Activation of the Rat Liver Glucocorticoid–Receptor Complex†

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ABSTRACT: The rat liver glucocorticoid receptor has been activated using three procedures: heat, gel filtration, and dilution. With time after heat activation the steroid–receptor complex loses its capacity to bind to DNA–cellulose, while receptor activated by Sephadex G-25 and by dilution maintains DNA–cellulose binding capacity. The rates of steroid dissociation from nonactivated and activated receptor are essentially identical. However, nonactivated receptor is capable of rebinding steroid, while activated receptor has a reduced capacity to rebind steroid. The results of the gel filtration and dilution studies suggest that a low-molecular-weight factor(s) exists in rat liver cytosol which is involved in the process of activation.

The activation of glucocorticoid–receptor complexes has been investigated extensively in the past several years. Activation has been defined (Baxter et al., 1975; Kalimi and Beato, 1973) as the change(s) in the cytoplasmic steroid–receptor complex which permits its translocation to the nucleus. More specifically, this change involves, at least in part, the exposure of positive charges on the receptor, resulting in an increase in the affinity of the steroid–receptor complex for a variety of polyanions, including DNA–cellulose (Baxter et al., 1975; Milgrom et al., 1973; Kalimi et al., 1975). Numerous procedures have been described which enhance the activation process in cell-free systems: increased temperature and/or increased ionic strength (Higgins et al., 1973), and treatment with Ca^{2+} at low temperature (Kalimi et al., 1975) and theophylline (Cake and Litwack, 1975). Factors which reduce or prevent activation include Ca^{2+}, either at raised temperatures (Milgrom et al., 1973; Kalimi et al., 1975) or following heat activation, and mercurials (Milgrom et al., 1973; Rousseau et al., 1975). These studies have begun to provide insight into the molecular mechanisms of activation.

The model of activation of the steroid–receptor complex presented in the literature (e.g., Higgins, et al., 1973; Turnell et al., 1974) suggests that this process is unidirectional; i.e., once activated the complex maintains its capacity to bind to DNA, presumably until the steroid dissociates from the receptor. Until recently, the reversibility of activation has not been investigated. In a preliminary report (Goidl et al., 1976), we provided evidence which supported the concept of reversibility (referred to as deactivation). In the present, more detailed examination of this question, differences in the rebinding of steroid to activated and nonactivated receptor became apparent, in agreement with the findings of Kreiger et al. (1976). The procedures used to activate the complex usually result in no more than 50% of the population in the activated state. Consequently, using such a mixed population it is not possible to determine conclusively whether deactivation occurs as distinct from steroid dissociation and lack of rebinding to activated receptor.

In the course of these studies, it was found that gel filtration on Sephadex G-25 and dilution of the cytosol also resulted in the activation of receptor. After either of these activation procedures, however, the receptor remained in the activated state; that is, DNA–cellulose binding capacity remained constant with respect to macromolecularly bound steroid. This suggested the possibility that an inhibitor of activation had been removed or diluted. In this report, we present data in support of such a modulating component as at least one factor in the process of activation of the rat liver glucocorticoid receptor.

Experimental Procedures

Preparation of Cytosol. Livers from adrenalectomized male CD Sprague-Dawley rats were homogenized in an equal volume of TSM buffer (50 mM Tris-HCl[1], pH 8.0, at 0 °C, 0.25 M sucrose, 3 mM $MgCl_2$) and cytosol prepared as previously described (Cake and Litwack, 1975). The cytosol was incubated for 3 h at 0–4 °C with [^{3}H]dexamethasone (New England Nuclear; 22.6 Ci/mmol) at a final concentration of 30 nM in the presence or absence of an 1000-fold excess of nonradioactive dexamethasone dissolved in ethylene glycol. Cytosol was stored in the vapor phase of liquid nitrogen without significant loss of specific steroid binding for 2–3 months.

Specific Binding of Steroid to Protein. Specific steroid binding was determined by three different procedures: (1) the dextran-coated charcoal technique (Beato et al., 1972), (2) gel filtration on Sephadex G-25 (1.5 × 6 cm) using TSM buffer as eluent, and (3) the DEAE-cellulose filter procedure (Baxter et al., 1975). Specifically bound steroid represents the difference in [^{3}H]dexamethasone binding in the presence and absence of 1000-fold excess of nonradioactive dexamethasone.

