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Steroid-binding Properties and Stabilization of Cytoplasmic Glucocorticoid Receptors from Rat Thymus Cells

By PHILIP A. BELL* and ALLAN MUNCK
Department of Physiology, Dartmouth Medical School, Hanover, N.H. 03755, U.S.A.

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1. A competitive binding assay was adapted for determination of the specific binding of glucocorticoids to cytoplasmic receptors from rat thymus cells. The steroid–receptor complexes prepared by incubation of a cytoplasmic fraction from rat thymus cells with [1,2-$^3H_2$]cortisol or with [1,2,4-$^3H_3$]triamcinolone acetonide had rates of dissociation at 37°C similar to those from intact cells. 2. The cytoplasmic receptor was unstable at 3°C, but the rate of inactivation was decreased in the presence of 2.5mM-EDTA. The steroid–receptor complex was stable. 3. Rate constants for association and for dissociation, and association constants, were determined for the interactions of cortisol, cortexolone, dexamethasone and triamcinolone acetonide with the cytoplasmic receptor at 3°C. Differences in the association constants for different steroids could largely be accounted for by the differences in the rate constants for dissociation, but the rate constants for association did not vary greatly; the implications of these findings for the nature of the steroid-binding site are discussed. 4. A cytoplasmic fraction prepared from cells which had been incubated at 37°C under anaerobic conditions bound much less [1,2-$^3H_2$]cortisol than did a fraction from aerobic cells, but the binding capacity was restored after exposure of the anaerobic cells to O_2. 5. The specific binding of [1,2-$^3H_2$]-cortisol to intact thymus cells incubated aerobically was not affected by the presence of 0.1mM-cycloheximide, nor did this concentration of cycloheximide inhibit the recovery of specific binding observed when anaerobic cells were transferred to an aerobic atmosphere. 6. The energy dependence of specific binding of cortisol to the receptor is discussed with reference to possible mechanisms.

In previous studies from this laboratory, an obligatory role has been demonstrated for the binding of glucocorticoids to specific receptors in intact rat thymus cells before the effects of active steroids on glucose transport can be initiated (Munck & Brinck-Johnsen, 1967, 1968; Wira & Munck, 1970; Munck & Wira, 1971). It has also been shown that both nuclear and cytoplasmic receptors can be extracted from cells with or without cortisol pretreatment by disruption by hypo-osmotic shock in 1.5mM-$MgCl_2$, and that these receptors are at least in part proteins (Wira & Munck, 1970; Munck & Wira, 1971). The work reported here represents a logical extension of those earlier studies; the physicochemical parameters of the interactions between glucocorticoids and the isolated cytoplasmic receptor have been investigated.

These investigations were aided by the use of an assay for steroid–macromolecular complexes that was based on the well-known principle that dextran-coated charcoal will absorb free, but not bound, steroid (Murphy, 1967; Milgrom & Baulieu, 1969). Studies of this nature are also greatly simplified by the fact that thymus cells do not metabolize cortisol, nor do they reduce cortisone to cortisol, so that cortisone is biologically inactive in isolated thymus cells (Munck & Brinck-Johnsen, 1968; Munck *et al.*, 1972).

A preliminary account of part of this work has appeared (Bell & Munck, 1971), and related studies have also been reported recently (Schaumberg, 1972).

* Present address: Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Milton Road, Cambridge CB4 1XJ, U.K.

Experimental

Materials

[1,2-$^3H_2$]Cortisol (42Ci/mmol), [1,2-$^3H_2$]cortisone (51Ci/mmol) and [1,2-$^3H_2$]cortexolone (17,21-dihydroxy[1,2-$^3H_2$]pregn-4-ene-3,20-dione; 35Ci/mmol) were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). [1,2,4-$^3H_3$]Dexamethasone (9α-fluoro-16α-methyl-11β,17,21-trihydroxy[1,2,4-$^3H_3$]pregna-1,4-diene-3,20-dione; 12Ci/mmol) was from Schwarz Bioresearch (Orangeburg,

N.Y., U.S.A.) and [1,2,4-$^3H_3$]triamcinolone acetonide (11β,21-dihydroxy-9α-fluoro[1,2,4-$^3H_3$]pregna-1,4-diene-3,20-dione-16α,17α-acetonide; 9.5 Ci/mmol) was a generous gift from Dr. F. Rosen. Triamcinolone acetonide was obtained from E. R. Squibb and Sons (New Brunswick, N.J., U.S.A.), and other non-radioactive steroids were from Calbiochem (Los Angeles, Calif., U.S.A.). Dextran 60 and cycloheximide were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and all other reagents were from Fisher Scientific Co. (Fair Lawn, N.J., U.S.A.) with the exception of reagents for the determination of glucose by the glucose oxidase method (Glucostat), which were purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

Methods and Results

General methods

Incubations were carried out in Neoprene-stoppered 10ml or 25ml Erlenmeyer flasks or in 10ml tubes, shaken in a Dubnoff metabolic incubator at approx. 100 oscillations/min.

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Protein Standard Solution, Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) as standard.

Radioactivity was measured for 0.05 or 0.5ml aqueous samples in 10ml of Bray's scintillation fluid (Bray, 1960) in polythene vials in a Packard Tri-Carb model 3310 liquid-scintillation spectrometer. Efficiency for unquenched tritium was 25%, and corrections for quenching were applied with *n*-hexa[^{3}H]decane as internal standard.

Aqueous solutions of non-radioactive steroids were prepared by measuring the E_{250} and taking $\epsilon_{250} = 1.4 \times 10^4 M^{-1} \cdot cm^{-1}$. Steroids were added to incubation mixtures as aqueous solutions at 10–100 × final concentration.

Preparation of cell suspensions and cytoplasmic fractions

Thymus cell suspensions were prepared in Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), from adrenalectomized male rats (Charles River CD strain, 180–220g) as described previously (Munck, 1968). Buffers and cell suspensions, which contained no added glucose, were equilibrated with O_2+CO_2 (95:5) for aerobic experiments and with N_2+CO_2 (95:5) for anaerobic experiments. Cell concentrations were measured by a standard microhaematocrit procedure, and were generally in the range 0.28–0.32ml of packed cells/ml of cell suspension. Cytoplasmic fractions for use in most of the binding experiments described below were prepared by first incubating a cell suspension aerobically at 37°C for 5min. The suspension was rapidly cooled to 3°C, and added, with vigorous agitation, to 5vol. of 1.5mM-$MgCl_2$ at 3°C. This diluted suspension was incubated with shaking at 3°C for 5min, a procedure which breaks cell membranes and results in discharge of cytoplasmic contents without disruption of nuclear structure (Wira & Munck, 1970), and was then centrifuged for 3 min at about 1500g_{av}. The low-speed supernatant from this procedure, subsequently referred to as the cytoplasmic fraction, was generally diluted with Tris–HCl buffer, to give a final buffer concentration of 10mM, and the pH was adjusted to 7.5. The protein concentration was approx. 2mg/ml.

