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# Activation of Progesterone Receptor by ATP

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Progesterone-receptor complex from freshly prepared hen oviduct cytosol acquired the ability to bind to isolated nuclei, DNA-cellulose and ATP-Sepharose when incubated with 5–10 mM ATP at 4 °C. The extent of this ATP-dependent activation was higher when compared with heat-activation achieved by warming the progesterone-receptor complex at 23 °C. The transformation of progesterone-receptor complex which occurred in a time-dependent manner was only partially dependent on hormone presence. The ATP effect was selective in causing this transformation whereas ADP, AMP and cAMP failed to show any such effect. The non-hydrolizable analogs of ATP, adenosine 5′-[α,β-methylene]triphosphate and adenosine 5-[β,γ-imido]triphosphate were also found to be ineffective. Presence of 10 mM sodium molybdate blocked both the ATP and the heat-activation of progesterone-receptor complex. $Mn^{2+}$ or $Mg^{2+}$ had no detectable effect on the receptor activation but the presence of $Ca^{2+}$ increased the extent of ATP-activation slightly. EDTA presence (> 5 mM) decreased the extent of receptor activation by about 40% and was, therefore, not included in the buffers used for activation studies. Divalent cations were also ineffective when tested in the presence of 1–5 mM EDTA. The steroid-binding properties of progesterone-receptor complex remained intact under the above conditions when analyzed for steroid-binding specificity and Scatchard analysis. However, the ATP-activated progesterone-receptor complex lost the ability to aggregate when tested on low-salt sucrose gradients. ATP was equally effective in activating the rat-uterine estradiol-receptor complex at 4 °C and influenced the transformation of 4-S receptor form into a 5-S form when analyzed on sucrose gradients containing 0.3 M KCl. The presence of ATP also increased the rate of activation of progesterone-receptor complex at 23 °C. These findings suggest a role for ATP in receptor function and offer a convenient method of studying the process of receptor activation at low temperature and mild assay conditions.

Steroid hormones are known to initially interact with their target cells to form steroid-receptor complexes at physiological temperatures. The complexes so formed are located in the cytoplasm from where they translocate into the nucleus [1]. On the other hand, upon their extraction from the target tissue cytosol, the steroid receptors have very little affinity to bind to nuclear sites. This nuclear binding capacity can be acquired *in vitro* under high ionic conditions [2–4] or by incubating the receptor preparations at elevated temperatures [2,3] in the presence of hormone. The avian progesterone-receptor complex 'activated' by such treatments is then able to bind to isolated nuclei or chromatin [5–7], DNA-cellulose [8], phosphocellulose [9] and ATP-Sepharose [10]. Although it is generally assumed that the binding *in vivo* of steroid induces conformational change(s) in the receptor molecule leading to the activation of the latter, the knowledge about the physico-chemical changes occurring during receptor activation has remained limited. Recently, activation of steroid hormone-receptor complexes in intact target cells has been described at physiological conditions [11,12].

An interaction between steroid receptors and ATP has been demonstrated by use of affinity chromatography [13–16]. While the functional significance of this ATP binding has remained obscure, it has been shown that ATP-Sepharose binding is a property of an activated steroid receptor [10,16]. In addition, preliminary reports from this laboratory have shown an ATP-dependent activation of glucocorticoid-receptor complex from rat liver [17,18]. In the present study, we present evidence that treatment of steroid-receptor complexes with ATP at 4 °C can induce transformation of non-activated progesterone-receptor and estradiol-receptor complexes to activated receptor forms that show increased affinity for nuclei, DNA-cellulose and ATP-Sepharose. ATP activation is a mild procedure and is carried out at low temperature, and therefore offers a convenient means of studying receptor activation.

*Note.* The term activation refers to an alteration *in vitro* of cytosol receptor to a form which binds to isolated nuclei, DNA-cellulose or ATP-Sepharose.

## MATERIALS AND METHODS

All reagents were of analytical grade and were made up in deionized water. All procedures were carried out at 4 °C unless indicated otherwise. Nucleotides, thioglycerol, activated charcoal, and progesterone were from Sigma; and sodium molybdate was purchased from Merck. [$^3$H]R5020 (70–80 Ci/mmol) and [2,3,6,7(n)-$^3$H]estradiol (98.5 Ci/mmol) were obtained from New England Nuclear.