DNA–Cellulose Binding Assay. The binding of glucocorticoid receptor to DNA–cellulose was determined by the procedure of Kalimi et al. (1975) using saturating amounts of DNA–cellulose. The DNA–cellulose (P-L Biochemical) is native calf thymus DNA adsorbed onto a cellulose matrix. Two-hundred microliters of a 25% slurry of DNA–cellulose and 100 μL of cytosol were incubated at 0–4 °C for 45 min. At

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[1] Abbreviations used are: HTC, hepatoma tissue culture; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

TABLE I: Effect of Washing on DNA-Cellulose Binding.

Procedure	DNA Binding (dpm/500 μL) Total[a]	Nonspecific[b]	Specific[c]
3× wash	18 700	30	18 670
No wash	26 200	5900	20 300
Cellulose	4 600	3900	700
Difference[d]	21 600	2000	19 600
(Wash/difference[d]) × 100	86.6	1.5	95.3

[a] Cytosol was incubated with 30 nM [^{3}H]dexamethasone for 3 h at 0 °C prior to heat activation. [b] Cytosol was incubated with 30 nM [^{3}H]dexamethasone + 30 μM nonradioactive dexamethasone for 3 h at 0 °C prior to heat activation. [c] Specific binding represents the difference between total and nonspecific binding. [d] No wash − cellulose.

the end of the incubation, 2 mL of cold TE buffer (10 mM Tris-HCl, pH 8.0, at 0 °C, 1 mM EDTA) was added. The samples were washed three times with the same buffer. The final pellet was suspended in 0.8 mL of TE buffer and 0.5 mL was taken for determination of radioactivity as previously described (Weinstein et al., 1967). DNA was determined by the method of Burton (1956) using salmon sperm DNA as standard. The data presented represent the mean of at least three experiments, except where indicated.

Results

DNA-Cellulose Assay. The activation of the glucocorticoid receptor has been measured by its ability to bind to DNA-cellulose using the procedure of Kalimi et al. (1975). Studies by Kalimi and his co-workers (Kalimi and Beato, 1973; Kalimi et al., 1975) and in our laboratory (unpublished observations) indicate that the interaction of the activated receptor with DNA-cellulose is similar to the binding of receptor to nuclei. To confirm that all of the activated receptor was indeed binding to the DNA-cellulose, the following experiment was performed. Activated receptor was incubated with DNA-cellulose for 45 min at 0 °C, the DNA-cellulose was pelleted (DNA-1), and the supernatant was incubated with fresh DNA-cellulose for 45 min at 0 °C (DNA-2). Both pellets were washed and aliquots were taken for counting. Approximately 90% of the activated receptor was bound to DNA-1 and the remainder was bound to DNA-2, indicating that the procedure measures about 90% of the activated receptor. This result is comparable to the removal of activated cytosol receptor by saturating levels of homologous nuclei in the HTC cell system (Higgins et al., 1973) and in the rat liver system (Atger and Milgrom, 1976a,b).

In the assay procedure described, the DNA-cellulose pellets are washed three times before counting. Simons et al. (1976) have reported a rapid rate of dissociation of activated HTC cell receptor from DNA-cellulose which results in significant under estimates of the activated form in washed DNA-cellulose pellets. We have compared washed pellets with unwashed pellets and included binding to cellulose alone as background. Our results (Table I) indicate that only about 5% of the [^{3}H]dexamethasone specifically bound to receptor is lost during the wash procedure, while the nonspecifically bound radioactivity is reduced by 99% by the washes. Consequently, the use of the wash procedure is well justified in our system.

Assay of [^{3}H]Dexamethasone-Receptor Complex. Higgins et al. (1973) have reported that the charcoal adsorption procedure does not accurately measure steroid specifically bound to receptor in the activated state. We have compared three methods for determining steroid-receptor complex: dextran-coated charcoal adsorption, gel filtration on Sephadex G-25, and retention on DEAE-cellulose filters (see Experimental Procedures). The data presented in Table II clearly show that dextran-coated charcoal and gel filtration gave essentially identical values for both activated and nonactivated [^{3}H]-dexamethasone-receptor complex. DEAE-cellulose filters, however, underestimated the amount of receptor specifically bound to steroid. Having established the reliability of the dextran-coated charcoal procedure, the ease and rapidity of this method, as compared with gel filtration, justified the use of the dextran-coated charcoal procedure in the present study.