For experiments in which the dependence of the steroid-binding ability of the cytoplasmic fraction on aerobic or anaerobic incubation of the cell suspension was being investigated, the cell suspension was either incubated aerobically for 95min, or aerobically for 5min, anaerobically for 30min, and again aerobically for a further 60min, all at 37°C. In both cases, 2ml portions of cell suspension were withdrawn after 5, 35, 65 and 95min of incubation and cytoplasmic extracts were prepared as described above.

Steroid binding

Competitive binding assay for specific steroid binding by cytoplasmic fractions. Portions of a thymus cell cytoplasmic fraction were incubated with 5nM-[1,2-$^3H_2$]cortisol for 75min at 3°C, with or without 10μM non-radioactive cortisol. Magnesium chloride (1.5mM) and a solution of bovine serum albumin (2.5 mg/ml) were also incubated with 5nM-[1,2-$^3H_2$]cortisol. At the end of the incubation period, multiple 0.5ml portions of all solutions were dispensed into plastic centrifuge tubes (1.5ml capacity) that contained 0.5ml of a dextran-coated charcoal suspension (1.0g of Norit A charcoal and 0.1g of Dextran 60 in 100ml of water), vigorously agitated for 10s on a vortex mixer, and incubated at 3°C. Duplicate tubes from each treatment were centrifuged, at intervals of up to 2h, for 2min in an Eppendorf model 3200 centrifuge. Of the supernatant 0.5ml from each tube was counted.

The results obtained are shown in Fig. 1. Almost all [1,2-$^3H_2$]cortisol was adsorbed from incubation mixtures containing 1.5mM-$MgCl_2$ or bovine serum albumin by dextran–charcoal within 30min, only a residual background value remaining. When a thymus cell cytoplasmic fraction was incubated with 5nM-[1,2-$^3H_2$]cortisol in the presence of 10μM-cortisol the amount of radioactivity not adsorbed by charcoal also fell to background values within 30min. Only when the cytoplasmic fraction was incubated with 5nM-[1,2-$^3H_2$]cortisol was a considerable proportion of the initial radioactivity retained in the

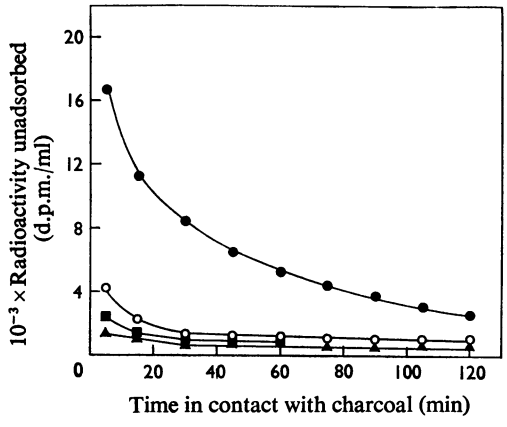

Fig. 1. *Adsorption of* [1,2-$^3H_2$]*cortisol by dextran–charcoal at* 3°C

[1,2-$^3H_2$]Cortisol (5nM) was equilibrated with (1) 1.5mM-$MgCl_2$ (■), (2) bovine serum albumin (2.5mg/ml; ▲), or with a thymus cell cytoplasmic fraction, (3) alone (●) or (4) in the presence of 10μM non-radioactive cortisol (○), before treatment with a suspension of dextran-coated charcoal for various periods of time.

supernatant, and it declined exponentially. We conclude that this slow exponential decline represents dissociation of a strongly bound fraction that is saturable. The initial, but rapid, exponential decline in unadsorbed radioactivity during the first 30min for samples containing 10μM-cortisol may represent the rapid dissociation of a non-saturable non-specific fraction. Subtraction of the values for unadsorbed radioactivity for samples containing both [1,2-$^3H_2$]cortisol and 10μM unlabelled cortisol from the corresponding values for samples containing only 5nM-[1,2-$^3H_2$]cortisol gave values conforming to a simple exponential curve. These values were plotted semi-logarithmically, and the regression line was calculated by the method of least squares. The correlation coefficient for the regression line was 0.997 and the time-constant for the exponential process was 67min, corresponding to a rate constant at 3°C of 1.49×10^{-2}min^{-1}.

Other active glucocorticoids also give a charcoal dissociation curve similar to that for [^{3}H]cortisol but with different rate constants; inactive steroids, such as cortisone, however, do not give such a fraction. The charcoal dissociation curve for the cytoplasmic fraction incubated with 5nM-[1,2-$^3H_2$]-cortisone is not significantly different from that for the same cytoplasmic fraction incubated with both 5nM-[1,2-$^3H_2$]cortisone and 10μM-cortisone, which in turn is not significantly different from the curve for the cytoplasmic fraction incubated with both 5nM-[1,2-$^3H_2$]cortisol and 10μM-cortisol (assuming equal specific radioactivities).

These observations formed the basis of an assay for the strongly bound fraction. In the standard binding assay, parallel incubations of the cytoplasmic fraction with the radioactive steroid were performed, with or without 10μM non-radioactive steroid. At the end of the incubation, replicate 0.5ml amounts were dispensed into charcoal as above and centrifuged after a fixed period of time, generally 15min. Specific binding was measured as the difference in radioactivity present after dextran–charcoal treatment of the cytoplasmic fraction incubated in the presence and absence of 10μM-steroid. Blank values were directly proportional to the concentration of ^{3}H-labelled steroid initially present, and were not affected by the presence of non-radioactive steroids up to 0.1mM. Protein concentrations were not significantly affected by charcoal treatment.