*Buffers.* Buffer A contained 10 mM Tris/HCl, 12 mM monothioglycerol, 0.01 M KCl and 20% (v/v) glycerol pH 8.0. Buffer B was the same as buffer A except it contained 1 M KCl. Buffer C contained 10 mM Tris/HCl, 12 mM monothioglycerol pH 8.0.

*Preparation of ATP-Sepharose and DNA-Cellulose.* ATP was covalently linked to Sepharose-4B as described previously [13,14] and our preparations contained 7–10 μmol of nucleotide/ml of packed Sepharose as determined by phosphate analysis [19]. DNA-cellulose was prepared according to the method of Alberts and Herrick [20]. Calf thymus

DNA (Sigma, type II) was linked to Cellex-410 (Bio-Rad) and preparations contained 1.5–2 mg DNA/ml packed DNA-cellulose as quantitated by the method of Burton [21]. DNA-cellulose was stored as a slurry and was hydrated in buffer C for two days at 4 °C before use.

*Preparation of Progesterone Receptor.* Freshly excised oviducts from white Leghorn hens were obtained from a local produce company. The tissue was rinsed with cold 0.9 % NaCl, transferred to 2 vol. (w/v) of buffer [40 mM Tris/HCl, 12 mM thioglycerol and 10 % glycerol (v/v) pH 8.0] and homogenized with a Tissumizer (Tekmar model SDT). The homogenate was centrifuged at $12000 \times g$ for 10 min followed by $150000 \times g$ for 90 min. The resulting supernatant ('cytosol') containing progesterone receptor was used immediately for studies on receptor activation.

*Preparation of Estradiol Receptor.* Female albino rats of Yale strain (18–20 days) were obtained from Spartan (Haslett, MI). The rats were killed by cervical dislocation, uteri stripped of fat, excised and minced. The tissue was then homogenized in 2 vol. (w/v) of the homogenization buffer (described earlier) and the cytosol was obtained by centrifugation at $12000 \times g$ for 10 min followed by $150000 \times g$ for 90 min.

*Activation of Cytosol Progesterone-Receptor Complex.* The freshly prepared hen oviduct cytosol was incubated with 10 nM [$^3$H]R5020, a non-metabolizable analog of progesterone, for 2 h at 4 °C to form [$^3$H]R5020-receptor complex in a total volume of 1–2 ml. Following this, the mixtures were treated with different concentrations of ATP (prepared in 10 mM Tris/HCl, and pH readjusted to 8.0) and incubated at 4 °C for 40–60 min. Parallel samples in triplicate containing steroid-receptor complexes were incubated at 23 °C with and without ATP to compare the extent of receptor activation. The control samples contained steroid-receptor complexes incubated at 4 °C with buffer C and no ATP. The extent of activation of steroid-receptor complexes was then measured by determining their uptake by isolated nuclei, and binding to DNA-cellulose or ATP-Sepharose.

*Nuclear Binding Assay.* The procedures for determining the nuclear uptake of [$^3$H]R5020 were as described previously [6, 7]. The cytosol [$^3$H]R5020-receptor complexes activated by either an exposure to 23 °C or treatment with ATP were incubated with hen oviduct nuclei prepared by the methods of Spelsberg et al. [22] and each assay tube contained 50–100 µg DNA [21]. After 1 h gentle shaking at 4 °C, the nuclei were washed twice with 3–5 ml buffer (0.01 M Tris/HCl, 0.001 M $MgCl_2$ and 10 % glycerol pH 7.5) and centrifuged at $1000 \times g$ for 10 min. Each pellet was resuspended in 2 ml of scintillation fluid (Beckman Bio-Sol BBS-3, Spectrafluor PPO-POPOP and toluene, 2:1:15, v/v/v) and transferred to a vial containing 3 ml of the same fluid for measurement of radioactivity.