TABLE II: Comparison of Methods for Determining Specific Steroid Binding.

State of activation[a]	Charcoal (SD)	Sephadex G-25 (SD)	DEAE-cellulose filter
Nonactivated	81 000 (3000)	78 700 (8600)	44 261
Activated	82 300 (4300)	83 600 (6000)	49 337

[a] p = 0.55 for nonactivated sample and 0.45 for activated sample.

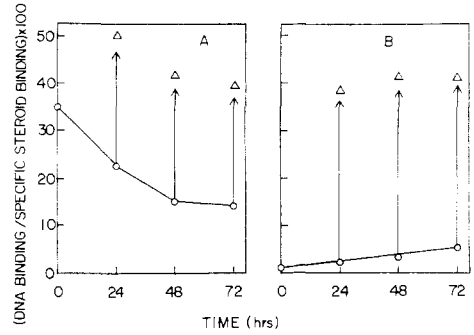

FIGURE 1: Heat activated receptor. (A) Deactivation of heat activated receptor. [^{3}H]Dexamethasone-labeled cytosol was incubated at 25 °C for 30 min, and aliquots were taken for specific steroid binding and DNA-cellulose binding at the times indicated while being stored at 0-4 °C. Arrows indicate times at which aliquots were taken for reactivation by heat treatment. (○) Deactivation; (△) reactivation by heat treatment. Percent of initial specific steroid binding was 92, 78, and 58% at 24, 48, and 72 h, respectively. (B) Activation of nonactivated receptor. [^{3}H]Dexamethasone-labeled cytosol was maintained at 0-4 °C as a control for the heat-activated samples. Aliquots were taken as described above for specific steroid binding and DNA-cellulose binding and for activation by heat treatment (arrows). (○) Activation of nonactivated cytosol; (△) activation by heat treatment. Percent of initial specific steroid binding was 112, 102, and 77% at 24, 48, and 72 h, respectively.

Reversibility of Heat Activation. To determine whether, once activated by heat, the receptor remained in the activated state, [^{3}H]dexamethasone-labeled cytosol was activated by heating at 25 °C for 30 min and aliquots were taken at various times for determination of specific steroid binding and DNA-cellulose binding. Figure 1A shows the apparent deactivation of receptor with time at 0-4 °C after heat activation. Activation by heat generally resulted in 30-40% of the steroid-receptor complex being bound to DNA-cellulose at zero time (immediately following activation). After 72 h, only 14% of the receptor remained activated. The conclusion that the loss of DNA-cellulose binding capacity was independent

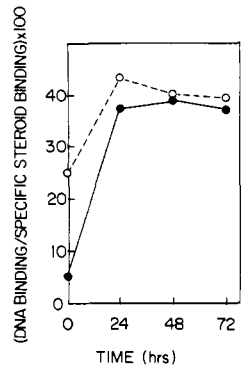

FIGURE 2: Activation and deactivation by Sephadex G-25 treatment. [^{3}H]Dexamethasone-labeled cytosol (500 μL) was applied to a column of Sephadex G-25 (1.5 × 6 cm) and eluted with TSM buffer containing 30 nM [^{3}H]dexamethasone. After discarding the first 3 mL, the next 2.5 mL was taken as macromolecular bound steroid. Aliquots were taken for determination of specific steroid binding and DNA-cellulose binding. The remainder was stored at 0–4 °C and assayed again at the indicated times. The control cytosol was diluted with TSM buffer to 10% with respect to cytosol, comparable to the dilution resulting from the chromatographic procedure. (○) Sephadex G-25 activated cytosol; (●) control cytosol. Percent of initial specific steroid binding was 78, 57, and 27% at 24, 48, and 72 h, respectively for the Sephadex G-25 sample and 95, 73, and 70% for the diluted sample.