Values thus obtained were either used directly on a comparative basis, or were extrapolated to zero time in contact with dextran–charcoal by using the value of the rate-constant for dissociation in the presence of charcoal derived as described below. The magnitude of the specific fraction determined by the charcoal method agrees well with measurements made from gel chromatography (C. R. Wira & A. Munck, unpublished work).

Linearity of competitive binding assay. A range of dilutions of a thymus cell cytoplasmic fraction was prepared by dilution with 1.5mM-$MgCl_2$. Portions (1ml) of each dilution were incubated with 5.4nM-[1,2-$^3H_2$]cortisol for 75min at 3°C, and then treated with dextran–charcoal as described above. The correlation coefficient of the regression line relating specific binding to protein concentration was 0.994.

Relation of cytoplasmic binding fraction to receptors in intact cells: dissociation of ^{3}H-labelled steroids from the cytoplasmic fraction and from intact cells. A cytoplasmic fraction was incubated with 5nM-[1,2-$^3H_2$]cortisol or with 5nM-[1,2,4-$^3H_3$]triamcinolone acetonide at 3°C. Two groups of flasks were used for each steroid, one group also containing the same steroid (non-radioactive, 10μM) from zero time. After 75min, non-radioactive steroid was also added to the other group of flasks to give a final concentration of 10μM. Both groups of flasks were then warmed to 37°C for various times, followed by rapid cooling back to 3°C. Samples were immediately withdrawn for measurement of binding by the dextran–charcoal method.

5nM-[1,2-$^3H_2$]Cortisol and 5nM-[1,2,4-$^3H_3$]triamcinolone acetonide were also incubated with a thymus cell suspension at 37°C. After equilibration (20min) samples of the cell suspension were diluted about

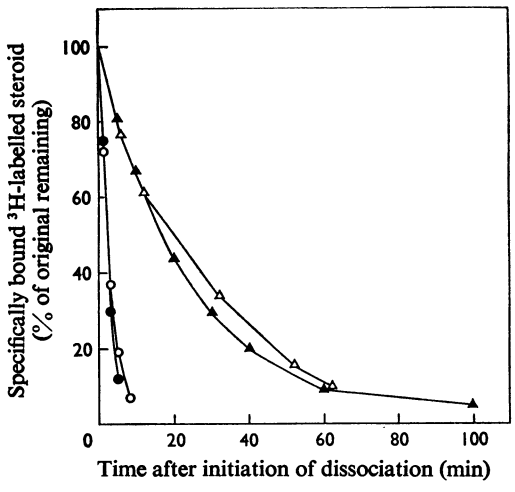

Fig. 2. *Dissociation curves for cortisol and triamcinolone acetonide from whole thymus cells or from the isolated cytoplasmic receptor at* 37°C

Dissociation from the isolated cytoplasmic receptor of [1,2-$^3H_2$]cortisol (●) or [1,2,4-$^3H_3$]triamcinolone acetonide (▲) was initiated by the addition of the appropriate unlabelled steroid at 10μM; dissociation of [1,2-$^3H_2$] cortisol (○) or [1,2,4-$^3H_3$]triamcinolone acetonide (△) from intact thymus cells was initiated by 50-fold dilution into fresh buffer.

50-fold with Krebs–Ringer bicarbonate buffer at 37°C. Samples were withdrawn at intervals from the diluted suspension for measurement of the radioactivity associated with the cells according to the procedure of Munck & Brinck-Johnsen (1968).

The rates of dissociation determined for the cytoplasmic fraction by displacing previously bound ^{3}H-labelled steroid with an excess of non-radioactive steroid are compared in Fig. 2 with the rates of dissociation of the same steroids from receptors in intact cells, determined by a dilution technique, and it can be seen that there is good agreement between the two methods. These observations indicate that the strongly bound, saturable, fraction measured by the dextran–charcoal technique represents the population of specific glucocorticoid receptors already described for intact thymus cells (Munck & Brinck-Johnsen, 1968). The specificity of the strongly bound fraction measured by the competitive binding assay, and its saturability, also indicate that this fraction corresponds to the cytoplasmic receptor present in intact cells.

Association of steroids with the cytoplasmic receptor: inactivation of receptor. Parallel incubations of a cytoplasmic fraction were performed with a given concentration of ^{3}H-labelled steroid, with or without 10μM non-radioactive steroid, and with other additions as appropriate. After incubation, 0.5ml portions were withdrawn and assayed for binding by the dextran–charcoal method.

Preliminary experiments showed that with [1,2-$^3H_2$]cortisol at concentrations from 5 to 20nM, equilibrium was reached after 75–90min of incubation at 3°C. However, if measurements of binding were made at times beyond 75min, a decrease in the concentration of bound steroid was observed, such that by 8h only 29% of the maximum remained bound (Fig. 3*a*). With 20nM-[1,2,4-$^3H_3$]dexamethasone, maximum binding was not attained until 3–4h, and 66% of the maximum remained bound at 8h. With 20nM-[1,2,4-$^3H_3$]triamcinolone acetonide, maximum binding was reached after 4h, and remained at this level at least to 8h. Similar results were obtained at 25°C, although the time-scale was different (Fig. 3*b*). These findings indicate that the free receptor or the steroid–receptor complex or both were in some way being inactivated, and that the complex could be stabilized completely by triamcinolone acetonide or partially by dexamethasone. Complete stabilization of the complex was also attained when saturating concentrations of [1,2-$^3H_2$]-cortisol were used, and the association was still reversible since addition of an excess of unlabelled cortisol caused dissociation of [1,2-$^3H_2$]cortisol from the complex (Fig. 4).

When the cytoplasmic fraction was pre-incubated for various times at 3°C before addition of [1,2-$^3H_2$]-cortisol, inactivation of the free receptor could clearly be demonstrated (Fig. 5). The rate constant for inactivation was 6.5×10^{-3}min^{-1} (k_3). A considerable degree of stabilization was achieved by the addition of 2.5mM-EDTA to the cytoplasmic fraction immediately after preparation; the rate constant was decreased to 1.6×10^{-3}min^{-1}. Warming the cytoplasmic fraction to 37°C for 10min before addition of ^{3}H-labelled steroid abolished all specific binding.

