors in thymus cells (Munck and Brinck-Johnsen, 1968). The activation rate constant, \( k_2 \), was determined from results of a temperature jump experiment at 37 °C with cortisol (Wira and Munck, 1974).

Finally, the ratio \( k_3/k_{11} \), which largely determines the steady state value of the ratio \([HR'n]/[HR']\) (see Appendix), was calculated from the value of this ratio in Fig. 1. Absolute values of \( k_3 \) and \( k_{11} \) were selected to make nuclear binding faster than activation, as indicated by our temperature jump experiments above. We have set \( k_3 = 10k_{11} \), but any value between 5\( k_{11} \) and infinity (with a corresponding change in \( k_{11} \)) would make almost no perceptible changes in the predictions of the model. As shown in the Appendix, regardless of their values, \( k_3 \) and \( k_{11} \) affect only the distribution of complexes between the forms HR' and HR'n, not the total in these two forms, and have no influence on the overall kinetic predictions of the model. Thus, HR' and HR'n can be combined into one population as in Fig. 7. In effect, then, \( k_3 \) and \( k_{11} \) can be replaced by a single equilibrium constant \( K_3 = k_3/k_{11} \). The total number of independent constants in the model is thus 4.

**Kinetic and Steady State Predictions of the Cyclic Model**—

Fig. 8 depicts the predictions of the model in a variety of simulated experiments with TA and cortisol at 37 °C. Results given by the model are shown by the curves. Wherever possible, we have superimposed experimental values. Mathematical procedures for obtaining solutions from the set of differential equations that describe the model are given in the Appendix. Fig. 8a compares the time course of association of TA predicted by the model with the results in Fig. 1. Both qualitatively and quantitatively, the agreement between model and experiment is excellent. It should be emphasized that the only experimental data from Fig. 1 that were used for the model are the 30-min values for HR' and HR'n, from which \( k_3/k_{11} \) was determined. The structure of the model and the other three constants were derived from entirely different experiments. The time course of HR predicted by the model falls slightly below the data from Fig. 1, but lies well within the experimental range normally observed. Similarly, the steady state value of about 4 given by the model for \([HR']/[HR']\) is higher than those for TA in Table I, but slightly lower than those in Figs. 3 and 4.

Fig. 8e shows the time course of association predicted for cortisol. These curves fall within the range of results for HR and HR' given as a function of time in Fig. 4b, and shown after reaching a steady state in Fig. 3a (11-min points) and Table I. They also fall within the range of steady state ratios of nuclear (no TX) to total cytosolic complexes in Table II.

A remarkable consequence of the model is that, although it has no provision for steroid-specific allosteric control of activation, the steady state value of \([HR']/[[HR']]\) it predicts for cortisol is different from that for TA, just as we find experimentally. In fact, the ratios predicted for both steroids agree quantitatively with the results in Fig. 4. With appropriate values of \( k_{11} \), similar agreement can be obtained with the experimentally determined ratios for dexamethasone and corticosterone.