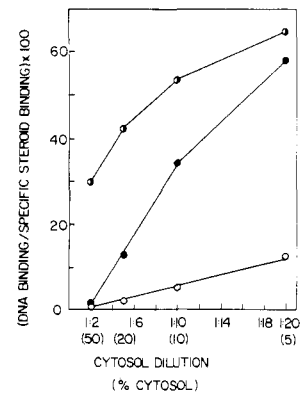

FIGURE 3: Effect of dilution on activation. [^{3}H]Dexamethasone-labeled cytosol was diluted with TSM buffer containing 30 nM [^{3}H]dexamethasone to the indicated levels with respect to cytosol and a portion incubated at 25 °C for 30 min. Specific steroid binding and DNA-cellulose binding were determined at zero time and again for the nonactivated samples after 24 h at 0–4 °C. (○) Nonactivated, t_0; (◑) heat activated, t_0; (●) nonactivated, t_{24}. Percent of initial specific steroid binding after 24 h was 139, 101, 102, and 87 for 50, 20, 10, and 5% cytosols, respectively.

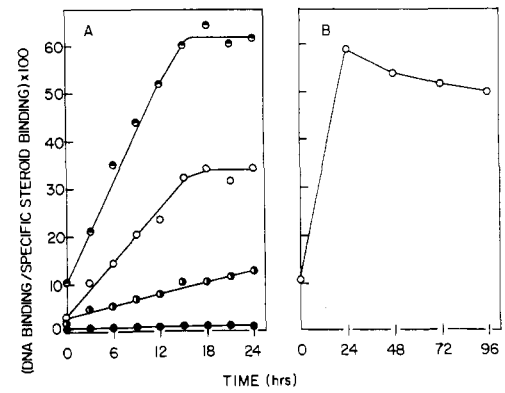

FIGURE 4: Time course of activation and deactivation after dilution. [^{3}H]Dexamethasone-labeled cytosol was diluted with TSM buffer to the indicated levels with respect to cytosol. Specific steroid binding and DNA-cellulose binding were determined at the indicated times. Samples were maintained at 0–4 °C at all times. (A) Activation. Data represent the mean of two experiments. (●) Fifty percent cytosol; (◑) 20% cytosol, (○) 10% cytosol; (◓) 5% cytosol. Percent of initial specific steroid binding remaining at 24 h was 132, 106, 86, and 65% for 50, 20, 10, and 5% cytosols, respectively. (B) Deactivation of 5% cytosol. Percent of initial specific steroid binding was 83, 45, 33, and 28% at 24, 48, 72, and 96 h, respectively.

of dissociation of [^{3}H]dexamethasone from receptor seemed reasonable, since the DNA-cellulose bound receptor is expressed as a percentage of the receptor specifically bound to steroid at any given time. As previously reported (Milgrom et al., 1973; Kalimi et al., 1975), nonactivated receptor became activated very gradually with time at 0–4 °C (Figure 1B). Both nonactivated receptor and receptor activated by heat at zero time could be reactivated at any time during the course of these studies (Figure 1A,B, arrows) and the deactivation of reactivated samples was essentially the same as that shown in Figure 1A. However, when cytosol was repeatedly activated every 24 h, the total amount of receptor capable of binding to DNA-cellulose never exceeded the original amount of steroid-receptor complex (data not shown). This finding suggests that at each round of activation only previously nonactivated receptor becomes activated.

Activation by Gel Filtration. The removal of free steroid by passage over a column of Sephadex G-25 resulted in the activation of receptor, as seen in Figure 2. Activation by Sephadex G-25 was as efficient as heat activation, generally resulting in 25–30% of the specifically bound steroid in the activated state. Unlike heat activation, however, Sephadex G-25 activated receptor was still activated at 24 h and, in fact, the degree of activation was often greater at 24 h than at zero time. The chromatographic procedure results in a 1:5 dilution of the cytosol. When the Sephadex G-25 activated sample was concentrated fivefold, the degree of activation was only marginally reduced (data not shown). Activation could also be effected by Bio-Gel P4, Sephadex G-15, and Sephadex G-50 (Cake et al., 1976). These results suggested the possibility that the mechanism of activation involves the removal of small molecule(s).