Rate of association of cortisol with the cytoplasmic receptor. Detailed measurements were made of the binding of [1,2-$^3H_2$]cortisol to the receptor during the first 20min of incubation at 3°C. The rate of binding was linear for the first 8min and then decreased. From measurements of the initial rate of binding at different concentrations of [1,2-$^3H_2$]-cortisol, and by using a value for total receptor concentration determined with a saturating concentration of [1,2-$^3H_2$]cortisol, the rate constant for association (k_1) for cortisol at 3°C was found to be 8.6×10^5M$^{-1}\cdot$min^{-1}. Since the value for total receptor concentration is an underestimate because of inactivation during the incubation, this value represents an upper limit.

Rates of dissociation of glucocorticoids from the steroid–receptor complex. A cytoplasmic fraction was

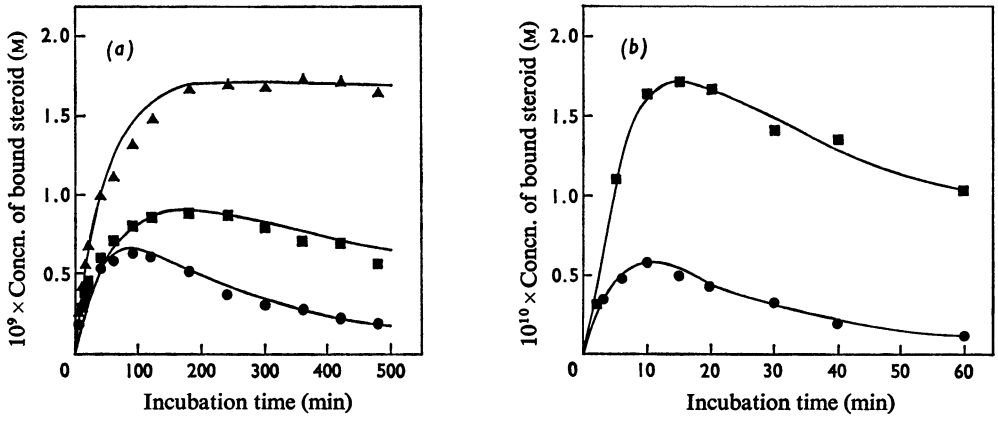

Fig. 3. *Association of steroids with the isolated cytoplasmic receptor*

Association of [1,2-$^3H_2$]cortisol (●), [1,2,4-$^3H_3$]dexamethasone (■) or [1,2,4-$^3H_3$]triamcinolone acetonide (▲) with the cytoplasmic receptor was determined with the dextran–charcoal binding assay, (*a*) at 3°C, with all steroids at 20nM, and (*b*) at 25°C with steroids at 6nM. Curves were fitted to the results in (*a*) by using eqn. (2) as described in the text and the Appendix.

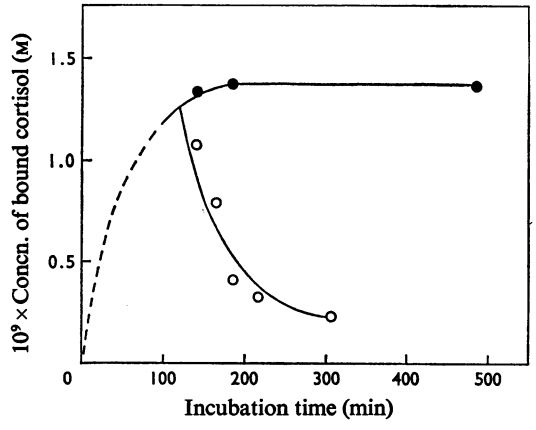

Fig. 4. *Binding of* 0.1μM-[1,2-$^3H_2$]*cortisol to the cytoplasmic receptor at* 3°C

Samples were withdrawn for measurement of binding by the dextran–charcoal method from flasks in which a thymus cell cytoplasmic fraction was incubated with 0.1μM-[1,2-$^3H_2$]cortisol alone (●) or after the addition of 20μM-cortisol at 120min (○).

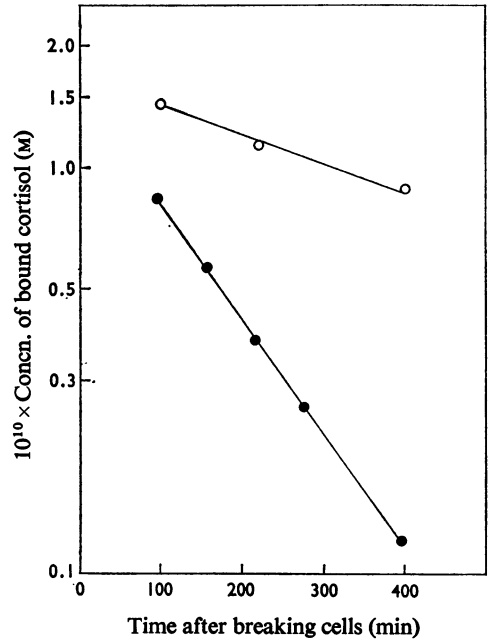

Fig. 5. *Inactivation of the cytoplasmic receptor at* 3°C

At various times after the preparation of the cytoplasmic fraction, 5nM-[1,2-$^3H_2$]cortisol was added and incubated for 75min at 3°C. The concentration of specifically bound [1,2-$^3H_2$]cortisol was determined with dextran–charcoal. Samples contained no EDTA (●) or 2.5mM-EDTA (○).

incubated with the appropriate ^3H-labelled steroid at 3°C, in two groups of flasks, one group also containing the same steroid (non-radioactive, 10μM) from zero time. After 75min non-radioactive steroid was also added to the other group of flasks to give a final concentration of 10μM, and samples were

withdrawn from both groups at successive intervals of time for measurement of binding by the dextran–charcoal method.

For determination of the rates of dissociation at 37°C, flasks in both groups were warmed to 37°C for various periods of time after the addition of 10 μM-steroid, the flasks were rapidly cooled to 3°C, and then assayed in the usual way. In some experiments the incubation mixture containing the cytoplasmic [1,2-$^3H_2$]cortisol–receptor complex was warmed to 25°C for 10 min at the end of 75 min of incubation at 3°C, and was cooled to 3°C again before addition of 10 μM-cortisol. Values for specifically bound steroid were plotted semilogarithmically against time after initiation of dissociation by displacement, and rate constants were determined from the regression lines fitted to the data by the method of least squares.

Rate constants for dissociation (k_2) determined by this method for the complexes of several glucocorticoids and for the antiglucocorticoid cortexolone are listed in Table 1.