*Measurement of Activation by Binding to Affinity Resins.* The extent of receptor activation was measured by using both batch assays and column chromatography procedures. In a batch assay, the affinity resin was taken in small ($\approx$ 1.5 ml) aliquots in culture tubes (13 × 100 mm) and equilibrated with buffer A. The activated receptor-complexes were then transferred to these tubes and the contents mixed continuously for 30 min–1 h over an Ames aliquot mixer. Subsequently, the tubes were centrifuged at $1000 \times g$ for 10 min and the supernatant discarded. The pellets containing ATP-Sepharose-bound steroid-receptor complexes were washed twice with 2 ml of buffer A. The complexes were then recovered by treating the washed pellets with buffer B, mixing the contents for 15 min over aliquot mixer and centrifugation at $1000 \times g$ for 10 min. Aliquots of supernatants were used to measure radioactivity due to steroid-receptor complexes. The contribution of radioactivity due to free steroid or non-specific steroid binding was calculated to be less than 5 %. The control values were obtained by measuring the radioactivity from aliquots containing steroid-receptor complexes which were kept at 4 °C without ATP treatment.

In column chromatography measurements, packed 1–2 ml ATP-Sepharose or DNA cellulose columns were equilibrated with buffer A. Aliquots containing steroid-receptor complexes (0.5–1 ml) were applied and the columns washed with 20 column volumes of buffer A. The adsorbed complexes were eluted with buffer B and fifteen 1–5-ml fractions were thus collected. Aliquots (0.5 ml) were used to determine the radioactivity.

In batch assays and in column chromatography procedures utilizing ATP-Sepharose, the activated steroid-receptor complexes were treated with Dextran-coated charcoal pellets (0.5 % of Norit A, 0.05 % Dextran T-70 made in 10 mM Tris/HCl pH 8.0) to remove free ATP, which if left unremoved, competitively reduces the binding of receptor to ATP-Sepharose.

*Sucrose Gradient Analysis.* Linear 5–20 % sucrose gradients (4.4 ml) were prepared in buffer containing 10 mM Tris, 12 mM thioglycerol and either 0–0.1 M KCl or 0.3 M KCl, using a Beckman gradient former. Samples (0.2 ml) containing steroid-receptor complexes with and without activation treatments were layered onto the gradients. Receptor samples were diluted with buffer C to lower the glycerol concentration. The gradients were centrifuged at $150000 \times g$ either for 16 h in a Beckman L-75 ultracentrifuge using SW 50.1 rotor or for 3 h on Sorval OTD-65 ultracentrifuge in a TV-865 vertical rotor. [$^{14}$C]Ovalbumin [23] was layered on a separate gradient as a standard for the determination of sedimentation coefficients [24]. The fractions were collected by piercing the bottom of each tube.

*Steroid Binding Assays.* Charcoal adsorption assays were performed to monitor the total and non-specific steroid binding. Triplicate aliquots (0.1 ml) from receptor preparations were incubated with 10 nM [$^3$H]R5020, or [$^3$H]estradiol for 2 h at 4 °C in a total volume of 0.5 ml. The data were corrected for non-specific binding which was determined by including excess of unlabeled R5020 or diethylstilbestrol for progesterone and estradiol receptor respectively. An equal volume of Dextran-coated charcoal suspension was added to each tube and the contents mixed gently. The mixtures were centrifuged at $1000 \times g$ for 10 min after a 5-min incubation at 4 °C. The supernatants were used to measure the radioactivity [13, 14].

Radioactivity was determined by combining aqueous samples with 5 ml scintillation fluid consisting of toluene and spectrafluor (Amersham) (1000:42, v/v).

*Statistical Evaluation of the Results.* The results were expressed (wherever appropriate) as the mean $\pm$ S.E.M. of triplicate determinations in a single experiment. Each experiment was repeated 3–6 times with identical results.

## RESULTS

### *Effect of ATP on the Activation of Progesterone-Receptor Complex*

In cell-free systems, activation of cytosol steroid-receptor complexes can be accomplished under a variety of experi-