Activation by Dilution. The nonactivated sample in Figure 2 was diluted with TSM buffer containing 30 nM [^{3}H]dexamethasone to 10% with respect to cytosol for comparison with the Sephadex G-25 treated sample, which is similarly diluted by the chromatographic procedure. It became apparent after several experiments that the sample which had been diluted but not chromatographed consistently resulted in a marginally higher level of activation at zero time than that observed in undiluted samples (50% cytosol). By 24 h, however, the diluted nonchromatographed sample showed a significantly greater degree of activation than undiluted samples (see Figure 2). To determine whether dilution by itself could indeed cause activation of the receptor, a series of dilutions with TSM buffer was made on the standard 50% cytosol, and specific steroid binding and DNA-cellulose binding was measured at zero time and at 24 h. Figure 3 shows that the degree of activation at 0–4 °C increased with dilution when measured at zero time. At 24 h, activation was markedly enhanced with increasing dilution. Heat activation of the diluted samples augmented the zero-time activation level. When heat-activated samples were diluted immediately before the DNA-cellulose assay, the degree of activation was only marginally increased over the level attained by heat treatment alone (data not shown), suggesting that dilution affects the state of activation of the steroid-receptor complex rather than its binding to DNA-cellulose. The time course of activation by dilution is shown in Figure 4A. Not

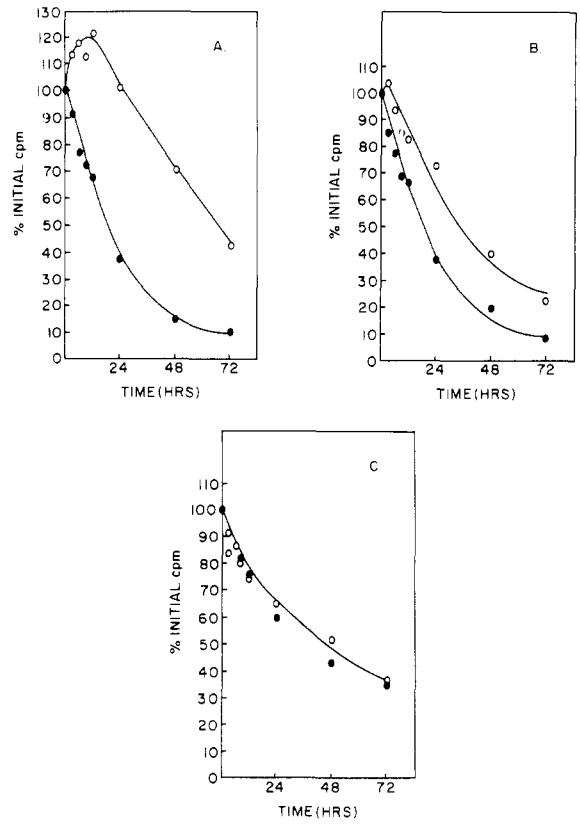

FIGURE 5: Dissociation and rebinding of steroid to receptor. [^{3}H]Dexamethasone-labeled cytosol was maintained in the nonactivated state or activated by heat (25 °C for 30 min) or Sephadex G-25 treatment. Unlabeled dexamethasone in ethylene glycol was then added to half of each sample at a final concentration of 30 μM (●). An equal volume ($^1/_{100}$ sample volume) of ethylene glycol was added to the other half of each sample (○). All samples were maintained at 0-4 °C. Aliquots were taken at the indicated time for determination of specific steroid binding. (A) Nonactivated. Rate constant for dissociation (k_d) = 0.0379 ± 0.0015/h. (B) Heat activated. k_d = 0.0354 ± 0.0021/h. (C) Sephadex G-25 activated. k_d = 0.0167 ± 0.0019/h.

only the degree of activation but the rate of activation increased with increasing dilution. This figure also demonstrates that the final degree of activation reached with time attained an equilibrium which is determined by the degree of dilution of the cytosol. This conclusion was also supported by the study of the heat activation before and after dilution (data not shown). It can also be seen in Figure 4A that the $t_{1/2}$ for the different equilibrium states is similar, about 6 h, suggesting that the rate constants are approximately the same. In addition, preliminary studies at very high dilutions of cytosol suggest that there may be a maximal rate of activation (data not shown). Figure 4B shows that in a 5% cytosol the DNA-cellulose binding capacity is lost only in proportion to the loss of macromolecularly bound steroid, indicating that deactivation did not occur.