The dissociation rate constant for cortisol at 3°C was also determined before and after the steroid–receptor complex had been warmed to 25°C. [1,2-$^3H_2$]Cortisol had originally been incubated either with the cytoplasmic fraction at 3°C or with whole cells at 3°C before preparation of the cytoplasmic fraction. In both cases the rate constant for dissociation at 3°C was decreased by previous heating (Table 2).

Fitting of theoretical curves to association data. Utilizing the data obtained above for the rate constant for inactivation of free receptor (k_3; Appendix) and for the rate constants for dissociation (k_2), the data in Fig. 3(*a*) were fitted to an expression for binding based on the relationships:

$$H+R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HR \quad \text{and} \quad R \xrightarrow{k_3} R' \qquad (1)$$

where H is steroid hormone, R is receptor, HR is steroid–receptor complex, R′ is the inactivated form of R, and k_1, k_2 and k_3 are rate constants. The expression is:

$$[HR] = \frac{k_1[H]_0[R]_0}{\sqrt{\theta^2-4k_2k_3}} \times \left[e^{\frac{t}{2}(-\theta+\sqrt{\theta^2-4k_2k_3})} - e^{\frac{t}{2}(-\theta-\sqrt{\theta^2-4k_2k_3})}\right] \qquad (2)$$

where $\theta = k_1[H]_0+k_2+k_3$.

The derivation of this equation is given in the Appendix. Best fit was achieved by varying the values for k_1, the association rate constant, and $[R]_0$, the receptor concentration at zero time. $[H]_0$ is the steroid concentration at zero time. The lines of best fit, which are shown in Fig. 3(*a*), were obtained for cortisol when $k_1 = 5\times10^5\,M^{-1}\cdot min^{-1}$ and $[R]_0 =$ 2.5 nM, for dexamethasone when $k_1 = 4\times10^5\,M^{-1}\cdot min^{-1}$ and $[R]_0 = 2.3$ nM, and for triamcinolone acetonide when $k_1 = 7\times10^5\,M^{-1}\cdot min^{-1}$ and $[R]_0 =$ 2.6 nM. Since $k_{assoc.} = k_1/k_2$, these values would correspond to association constants of $3.3\times10^7\,M^{-1}$, $1.3\times10^8\,M^{-1}$ and $2.7\times10^9\,M^{-1}$ for the complexes with cortisol, dexamethasone and triamcinolone acetonide respectively at 3°C.

Table 1. *Rate constants for dissociation (k_2) for steroid–cytoplasmic receptor complexes*

For experimental details see the text and the Appendix.

Steroid	Rate constant for dissociation (min^{-1}) At 3°C	At 37°C
Cortexolone	3.33×10^{-2}	—
Cortisol	1.53×10^{-2}, 1.74×10^{-2}	0.43
Dexamethasone	3.13×10^{-3}	0.15
Triamcinolone acetonide	2.59×10^{-4}	3.9×10^{-2}

Table 2. *Effect of prewarming cortisol–receptor complexes on the subsequent dissociation rates at 3°C*

[1,2-$^3H_2$]Cortisol–cytoplasmic receptor complexes were prepared by incubation of 25 nM-[1,2-$^3H_2$]-cortisol at 3°C with a cytoplasmic fraction (Expt. 1) or with whole cells before preparation of a cytoplasmic fraction (Expt. 2). Complexes were warmed to 25°C for 10 min (Expt. 1) or 7 min (Expt. 2), subsequently cooled to 3°C, and dissociation was initiated by displacement with 10 μM-cortisol. Controls were maintained at 3°C throughout.

	$10^2\times$Rate constant for dissociation at 3°C (min^{-1}) Prewarmed samples	Controls
Expt. 1	1.28	1.74
Expt. 2	1.37	1.74

Equilibrium binding studies. Despite the fact that inactivation of the receptor meant that true equilibrium could not be reached, when apparent equilibrium binding, i.e. maximum binding, was measured with the competitive binding assay for a range of concentrations of [1,2-$^3H_2$]cortisol, the results did give a straight-line Scatchard plot, as shown in Fig. 6. The regression line derived by the method of least squares had a correlation coefficient of 0.98 and gave a value for the association constant of $1.7\times10^7\,M^{-1}$ and a total receptor concentration of 2.05 nM.

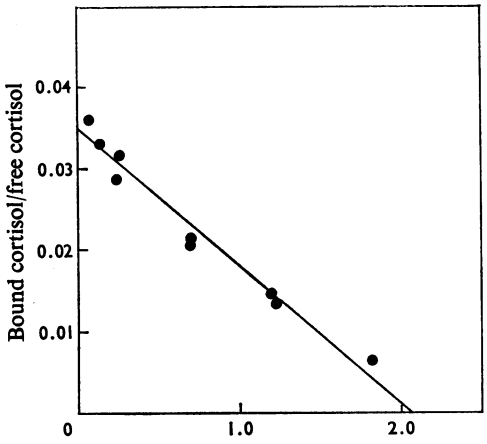

Fig. 6. *Scatchard plot for binding of* [1,2-$^3H_2$]-*cortisol to the cytoplasmic receptor at* 3°*C*

The concentration of specifically bound [1,2-$^3H_2$]-cortisol was determined with dextran–charcoal.

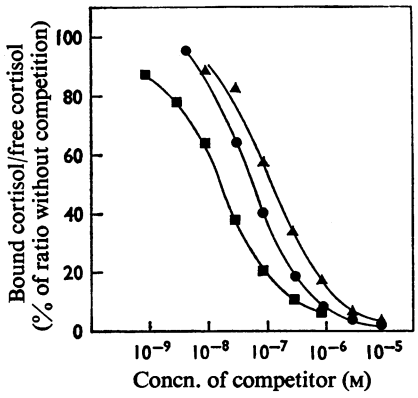

Fig. 7. *Competition by other steroids for binding of* [1,2-$^3H_2$] *cortisol to the cytoplasmic receptor at* 3°*C*

The cytoplasmic fraction was equilibrated with 5nM-[1,2-$^3H_2$]cortisol and with various concentrations of non-radioactive cortisol (●), dexamethasone (■) or cortexolone (▲); binding was measured by using dextran–charcoal.