The differences given by the model between \([HR']/[[HR']]\) for TA and cortisol are purely dynamic (i.e. nonequilibrium) consequences of the choice of \( k_{11} \) as the rate constant governing regeneration of R from HR' and HR'n: the smaller this rate constant, the greater the lifespan, and thus the concentrations, of HR' and HR'n in relation to HR (the lifespan of which depends mainly on \( k_3 \)), and consequently the higher the ratio \([HR']/[[HR']]\) (see also Appendix). Potent glucocorticoids such as TA and dexamethasone, with relatively slow rates of dissociation (i.e. small \( k_{11} \)) compared to cortisol and corticosterone, will therefore maintain a larger proportion of glucocorticoid-receptor complexes in the activated forms HR' and HR'n, and thus, presumably, generate greater biological
Fig. 8. Kinetic and steady state behavior of the cyclic model. As outlined in the Appendix, the set of differential equations describing the model in Fig. 6 has been solved for various initial conditions by an iterative procedure to give the time courses shown, and algebraically for steady state values. The ordinates are in arbitrary units of concentration, set to 10 for the maximum value illustrated. Rate constants used for all the calculations are: $k_1 = 0.01 \text{ nM}^{-1} \text{ min}^{-1}$, $k_2 = 1 \text{ min}^{-1}$, $k_3 = 10 \text{ min}^{-1}$, $k_4 = 3.5 \text{ min}^{-1}$. In addition, for TA (a–d), $k_1 = 0.05 \text{ min}^{-1}$, and for cortisol (e–h), $k_3 = 0.34 \text{ min}^{-1}$. Hormone concentration $[H]$ is assumed constant throughout each time course. For association (a, e), $[H] = 15 \text{ nM}$. For dissociation and temperature jump (b, c, f, g), $[H] = 0$. In addition, the dashed line in b represents TA dissociation from whole cells with $[H] = 0.5 \text{ nM}$, following a 50-fold dilution of the cell suspension. (Because a 50-fold dilution gives only about a 30-fold reduction in free hormone concentration (Munck and Brinck-Johnsen, 1968), the concentration used after dilution was 30 times lower, 0.5 nM, than the 15 nM assumed for the initial equilibration.) All values computed for the model are shown as continuous or dashed curves, identified as representing HR, HR', etc. Curves for whole cell are the sum of values for HR, HR', and HR. Superimposed on the curves are data from experiments at 37 °C. These data are taken from the following sources: a, from Fig. 1; b, TA dissociation from rat thymus cells (Bell and Munck, 1973); c, HR' from a temperature jump experiment with dexamethasone (Cidlowski and Munck, 1980); d, steady state TA binding to whole mouse fibroblasts, recalculated from Hackney et al. (1970); e, cortisol dissociation from whole rat thymus cells (Munck and Brinck-Johnsen, 1968); f, cortisol dissociation from whole rat thymus cells (Munck and Brinck-Johnsen, 1967, 1968); g, HR' from a temperature jump experiment with cortisol (Wira and Munck, 1974); h, steady state cortisol binding to whole rat thymus cells (Munck and Brinck-Johnsen, 1968). The maximum value for each set of experimental data has been scaled approximately to the maximum value of the corresponding curve from the model. In a, the scale is based on the 30-min value of HR'.
activity and exhibit higher efficacy than the natural glucocorticoids. Dissociation curves predicted by the model for TA and cortisol are shown in Fig. 8, b and f. In this case, agreement between the model and experiment is by design, since the values of $k_1$ were derived from these data. In Fig. 8b, we include two dissociation curves predicted for whole cells by the model: the solid line is calculated for dissociation after an infinite dilution (i.e. with hormone concentration $[H] = 0$) and the dashed line is calculated for dissociation after a 50-fold dilution, which is how the experimental points were obtained. Comparison of the rates of decay of $HR'$ and HR in Fig. 8, b and f, illustrates the point made above, namely, that the rate for $HR'$ depends on $k_1$ and that for HR depends mainly on $k_2$.

For the temperature jump experiments simulated in Fig. 8, c and g, we have assumed that the initial values of HR' and HR are zero, and have consequently adjusted the experimental values shown for HR' by subtracting the original 0-s values, which were of about the same magnitude as shown in Fig. 2. Since our present experimental result with TA at 37 °C (Fig. 2a) has the temperature artifact due to slow warming mentioned above, we have superimposed on Fig. 8c the results of an earlier temperature jump experiment with dexamethasone. The agreement with the model is not perfect, but well within the variability for this kind of experiment. The rate of appearance of HR' in the model is determined largely by the value of $k_0$, which was chosen to fit the temperature jump data with cortisol in Fig. 8g.

Fig. 8, c and g, displays the consequence of choosing a finite value of $k_0$ ($k_0 = 10k_2$) or, the time course curves for the model. For about the first 10 s, the curve for HR' lies below that for HR. Such a slight delay in the appearance of HR' would be undetectable by the methods used in Fig. 2. From tests of the model with a series of values of $k_0$ (and appropriate values of $k_2$ to keep $[HR']/[HR]$ constant), we have concluded that for the delay to be experimentally detectable $k_0$ would probably have to be less than $5k_2$. Even a value as low as $2k_0$, which gives a pronounced delay in HR' in a temperature jump time course with the model, causes only minor changes in association and dissociation time courses and no change in steady state predictions of the model. With an infinite value for $k_0$, the only difference would be that the curves for HR' and HR would rise from the origin in their final proportions, with no delay in the rise of HR'.

Steady state values for TA and cortisol given by the model are shown in Fig. 8, d and h. In addition to agreeing well with experimental data for steady state binding of the steroids to whole cells, they illustrate a number of points. In the first place, the values are indistinguishable from those that would be generated by an equilibrium model, and yield linear Scatchard plots, a conclusion similar to that reached by Närøy et al. (1980) from an analysis of steady state predictions of a general cyclic model. Because H binds to R with rate constant $k_1$ and dissociates from all species with rate constant $k_3$, the model in Fig. 6 gives an apparent association constant identical with the equilibrium association constant $k_1/k_3$. That is not generally the case with cyclic models, however (Närøy et al., 1980).