Rate of Steroid-Receptor Dissociation and Rebinding. In the course of these studies it became evident that, although there was considerable variation from one experiment to the next, specific steroid binding in activated cytosol consistently decreased at a much faster rate than in nonactivated cytosol. Consequently, the rate of steroid dissociation from activated and nonactivated receptor was compared. Cytosol labeled with [^{3}H]dexamethasone was activated by heat or Sephadex G-25 and 1000-fold excess cold dexamethasone was added to half of each sample. An equal volume of ethylene glycol (vehicle) was added to the other half. Aliquots were then taken at various times for determination of specific steroid binding. In the presence of excess cold steroid, both heat activated cytosol and nonactivated cytosol lost [^{3}H]dexamethasone at the same rate (Figure 5A,B), while the loss from Sephadex G-25 activated receptor occurred at a slightly slower rate (Figure 5C). In the absence of excess cold steroid (ethylene glycol alone), however, a marked difference was observed in steroid binding between activated and nonactivated samples. An initial increase in specifically bound [^{3}H]dexamethasone was routinely observed in nonactivated samples regardless of the length of time allowed for complete binding. After 24 h, steroid was lost at a slow rate with 50% of the original steroid binding remaining after more than 2 days. In activated samples, the loss of steroid binding was much more rapid with less than 40% of the original steroid binding remaining after 2 days. These findings support the conclusion that the rate of dissociation of steroid from activated and nonactivated receptor is similar. However, nonactivated receptor is capable of rebinding free steroid at least initially, while activated receptor has a reduced capacity for rebinding of steroid.

Elimination of pH Effect in Removal of Low-Molecular-Weight Component. The activation of receptor by gel filtration (see Figure 2) suggested that the low-molecular-weight components of cytosol, which were separated from the macromolecular components by the chromatographic procedure, participate in the activation process. That this involvement of a small molecule(s) does indeed occur has been demonstrated in a previous report (Cake et al., 1976). Not only was the steroid-receptor complex activated by gel filtration, but this activation was prevented by the presence of the low-molecular-weight components of cytosol during gel filtration. As previously reported (Goidl et al., 1976), activation could also be prevented by diluting the [^{3}H]dexamethasone-receptor complex with unlabeled whole cytosol as compared to dilution with buffer. However, since the degree of activation of the receptor is affected by pH (Parchman, Goidl, and Litwack, unpublished observations), the following experiment was performed in order to distinguish between pH effects and the presence of small molecules, the removal of which presumably results in activation. A relatively large volume (15 mL) of [^{3}H]dexamethasone-labeled cytosol was applied to a column of Sephadex G-25 (10 mL bed volume) which had been equilibrated with buffer (50 mM Tris-HCl, 3 mM $MgCl_2$) at either pH 7.0 or 8.0 at 0 °C. Elution was carried out using the same buffer and 1-mL fractions were collected. As previously described (Cake et al., 1976), this procedure resulted in fractions, eluting immediately after the void volume, which were undiluted with respect to macromolecules but separated from small molecules. With continued elution, the small molecules began to elute and, eventually, fractions were obtained which contained both macromolecules and small molecules at essentially the same concentration as the applied cytosol. When the Sephadex G-25 column was equilibrated and eluted at pH 8.0 (Figure 6A), the early fractions containing macromolecules separated from small molecules were activated and able to bind to DNA-cellulose. With continued elution, the degree of activation of the [^{3}H]dexamethasone-receptor complexes declined. When the small molecules reached the concentration of the applied cytosol, as shown by the elution profile of KCl, the steroid-receptor complexes were virtually inactive with respect to DNA-cellulose binding. The pH of the eluted fractions also decreased from pH 8.0 to 7.0 as the small molecules began to elute, suggesting the possibility that the decrease in DNA-cellulose binding capacity might be due to the decrease in pH. However, as shown in Figure 6B, steroid-receptor complexes separated from small molecules were ac-

tivated even when the pH was maintained at pH 7.0 by equilibrating and eluting the Sephadex G-25 column with buffer at pH 7.0. In this case, the total amount of activated receptor was less than that observed at pH 8.0. It seems clear from this study that, although the pH of the system affects the degree of activation, a small molecule(s) is indeed involved in the state of activation of the glucocorticoid receptor.