Competition data were also obtained for cortisol by incubating the cytoplasmic fraction with 5nM-[1,2-$^3H_2$]cortisol and with various concentrations of non-radioactive cortisol, cortexolone or dexamethasone. The apparent equilibrium binding data thus obtained are shown in Fig. 7. The ratios, concentration of cortisol/concentration of competitor, applied to the concentrations required to decrease the original value for bound [1,2-$^3H_2$]cortisol/free [1,2-$^3H_2$]-cortisol by one-half, are thus a measure of the competition factors, which, compared to cortisol (1.0), are 0.45 and 3.9 respectively for cortexolone and dexamethasone. Since the apparent association constant for cortisol was $1.7\times10^7\,M^{-1}$, then the apparent association constant for cortexolone is $7.6\times10^6\,M^{-1}$ and for dexamethasone is $6.6\times10^7\,M^{-1}$. The apparent association constant for triamcinolone acetonide estimated by direct binding was $1.14\times10^8\,M^{-1}$ with a receptor concentration of 1.95nM (Fig. 8).

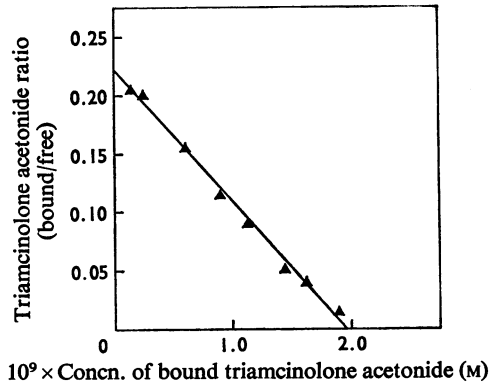

Fig. 8. *Scatchard plot for binding of* [1,2,4-$^3H_3$]-*triamcinolone acetonide to the cytoplasmic receptor at* 3°*C*

The concentration of specifically bound [1,2,4-$^3H_3$]-triamcinolone acetonide was determined with dextran–charcoal.

Dependence of specific binding on incubation conditions for cells. When cytoplasmic fractions were prepared from thymus cell suspensions that had been incubated for various periods at 37°C in an O_2 atmosphere, and were assayed for receptor content by incubation with 5nM-[1,2-$^3H_2$]cortisol at 3°C, a steady decline in the amount of cortisol bound was observed with the length of time of incubation of the cell suspension at 37°C (Table 3). Concomitant with this decrease was a marked increase in protein content of the cytoplasmic fraction, such that the decline in binding was even more marked when results were expressed relative to protein concentration. This increase in protein content was not observed when the cells were incubated at 3°C.

When cell suspensions were incubated in an atmosphere of N_2 a marked decrease in specific binding occurred, which was largely reversed when the cells were transferred to an O_2 atmosphere, although this effect was also less marked when results were

Table 3. *Influence of aerobic or anaerobic incubation of thymus cells on the subsequent cortisol-binding capacity of a cytoplasmic fraction*

Cells were incubated at 37°C for up to 95min. In Expt. 1 the gas phase was O_2+CO_2 (95:5); in Expt. 2 the gas phase was O_2+CO_2 (95:5) from 0 to 5min, N_2+CO_2 (95:5) from 5 to 35min and O_2+CO_2 (95:5) from 35 to 95min. Cytoplasmic fractions were prepared at the times indicated; binding was measured by using the dextran–charcoal assay after a 75min incubation with 5nM-[1,2-$^3H_2$]cortisol.

Incubation time for cells (min)	$10^{13}\times$Concn. of cortisol bound (mol bound/ml of incubate)		$10^{13}\times$Concn. of cortisol bound (mol bound/mg of protein)		Concn. of protein (mg/ml of incubate)	
Expt. no. ...	1	2	1	2	1	2
5	1.36	1.42	0.62	0.67	2.19	2.12
35	1.18	0.38	0.40	0.15	2.95	2.53
65	1.15	0.96	0.33	0.34	3.48	2.82
95	0.91	0.57	0.24	0.18	3.79	3.17

Table 4. *Effect of cycloheximide on specific binding of* [1,2-$^3H_2$]*cortisol by thymus cells*

In Expt. 1 cell suspensions were incubated at 37°C in O_2+CO_2 (95:5) with 66nM-[1,2-$^3H_2$]cortisol from zero time, and 0.1mM-cycloheximide was added at 8.25min. In Expt. 2 cell suspensions were incubated in N_2+CO_2 (95:5) with 29nM-[1,2-$^3H_2$]cortisol and 0.1mM-cycloheximide from zero time. After 20min incubation at 37°C, the gas phase was changed to O_2+CO_2 (95:5). In both experiments binding was measured by a dilution technique, and is expressed as the ratio of the steroid concentration specifically bound to the cells to the equilibrium concentration in the medium. The equilibrium concentration in the medium was 39nM in Expt. 1, 17nM in Expt. 2.

Sample no.	Incubation time (min)	Gas phase	Cycloheximide	Ratio of steroid concentration specifically bound to cells/equilibrium concentration in medium
Expt. 1				
1	8	O_2+CO_2 (95:5)	Absent	0.609
2	14	O_2+CO_2 (95:5)	Present	0.676
3	30	O_2+CO_2 (95:5)	Present	0.649
4	46	O_2+CO_2 (95:5)	Present	0.661
5	65	O_2+CO_2 (95:5)	Present	0.574
Expt. 2				
1	20	N_2+CO_2 (95:5)	Present	0.204
2	35	O_2+CO_2 (95:5)	Present	0.86

expressed in terms of protein concentration as a consequence of the rise in protein content with time. The binding of [1,2-$^3H_2$]cortisol to the cytoplasmic fraction prepared from anaerobic cells could not be increased by the addition to the cytoplasmic fraction of ATP at a final concentration of 0.1mM.

Effect of cycloheximide on binding of [1,2-$^3H_2$]*cortisol to intact cells.* Thymus cell suspension (1.5ml) was incubated at 37°C in an atmosphere of O_2+CO_2 (95:5) with glucose (1mg/ml) and 66nM-[1,2-$^3H_2$]cortisol. After 8.25min, cycloheximide was added in a small volume of buffer to give a final concentration of 0.1mM. Portions (0.14ml) were withdrawn at successive time-intervals before and after the addition of cycloheximide, and were diluted 50-fold into fresh buffer containing 0.1mM-cycloheximide, at 37°C. Samples of the diluted cell suspension were withdrawn at intervals for measurement of the radioactivity associated with the cells, and the results were analysed as described by Munck & Brinck-Johnsen (1968) to derive values for the fraction of radioactivity specifically bound to the cells immediately before dilution.