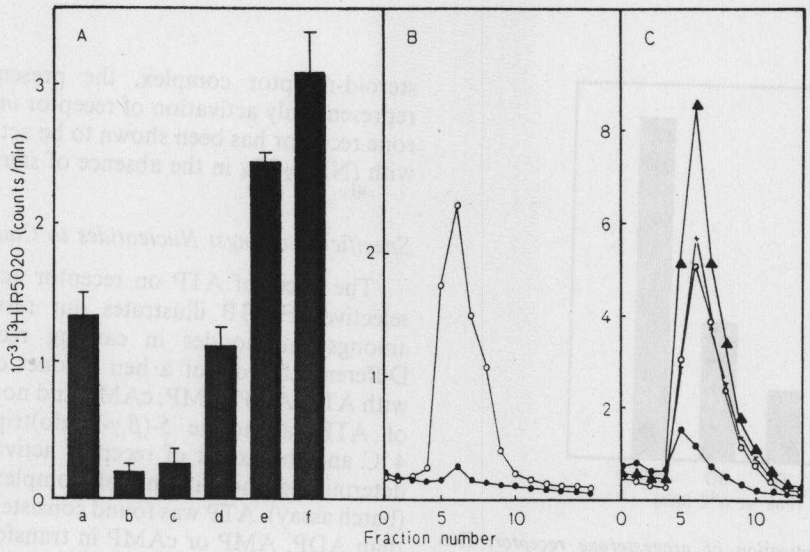


Fig. 1. *The effect of ATP on the activation of progesterone-receptor complex.* (A) The freshly prepared cytosol was labeled with 10 nM [$^3$H]R5020 for 2 h at 4 °C. Portions (0.4 ml) of this preparation were treated with buffer C (b) or with different concentrations of ATP (c: 1 mM; d: 2 mM; e: 5 mM; f: 10 mM) for 40 min at 4 °C in a total volume of 0.5 ml. Aliquots in triplicate (a) were also exposed to 23 °C for 40 min. The extent of receptor activation was measured by performing nuclear binding assays as described in Materials and Methods. (B) Aliquots (0.5 ml) containing progesterone-receptor complex were incubated with buffer C (●——●) or 10 mM ATP (○——○) for 40 min at 4 °C in a total volume of 1 ml. The complexes were mixed with 1 ml packed DNA-cellulose and the slurry was shaken for 30 min at 4 °C. Subsequently, the slurry was poured into columns which were washed with 20 vol. of buffer A. 15 1-ml fractions were collected and used to determine radioactivity. (C) The cytosol [$^3$H]R5020-receptor complexes were formed as described above and divided equally into four groups. The complexes (1 ml) were either activated by a 40 min incubation at elevated temperature (23 °C) or by treatment with 10 mM ATP at 4 °C or both. All the samples were treated with charcoal pellets to remove excess ATP. The complexes after such treatment were then chromatographed over 2 ml ATP-Sepharose columns pre-equilibrated with buffer A. 15 1.5-ml fractions were collected with buffer B. (●——●) 4 °C, no ATP; (×) 4 °C + 10 mM ATP; (○——○) 23 °C; (▲) 10 mM ATP, 23 °C

mental conditions like cytosol dilution, treatment with salts, alterations in pH or by brief incubation at elevated temperatures [2–4,7,9,25]. Once activated, the steroid-receptor complexes acquire the ability to bind to nuclei, DNA-cellulose or ATP-Sepharose [5,6,10,16]. In the present studies when freshly prepared [$^3$H]R5020-receptor complex was incubated with different concentrations of ATP at 4 °C for 40 min, it showed increased affinity to bind to isolated nuclei (Fig. 1 A). The effect of ATP on nuclear binding was observed at concentrations over 1–2 mM. The nuclear uptake of progesterone-receptor complex increased gradually exhibiting a maximum at 10 mM nucleotide which accounted for 45% of the total progesterone-receptor complex present as against a modest nuclear binding (4%) in the absence of ATP treatment. ATP treatment at 4 °C appeared to be more effective in causing receptor activation when compared with heat-activation (23 °C at 40 min) in the absence of the nucleotide.

A DNA-binding site for steroid receptors has been postulated [1,4,9,26]. It has been further shown that the DNA binding is a property of an activated receptor [8]. Fig. 1 B shows that the cytosol progesterone-receptor complex has very little affinity to bind to DNA-cellulose. A 40-min incubation of such a preparation with 10 mM ATP at 4 °C allowed a sizeable portion (25%) of the original receptor-complex to be retained on the DNA-cellulose column which could be successfully eluted with high-salt buffer. The observed increase in the DNA or nuclear binding of progesterone receptor could not be accounted for by an increase in the available steroid-receptor complexes following ATP treatment, since the nucleotide was added after the steroid binding by the receptor had reached its maximum.

Because of the efficiency and convenience of the technique, ATP-Sepharose binding has been used as a measure of receptor activation [10,16,27,28]. Identical results were obtained when activation of receptor was measured by its binding to isolated nuclei, DNA-cellulose or phosphocellulose [10,28]. Therefore it is believed that the results illustrated in the following experiments using ATP-Sepharose accurately reflect the receptor activation process as it occurs in a cell-free system [28].