Another steady state prediction of the cyclic model is that the ratios $[HR']/[HR]$, as well as the ratios of nuclear to total cytosolic binding, are independent of hormone concentration, in agreement with our results in Table I as well as with the experiments of Svec and Harrison (1979) and Bloom et al. (1980). HR' in relation to total cytosolic binding is somewhat larger for TA than for cortisol. In this respect, the model gives values intermediate between those obtained with and without Triton X-100 (Table II), and with and without partial extraction of nuclei (Ciidlowski and Munck, 1978, 1980); they are reasonably consistent with the results obtained by Svec and Harrison (1979) using Triton X-100. Finally, it is evident that the cyclic model yields HR' proportional to total receptor binding, and thus overcomes the difficulty that Raaka and Samuels (1983) suggested could arise with a model that includes irreversible steps.

**General Discussion**—In terms of number of constants and simplicity of reactions postulated, the model in Fig. 6 (or its equivalent in Fig. 7) is perhaps unique in the parsimony with which it accounts quantitatively for the behavior of glucocorticoid receptors in whole cells. Except in this sense, however, the success of the model in matching experiment does not establish its uniqueness, although it attests strongly to its plausibility. The model allows little flexibility in choice of values for rate constants; changes in constants by a factor of 2 can cause the results to deviate significantly from experimental values. Nevertheless, the data can always be accommodated by more complex models with more adjustable constants. For instance, we already noted that the reactions indicated by dashed arrows may include several steps. Similarly, the activation reaction could consist of combinations of reversible allosteric and irreversible covalent transformations. There is also evidence (Pratt et al., 1975) for greater complexity in the initial reaction of H with R at low temperature than shown in Fig. 6. As long as the overall kinetics of the reactions remain approximately as assumed in Fig. 6, however, inclusion of additional steps and other receptor species will leave the general behavior of the model unaltered.

The remarkable fidelity with which the model simulates the complex kinetics of association of TA in Fig. 8a and produces, in absolute as well as relative magnitude, the observed steady state values of $[HR']/[HR]$ for TA and cortisol, rests in good part on the assumption that activation is irreversible, coupled with the choice of $k_1$, as rate constant for formation of R from $HR'$ and HR'. These conditions are not indispensable, however; the data can be matched fairly well even by equilibrium models, but such models generally require the ad hoc assumption of steroid-specific allosteric control of activation, and the assignment of rate constants for reactions of hypothetical species such as the unoccupied activated receptor $R'$ (cf. Munck et al., 1972). Those complications make equilibrium models difficult to test rigorously.

Beyond accounting for the ratios $[HR']/[HR]$, the cyclic model leads to the conclusion that steroids with high dissociation rate constants $k_3$, should act as glucocorticoid antagonists, because they form low levels of $HR'$ (in relation to HR) and hence of HR'. To some extent this conclusion is already supported by the correlation observed by Raynaud et al. (1980) between steroid hormone antagonism and dissociation rate constants. According to the model, the antagonist properties of such steroids arise because in the cell the complexes $HR'$ and HR' decay too rapidly to accumulate. (The value of $k_1$ does not affect this argument because, as shown in the Appendix, relative steady state concentrations of HR, $HR'$, and HR' are independent of $k_1$, just as they are independent of $[H]$). This mechanism contrasts with that of the allosteric models discussed above, in which antagonism is due to inability of a steroid to form $HR'$. This latter mechanism would of course also give antagonist behavior if it were included in the cyclic model, as presumably, would whatever mechanism operates in the case of the antagonist dexamethasone 21-mesylate, which can form activated covalent steroid-receptor complexes (Simons et al., 1983).

The cyclic model in Fig. 6 can easily be expanded to include...
reactions for degradation and de novo synthesis of receptors. As explained above, we have omitted these reactions because they seem to play no role in the rapid kinetic phenomena that have concerned us here. Were the model to be applied to other steroid hormone receptor systems, such an omission might not be justified.