In another experiment, a cell suspension was prepared in an atmosphere of N_2+CO_2 (95:5). Two 1ml samples of this suspension were then incubated at 37°C under N_2, with 0.1mM-cycloheximide and with 29mM-[1,2-$^3H_2$]cortisol. After 20min specific binding was determined for one flask by the dilution technique described above; the remaining flask was incubated for a further 15min in an atmosphere of O_2+CO_2 (95:5) before specific binding was determined by the dilution technique.

The presence of 0.1mM-cycloheximide had little or

no effect on the magnitude of the fraction of [1,2-$^3H_2$]cortisol specifically bound by cells incubated aerobically; binding was still at or near normal values after 1h (Table 4). By contrast, incubation of cells for 20min in an N_2 atmosphere at 37°C, also in the presence of 0.1mM-cycloheximide, caused a large decrease in specific binding; binding, expressed as the ratio of the concentration of steroid specifically bound to the cells to the equilibrium concentration in the medium, was decreased to 0.204. Further, when O_2 was re-admitted, the ratio rose to 0.86 within 15min, even in the presence of 0.1mM-cycloheximide (Table 4).

Discussion

The studies reported here show that the glucocorticoid receptors previously identified in the cytoplasm of intact thymus cells (Munck & Wira, 1971) are also present in the cytoplasmic fraction obtained by rupture of cells not previously treated with glucocorticoids *in vitro*. The competitive binding assay with dextran-coated charcoal utilized in these studies is similar to that used by Baxter & Tomkins (1971) for the study of steroid receptors from hepatoma tissue culture cells; they, however, treated with charcoal for a brief period only, and regarded the values obtained for specific binding after charcoal treatment as being zero-time values. This approach is satisfactory when using [^{3}H]dexamethasone, as did Baxter & Tomkins, because of the relatively slow dissociation rate of this steroid from the receptor. With less strongly bound steroids such as cortisol or cortexolone, however, which have appreciable dissociation rates even at 3°C, it is necessary to use the technique reported here, to extrapolate back to zero time.

Accurate measurements of the association constants and rates of association of glucocorticoids with the cytoplasmic receptor were complicated by inactivation of the receptor. A good fit to the experimental data was obtained by using an expression (eqn. 2) for the rate of association which assumes that only free receptor is inactivated (for the derivation of eqn. 2, see the Appendix) and that receptor bound to steroid is stable, an assumption which is supported by the results which show stabilization of binding by saturating concentrations of cortisol; this stability is due to the fact that under these conditions any one receptor molecule is free for only a very short period of time. The stability of the complex of the receptor with triamcinolone acetonide at less than saturating concentrations is due to the stability of the individual complexes, which once formed practically do not dissociate. Thus, in general, increased stabilization represents a decreasing ability of the steroids to respond by rapid dissociation to disturbances of the equilibrium caused by inactivation of the free receptor. The observation that unlabelled cortisol will displace [1,2-$^3H_2$]cortisol from the receptor even when the latter is saturated demonstrates that when inactivation can be circumvented the receptor is capable of rebinding a cortisol molecule a second time. Our results differ from those obtained with mouse fibroblasts, where binding did not seem to be reversible (Pratt & Ishii, 1972; Ishii *et al.*, 1972).

Pratt & Ishii (1972) have reported degradation of the cytoplasmic receptor for glucocorticoids from mouse fibroblasts, and have also shown that triamcinolone acetonide has a stabilizing effect. Kirkpatrick *et al.* (1972) have presented similar evidence for inactivation of the glucocorticoid receptor from lymphosarcoma cells and stabilization by triamcinolone acetonide, and have suggested that the inactivation is more likely to represent a structural change in the receptor than enzymic digestion, since the incubations are conducted at low temperatures and since corticosteroids in general tend to accelerate rather than inhibit proteolysis *in vitro*. It is for this reason that we have referred to the process as inactivation rather than degradation, though firm conclusions on this point must await purification of the receptor.

The association constants reported here correlate with the biological effectiveness of cortisol and dexamethasone in inhibiting glucose uptake by isolated thymus cells (Kattwinkel & Munck, 1966; Munck & Brinck-Johnsen, 1968). Additionally, we have also found in preliminary experiments that the metabolic activity of triamcinolone acetonide appears to be two- to ten-fold that of cortisol, which also correlates with its higher association constant. Our results also confirm the findings of Munck & Brinck-Johnsen (1967, 1968), that cortexolone, which is biologically inactive in isolated thymus cells, binds to the receptor and competes with cortisol; it therefore can be considered an antiglucocorticoid.

When these results are considered together with the values for rate constants for dissociation and for association, it becomes apparent that the differences in binding affinity are determined largely by the differences in rate constants for dissociation, and that the association rates are very similar. The implications of these findings are that the groups that distinguish these steroids, particularly the 11β-OH and 9α-fluoro groups, do not come into play until the steroid has entered the binding site of the receptor, a conclusion consistent with the view of the steroid binding site as a hydrophobic pocket within the receptor, with polar groups strategically placed so as to form hydrogen bonds with the polar groups of the steroid (Munck *et al.*, 1972).

We previously reported (Munck *et al.*, 1972) that the cytosol–receptor complex prepared at 3°C will

be transferred to isolated nuclei at 3°C only if it has been prewarmed to 25°C and then cooled again to 3°C. The rate-limiting step is the temperature-dependent transformation of the complex, transfer of the transformed complex to the nucleus being extremely rapid even at 3°C. Two possible mechanisms to account for this transformation were suggested: one is an equilibrium mechanism in which the effect of the steroid is to change the position of equilibrium of the two forms of the receptor so as to favour the form with high affinity for the nucleus; the other mechanism is a kinetic one in which the steroid accelerates the rate of the temperature-sensitive transformation. An essential difference between these two mechanisms is that, in the equilibrium mechanism, the association constant for the interaction of steroid with the transformed receptor must be much greater than for the interaction with untransformed receptor, whereas in the kinetic mechanism the association constant remains unchanged. While it has not proved possible in the present work to determine the association constant for the transformed cortisol–receptor complex, we have been able to demonstrate that transformation results in a 21–26% decrease in the rate constant for dissociation. While this relatively small change in the rate constant for dissociation would not appear to make a significant difference to the association constant, it should be borne in mind that it is not possible to estimate the proportion of steroid–receptor complex that has been transformed; this difference might be the result of transformation of only a small proportion of the complex.