Fig. 1 C illustrates our results on activation of progesterone receptor by ATP as measured by receptor binding to ATP-Sepharose. Less than 10% of the total cytosol [$^3$H]R5020-receptor complexes adsorbed to ATP-Sepharose. A 40-min incubation of such preparations with 10 mM ATP at 4 °C resulted in an increased (5–7-fold) receptor binding to the affinity resin. A comparable ATP-Sepharose binding was observed upon heat-activation of cytosol receptor allowing about 50% of the original complexes to be retained and subsequently eluted from the columns. In addition, a complimentary effect was seen when ATP was included during the heat-activation procedure which resulted in a significant increase in the extent of receptor activation and binding of a majority (70–80%) of the complexes to ATP-Sepharose.

The transformation of non-activated cytosol complexes to an activated form occurred in a time-dependent manner. The maximum extent of activation was observed to be about a 60-min incubation of nucleotide (10 mM) with cytosol preparations as 4 °C (Fig. 2). Receptor activation has been shown to be influenced by the ionic strength of the incubation mixture or its ingredients [2–4]. The possibility was considered that activation of receptor could have resulted from an ionic strength generated by high concentration ATP. The ionic strength contribution by ATP even up to 1 M concentration was found to be minimal (not shown) and therefore suggests

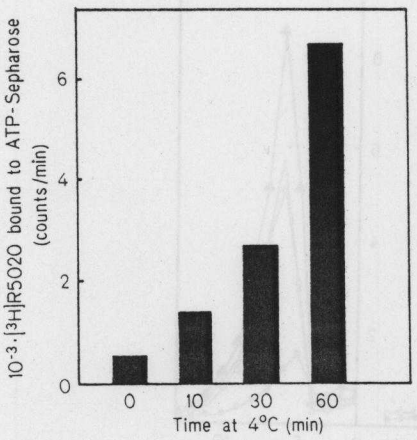


Fig. 2. *Time-course of ATP-activation of progesterone receptor.* Hen oviduct cytosol was complexed with 13 nM [$^3$H]R5020 for 90 min at 4 °C. The complexes were then incubated with and without ATP for the periods shown in the figure. Free nucleotide was removed by pelleted charcoal treatment and preparations mixed, centrifuged at $1000 \times g$ for 10 min. The supernatants were used immediately for chromatography over identical columns of ATP-Sepharose ($\approx$ 2 ml). 15 1-ml fractions were collected each with buffer A (low-salt) and buffer B (1 M KCl). The peak fractions eluted with 1 M KCl from each column were pooled and radioactivity expressed in as shown in figure

that the nucleotide must act via a selective mechanism to cause receptor activation.

*Requirement of Hormone Presence for ATP-Activation*

The steroid receptors *in vitro* undergo an activation process which is dependent on the presence of hormones [29]. Fig. 3A compares the hormone-dependence of progesterone receptor during its activation either by ATP-treatment at 4 °C or by an exposure to 23 °C. The results of this and following experiments using ATP-Sepharose were obtained by performing batch assays which proved convenient and allowed greater number of samples to be analyzed. At low temperature (4 °C), the cytosol receptor showed very little binding to ATP-Sepharose. A 60-min treatment of the receptor-complexes with 10 mM ATP at 4 °C or incubation at 23 °C resulted in an increased receptor binding to ATP-Sepharose. The extent of receptor-activation was significantly greater when preparations were treated with ATP than when incubated at 23 °C. However, if cytosol was incubated at 23 °C in the absence of hormone, cooled, and mixed with 10 nM [$^3$H]R5020 and followed by ATP-Sepharose assay, no activation was seen (open bars). The inability of receptor treated at 23 °C to be subsequently activated by ATP may be due to the effect of elevated temperature on receptor destruction. On the other hand, ATP treatment did not distinguish between occupied or unoccupied receptor and yielded preparations which contained almost equally activated receptor. The extent of receptor activation was, however, slightly lower in the absence of the steroid. ATP may also be stabilizing the unoccupied receptor in the absence of hormone and therefore account for the activation seen in Fig. 2. An increased steroid binding by ATP-treated unoccupied receptor has been reported [18]. This observation has important implications in that it may be difficult to envisage such a process of receptor activation in the absence of steroid in a biological system. Although studies are in progress to determine the influence of ATP *in vivo* on activation of progesterone receptor or steroid-receptor complex, the present studies could also represent only activation of receptor *in vitro*. Avian progesterone receptor has been shown to be activated by precipitation with $(NH_4)_2SO_4$ in the absence of steroid [7].