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APPENDIX

The cyclic kinetic model in Fig. 6 is represented by the following set of equations, in which t is time (minutes), and concentrations of all receptor species are in arbitrary units of hormone binding sites per unit volume:

\[
\begin{align*}
&[\text{Ro}] = [\text{R}] + [\text{HR}] + [\text{HR}'] + [\text{HR}'] \\
&d[\text{HR}] / dt = k_1[\text{HR}][\text{R}] - (k_+ + k_0)[\text{HR}] \\
&d[\text{HR}'] / dt = k_2[\text{HR}] + k_- [\text{HR}'] - (k_+ + k_0)[\text{HR}'] \\
&d[\text{HR}'] / dt = k_3[\text{HR}'] - (k_+ + k_3)[\text{HR}'] \\
&[\text{Ro}], \text{ the total concentration of hormone-binding sites, and [R], the free hormone concentration, are taken to be constants.} \text{ [Ro] acts as a scale factor, since division of Equations 1-4 by [Ro] transforms them into an equivalent set in the variables [R]/[Ro], [HR]/[Ro], [HR']/[Ro], and [HR']/[Ro]. For each of the computed results in Fig. 8, the vertical scale has been set by adjusting [Ro]; in } \alpha, \text{ for example, [Ro] was about 19. Substituting } [\text{R}] \text{ from Equation 1 into Equation 2 gives}
\end{align*}
\]

\[
d[\text{HR}] / dt = k_1[\text{HR}]/[\text{Ro}] - [\text{HR}'] - [\text{HR}'] - [\text{HR}'] \\
- (k_+ + k_0)[\text{HR}] \\
\]

With suitable initial conditions, Equations 3-5 determine the time dependence of [HR], [HR'], and [HR']. Steady state relations, obtained by setting Equations 3-5 equal to zero, are as follows:

\[
\begin{align*}
&[\text{HR}']/[\text{HR}] = k_0/(k_+ + k_0) \\
&[\text{HR}']/[\text{HR}] = (k_2/k_0)(k_+ + k_0)/(k_+ + k_0 + k_0) \\
&(\text{[HR]'} + [\text{HR}'])/[\text{HR}] = k_0/k_1 \\
&[\text{HR}] = k_0/k_1 \\
\end{align*}
\]

Equation 8 follows from Equations 6 and 7. Equations 6-8 show that the ratios of steady state concentrations of complexes are independent of k_0 and k_1. With the numerical values for rate constants in Fig. 8, [HR']/[HR] is approximated by k_0/k_0 = 2.9, and [HR']/[HR] by k_0(k_0/k_0)/(k_0 + k_0) or roughly k_0/k_0 = 1/(k_0). Equation 9 shows that steady state values of [HR], and therefore of [HR'] and [HR'], have the same functional dependence on [H] as they would for equilibrium binding according to the reaction H + R = HR, with apparent association constant k_0.

The time courses illustrated in Fig. 8 were obtained by solving Equations 3-5 simultaneously with a fourth order Runge-Kutta iterative procedure (Hornbeck, 1975), programmed for a Hewlett-Packard Model 41C calculator. The step interval used for integration was 0.1 min. Reduction of the interval to 0.05 min made negligible difference in the results. A closed solution, obtained for the time course of HR in Fig. 5, coincided with the iterative solution to three significant figures. Values chosen for rate constants and for [H] are given in Fig. 8. Initial values used for [HR], [HR'], and [HR'] in

Fig. 8 were: in a and e, zero for all complexes; in b and f, steady state levels of complexes; in c and g, [HR] = 10, [HR'] = [HR'] = 0.

If Equations 3 and 4 are added they give

\[
d[\text{HR}'] + [\text{HR}']/dt = k_1[\text{HR}] - k_0[\text{HR}'] + [\text{HR}'] \\
\]

This equation and Equation 5 can be viewed as having only two dependent variables, [HR] and [HR'] + [HR']. Equations 4 and 5 completely determine the time dependence of these two variables, as well as the steady state values in Equations 8 and 9. They are equivalent to Equations 3-5 except for treating HR' and HR' as one kinetic species, and represent the simplified model in Fig. 7. The conclusion that HR' and HR' in Fig. 6 can be lumped together in this way is unaffected by the choice of k_0 and k_0; it depends only on the fact that the model prescribes the same fate (return to R) with rate constant k_0 for both complexes. As long as HR' and HR' are not differentiated kinetically the model in Fig. 6 can therefore be reduced to that in Fig. 7, where the proportion of HR' to HR' can be set by an equilibrium or distribution constant K_0 = [HR']/[HR'].

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