Discussion

The effect of dilution on activation was first reported by Higgins et al. (1973). Dilution was considered as a condition which influenced protein association, suggesting the possibility that activation involves the disaggregation of the HTC cell glucocorticoid-receptor complex into subunits. These conditions also favor the dissociation of an inhibitor of activation from the complex. The first suggestion that an inhibitor of activation might exist in rat liver cytosol was made by Milgrom et al. (1973) on the basis of enhanced activation after partial purification of the receptor. This inhibitory effect was thought to be due to the direct interaction of nonreceptor macromolecules with the receptor (Milgrom and Atger, 1975). More recently, by studying the kinetics of activation of various dilutions of rat liver cytosol, these workers concluded that the rate of thermal activation is not dependent on the concentration of cytosol and, therefore, the mechanism of activation does not involve the interaction of receptor with another component of cytosol (Atger and Milgrom, 1976a). The increase in rate of activation upon dilution which we have observed is clearly inconsistent with their findings. Furthermore, the progesterone-receptor complexes from rabbit and guinea pig uterus are activated by dilution and gel filtration (Saffran et al., 1976) in a manner comparable to the results which we have presented. The presence of an inhibitor of activation became apparent (Simons et al., 1976) when the dexamethasone-receptor complex of HTC cells was diluted with homologous cytosol. Similar findings have also been observed in the nuclear binding of the uterine estradiol-receptor using either homologous or heterologous cytosol (Chamness et al., 1974).

The effect of dilution on activation presented in this report is consistent with a mechanism involving either disaggregation of protein subunits or dissociation of an inhibitor. That an inhibitor of less than 1500 molecular weight is involved in the process of activation is supported by the gel-filtration studies. Such a "modulator" presumably maintains the glucocorticoid receptor in rat liver cytosol in the nonactivated state. Activation occurs experimentally by removal of this component by gel filtration or dilution or by changing the equilibrium, possibly reversibly, by heat treatment. The inhibitory factor described by Simons et al. (1976) was associated with the macromolecular fraction of HTC cell cytosol, suggesting that both macromolecular and micromolecular modulating components may exist.

Because activated and nonactivated receptors behave differently with respect to steroid dissociation and rebinding, it has not been possible to distinguish between true deactivation and loss of steroid from the activated complex. Conclusive resolution of the question of reversibility of activation requires a homogeneous population of activated steroid-receptor complexes. To date, our attempts to obtain such a population have been unsuccessful. A recently published procedure for the separation of activated and nonactivated forms of the receptor (Atger and Milgrom, 1976b) may prove fruitful for these studies.

In addition to pursuing this problem, current experiments in our laboratory are directed toward identifying the low-molecular-weight component. The possibility that additional factors are indeed involved in the process of activation, such as conformational alterations of the steroid-receptor complex, is also under investigation.

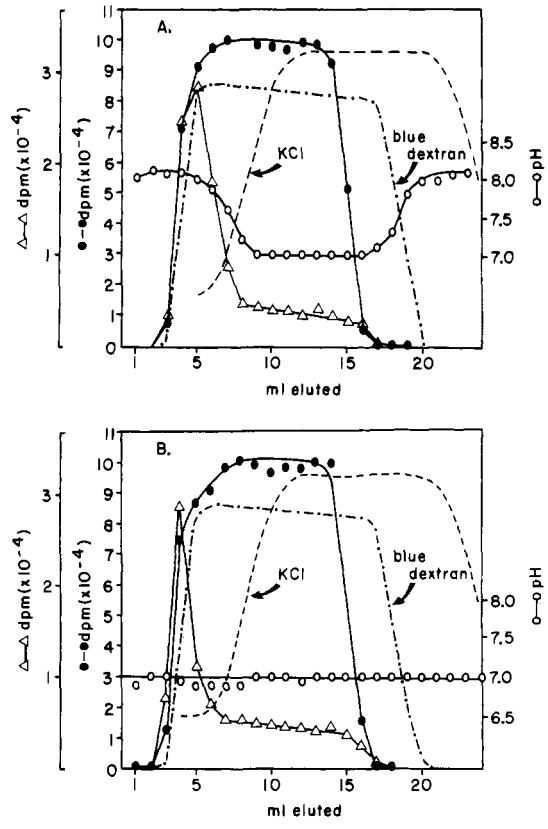

FIGURE 6: Removal of small molecules from cytosol at pH 8.0 and pH 7.0. [^{3}H]Dexamethasone-labeled cytosol (15 mL) was applied to a column of Sephadex G-25 (1.5 × 6 cm) and eluted with buffer (50 mM Tris-HCl, 3 mM $MgCl_2$), and 1-mL fractions were collected. Each fraction was assayed for specific steroid binding (●) and DNA-cellulose binding (△). The pH of each fraction was also determined (○). The columns were characterized using a solution of blue dextran and KCl in buffer at the appropriate pH. (A) Column equilibrated and eluted with buffer at pH 8.0. (B) Column equilibrated and eluted with buffer at pH 7.0.