*Specificity amongst Nucleotides to Cause Receptor Activation*

The effect of ATP on receptor activation appears to be selective. Fig. 3B illustrates our results on the specificity amongst nucleotides in causing receptor transformation. Different aliquots of a hen oviduct cytosol were incubated with ATP, ADP, AMP, cAMP and non-metabolizable analog of ATP [adenosine 5-($\beta$,$\gamma$-imido)triphosphate] for 1 h at 4 °C and the extent of receptor activation was measured by determining the binding of complexes to ATP-Sepharose (batch assay). ATP was found consistently to be more effective than ADP, AMP or cAMP in transforming the cytosol progesterone-receptor complex to an activated form. The ATP analogs were able to activate the receptor only slightly above the background level. Another non-metabolizable analog of ATP, adenosine 5′-[$\alpha$,$\beta$-methylene]triphosphate was also ineffective in transforming the receptor to an activated state (not shown). Our preliminary studies indicate that other nucleoside triphosphates may also promote receptor activation (Fig. 3B, inset). This would indicate that the hydrolysis of ATP, particularly of the terminal phosphate, may play some role in the process of receptor activation. On the other hand, the inability of ATP analogs to induce activation may suggest the involvement of specific structural arrangement between receptor and nucleotides that is not possible with the analogs. A phosphorylation-dephosphorylation mechanism has been suggested to determine the level of active receptor capable of steroid binding [30]. Whether a similar mechanism exists at the level of receptor activation is not clear at the moment. It is possible that in an analogous physiological condition, ATP metabolism may provide energy for the cytoplasmic steroid-receptor complex to translocate onto sites in the nucleus [13]. Although the ATP metabolism at 4 °C is slow, metabolism of only a fraction of the added nucleotide may actually be required either to provide energy or to phosphorylate the receptor protein which exists at much lower concentration. Conversely, free ATP may be binding to the receptor and altering its conformation by exposing certain groups which facilitate binding to nuclei, DNA-cellulose or ATP-Sepharose.

*Effect of Sodium Molybdate on ATP Activation*

Sodium molybdate has been shown to inhibit the salt or heat-activation of thymocyte glucocorticoid receptor [30] and temperature-activated avian progesterone receptor [28, 31, 32] and rat uterine receptor [33]. Fig. 3C demonstrates that molybdate (10 mM) when added prior to or with ATP, blocks the receptor activation. However, if the progesterone-receptor complex is incubated first with ATP for 1 h and then treated with molybdate, receptor activation still occurs. These results indicate that molybdate is also an inhibitor of ATP-dependent receptor activation and acts selectively on the non-activated form of the receptor and has no observable influence on an already transformed steroid-receptor complex. The effect of molybdate appears to be on the rate of activation rather than on the extent of activation of progesterone-receptor. When receptor preparations were incubated with ATP in the presence of molybdate for over 90 min – 2 h, a decrease in the inhibition of activation was observed (data not shown).