Munck & Brinck-Johnsen (1968) showed that for intact thymus cells at 37°C there is a correlation between ATP concentrations and the magnitude of specific cortisol binding. In the present work we have demonstrated that binding of cortisol to the cytoplasmic receptor from metabolically intact cells can proceed normally, but that when the cytoplasmic fraction is prepared from cells incubated anaerobically the amount of steroid bound is greatly decreased, and cannot be increased by addition of ATP to the cytoplasmic fraction. This inhibition of binding is reversible, since introduction of O_2 to cells previously incubated anaerobically results in an increase in the cortisol-binding properties of the cytoplasmic fraction. Even though a gradual decline in binding ability is noted with time for cells incubated aerobically, this may represent a gradual decrease in ATP concentration due to the absence of substrate (Young, 1969).

The specific binding of cortisol to intact cells under aerobic conditions is not affected by the presence of 0.1mM-cycloheximide for periods of up to 1h. The concentration of cortisol in this experiment was such that approximately half the receptor sites were occupied, and from the rate constant for association it can be calculated that if all receptor molecules were destroyed after complex-formation, and had to be replaced by protein synthesis *de novo*, all receptors would be destroyed in about 10min in the absence of protein synthesis. Transfer of cells to an anaerobic environment does result in almost total loss of specific binding within 20min, but this inhibition is readily reversible even in the presence of 0.1mM-cycloheximide, a concentration that inhibits protein synthesis in thymus cells by more than 90% within 5min (C. Hallahan, D. A. Young & A. Munck, unpublished work). These observations clearly indicate that destruction and synthesis of receptor proteins *de novo* do not occur at rates comparable with those at which the hormone enters and leaves the nucleus under aerobic conditions, and are not involved in the reversible disappearance of specific binding under anaerobic conditions.

These and other observations have led us to postulate a mechanism in which ATP, whether directly or indirectly, is necessary to convert the receptor from a form in which it is unable to bind cortisol into an active form (Munck *et al.*, 1972). This mechanism could be cyclic, in which case the ATP could supply the free energy for maintenance of a cycle in which the receptor forms a complex with the hormone, becomes inactivated after reaching the nucleus, and emerges to be reactivated and form a new complex. From studies of the energy dependence of the binding of triamcinolone acetonide to cultured mouse fibroblasts, Ishii *et al.* (1972) have proposed that the release of the receptor from the particulate fraction and its regeneration to a form capable of binding steroid again is energy-dependent. Despite the differences in the reversibility of binding of triamcinolone acetonide already referred to, our observations clearly support the concept of regeneration of receptor.

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References

Baxter, J. D. & Tomkins, G. M. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 932–937

Bell, P. A. & Munck, A. (1971) *Biochem. J.* **126**, 11P

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285

Ishii, D. N., Pratt, W. B. & Aronow, L. (1972) *Biochemistry*, **11**, 3896–3904

Kattwinkel, J. & Munck, A. (1966) *Endocrinology*, **79**, 387–390

Kirkpatrick, A. F., Kaiser, N., Milholland, R. J. & Rosen, F. (1972) *J. Biol. Chem.* **247**, 70–74

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Milgrom, E. & Baulieu, E. E. (1969) *Biochim. Biophys. Acta* **194**, 602–605

Munck, A. (1968) *J. Biol. Chem.* **243**, 1039–1042

Munck, A. & Brinck-Johnsen, T. (1967) *Excerpta Medica Found. Int. Congr. Ser.* **132**, 472–481

Munck, A. & Brinck-Johnsen, T. (1968) *J. Biol. Chem.* **243**, 5556–5565

Munck, A. & Wira, C. R. (1971) *Advan. Biosci.* **7**, 301–330

Munck, A., Wira, C., Young, D. A., Mosher, K. M., Hallahan, C. & Bell, P. A. (1972) *J. Steroid Biochem.* **3**, 567–578

Murphy, B. E. P. (1967) *J. Clin. Endocrinol. Metab.* **27**, 973–990

Pratt, W. B. & Ishii, D. N. (1972) *Biochemistry*, **11**, 1401–1410

Schaumberg, B. P. (1972) *Biochim. Biophys. Acta* **261**, 219–235

Wira, C. & Munck, A. (1970) *J. Biol. Chem.* **245**, 3436–3438

Young, D. A. (1969) *J. Biol. Chem.* **244**, 2210–2217

APPENDIX

Derivation of Expression for Concentration of Hormone–Receptor Complex

For the reaction system in which steroid hormone, H, associates with receptor, R, to give hormone–receptor complex, HR, and in which there is an accompanying inactivation of receptor to a form R′, then

$$H+R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HR \quad \text{and} \quad R \xrightarrow{k_3} R'$$

where k_1, k_2 and k_3 are rate constants.

Then

$$d[HR]/dt = k_1[H][R]-k_2[HR]$$

and

$$-d[R]/dt = k_3[R]+d[HR]/dt$$

If [HR] is small compared to [H], then [H] may be replaced by $[H]_0$, the zero time value of [H]. Elimination of [R] and its derivative from the resulting equations then gives

$$d^2[HR]/dt^2+\theta\, d[HR]/dt+k_2k_3[HR] = 0 \quad (1)$$

where

$$\theta = k_1[H]_0+k_2+k_3$$

Now when $t = 0$, $[HR] = 0$ and $d[HR]/dt$ becomes $k_1[H]_0[R]_0$, where $[R]_0$ is the zero-time value of [R]. It follows that the solution to eqn. (1) under these conditions becomes:

$$[HR] = \frac{k_1[H]_0[R]_0}{\sqrt{\theta^2-4k_2k_3}} \times \left[e^{\frac{t}{2}(-\theta+\sqrt{\theta^2-4k_2k_3})}-e^{\frac{t}{2}(-\theta-\sqrt{\theta^2-4k_2k_3})}\right] \quad (2)$$

When $k_3 = 0$ (in the absence of inactivation), this expression simplifies to the usual form,

$$[HR] = \frac{k_1[H]_0[R]_0}{k_1[H]_0+k_2}[1-e\{-t(k_1[H]_0+k_2)\}] \quad (3)$$