Acknowledgments

We thank Dr. Robert Small of the Biometrics Department of Temple University School of Medicine for his statistical analysis of the steroid dissociation and rebinding data. We also thank Dr. V. L. Schramm of the Department of Biochemistry for many helpful discussions.

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Sodium-Dependent Methyl 1-Thio-β-D-galactopyranoside Transport in Membrane Vesicles Isolated from *Salmonella typhimurium*†

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ABSTRACT: Membrane vesicles isolated from *Salmonella typhimurium* G-30 grown in the presence of melibiose catalyze methyl 1-thio-β-D-galactopyranoside (TMG) transport in the presence of sodium or lithium, as shown initially with intact cells by Stock and Roseman (Stock, J., and Roseman, S. (1971), *Biochem. Biophys. Res. Commun. 44,* 132). TMG-dependent sodium uptake is also observed, but only when a potassium diffusion potential (interior negative) is induced across the vesicle membrane. Cation-dependent TMG accumulation varies with the electrochemical gradient of protons generated as a result of D-lactate oxidation, and the vesicles catalyze D-lactate-dependent sodium efflux in a manner which is consistent with the operation of a proton–sodium exchange mechanism. Although the stoichiometry between sodium and TMG appears to be 1:1 when transport is induced by a potassium diffusion potential, evidence is presented which indicates that the relationship may exceed unity under certain conditions. The results are explained in terms of a model in which TMG–sodium (lithium) symport is driven by an electrochemical gradient of protons which functions to maintain a low intravesicular sodium or lithium concentration through proton–sodium (lithium) antiport.

Recent studies confirm the hypothesis that chemiosmotic phenomena, as postulated by Mitchell (1961, 1966, 1968, 1973), are responsible for respiration-linked active transport in membrane vesicles isolated from *Escherichia coli* (for reviews, see Harold, 1972, 1976; Ramos et al., 1976; Ramos and Kaback, 1977a,b; Kaback, 1976). Oxidation of electron donors which drive transport in the vesicles leads to the development of an electrochemical gradient of protons across the membrane, and it has been shown that this thermodynamic entity is composed of interconvertible electrical and chemical parameters according to the following relationship:

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - \left(\frac{2.3RT}{F}\right)\Delta pH \qquad (1)$$

where $\Delta\bar{\mu}_{H^+}$ represents the electrochemical gradient of protons, $\Delta\Psi$ denotes the electrical potential across the membrane, and ΔpH is the chemical difference in proton concentrations across the membrane ($2.3RT/F$ = 58.8 mV at room temperature).

Evidence has also been presented (Harold, 1972, 1976; Ramos and Kaback, 1977a,b; Kaback, 1976) which indicates that $\Delta\bar{\mu}_{H^+}$ or one of its components is the immediate driving force for the accumulation of a variety of different solutes and that accumulation of certain solutes most probably occurs via coupled movements with protons (i.e., symport). By this means, a substrate-specific membrane protein (i.e., a porter or carrier) translocates substrate with one or more protons, the substrate moving against and the proton(s) with their respective electrochemical gradients. The net result, substrate accumulation, is accomplished energetically by removing protons from the internal space.

One attractive conceptual aspect of the chemiosmotic hypothesis for bacterial active transport is its analogy to the mechanism suggested for sugar and amino acid transport in many eucaryotic cells (Crane, 1977). In these systems, an electrochemical gradient of sodium, rather than protons, is generated through the action of the membraneous sodium, potassium-dependent ATPase, and accumulation of sugars and amino acids occurs via coupled movements with sodium (this process is referred to traditionally as cotransport rather than symport).

Although it is almost certain that many bacterial transport systems catalyze proton–substrate symport, several instances have been reported in which the transport of a specific solute is dependent upon the presence of sodium or lithium ion (Drapeau et al., 1966; Wong et al., 1969; Thompson and MacLeod, 1971; Sprott and MacLeod, 1972; Harold and Baarda, 1967; Harold et al., 1970; Stock and Roseman, 1971;

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