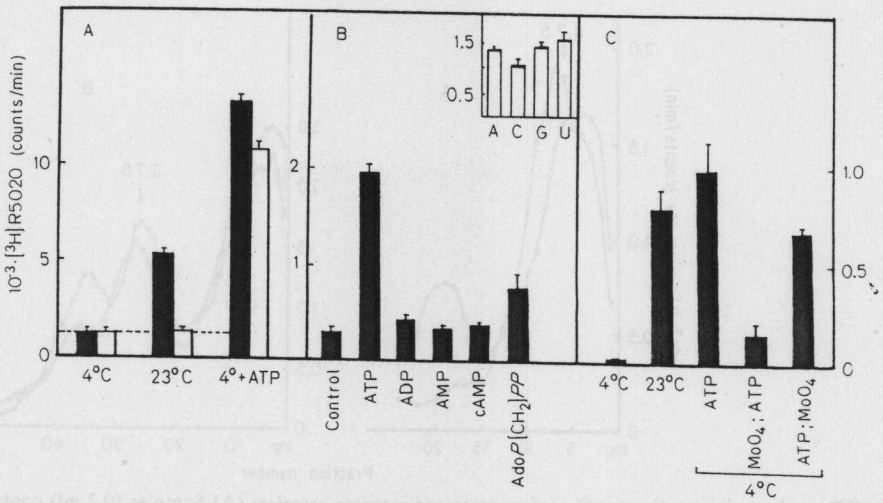


Fig. 3. *Nucleotide activation of progesterone receptor.* (A) Requirement of hormone for the ATP-activation of progesterone receptor. The freshly prepared hen oviduct cytosol was evenly divided into two groups. Cytosol receptor from group I was complexed with 8 nM [$^3$H]R5020 for 2 h at 4 °C and divided into three sub-groups. The duplicate aliquot (3 ml) from each sub-group was activated by a 1 h incubation at 23 °C or by treatment with 10 mM ATP at 4 °C. The control samples remained at 4 °C and contained no ATP. Group II was treated similarly except that the activation steps were performed prior to the addition of hormone. After these treatments, the contents of all the tubes from both groups were chilled and transferred to separate tubes containing pelleted Dextran-coated charcoal to remove excess steroid and ATP. The tubes were stirred briefly and centrifuged at 1000 × *g* for 10 min. The supernatant from each tube containing progesterone-receptor complexes was mixed separately with 2 ml packed ATP-Sepharose and the batch assays were performed to quantify the binding of complexes to ATP-Sepharose as described in Materials and Methods. (■) Receptor complex + treatment; (□) receptor + treatment + hormone. (B) Specificity amongst nucleotides in the activation of progesterone-receptor complex. Cytosol aliquots (2 ml) were incubated with 10 nM [$^3$H]R5020 for 2 h at 4 °C in a total volume of 3 ml and treated with different nucleotides (10 mM) for 1 h at 4 °C. A batch assay was used to determine the ATP-Sepharose binding capacity of progesterone-receptor complex. The nucleotides were removed prior to the incubation of receptor preparations with ATP-Sepharose and the procedures used were the same as described in the legend of Fig. 2. AdoP[$CH_2$]PP, adenosine 5′-[$\beta,\gamma$-imido]triphosphate; A, ATP; C, CTP; G, GTP; U, UTP. (C) Effect of sodium molybdate on the activation of progesterone-receptor complex. The progesterone-receptor complex (1 ml) formed as described earlier was either kept at 4 °C or heat/ATP-activated for 1 h. Sodium molybdate (10 mM) was included along with ATP (10 mM) during activation or added after ATP treatment was complete. 15 min after such treatment, the samples were charcoal-treated, centrifuged and the supernatant mixed with 2 ml packed ATP-Sepharose. The extent of receptor activation was determined by measuring the binding of progesterone-receptor complex to ATP-Sepharose using batch assay. $MoO_4$, sodium molybdate.

This observation is consistent with the findings of Nishigori et al. [34] who observed an inhibition of progesterone receptor activation by vanadate, a chemical similar to molybdate, to be on the rate of activation rather than on the maximum extent of receptor transformation.

### *Inhibition of the ATP-Dependent Activation*

In recent years, a number of chemical compounds have been identified which block receptor properties [6, 27, 28, 31, 32, 34–36]. These inhibitors have the potential to characterize site(s) on receptor involved in such processes as receptor activation, translocation and interaction of receptor complexes with nuclear constituents. The results of our attempts to identify inhibitors of ATP-activation of progesterone receptor are summarized in Table 1. The inhibitor concentrations used in the table represent those that have been found to be most effective in various studies and which do not destroy the steroid-receptor complexes. The compounds were added to the preparations that contained fully formed progesterone-receptor complexes. Charcoal adsorption assays were also performed to determine the effects of these compounds on the dissociation of the complexes. At the concentrations used and reported in Table 1, there was no loss of steroid from the steroid-receptor complexes. Pyridoxal 5′-phosphate, *o*-phenanthroline and sodium levamisole appeared to have no significant effect on receptor activation by ATP whereas sodium molybdate, tungstate and rifamycin AF/013 blocked receptor activation by more than 40%. Heparin and aurin

Table 1. *Chemical inhibitors of the process of progesterone-receptor activation by ATP*

Freshly prepared hen oviduct cytosol was complexed with 10 nM [$^3$H]-R5020 for 2 h at 4 °C. A series of tubes was set up (in duplicate) in a final volume of 0.5 ml and contained 0.15 ml of receptor complex, 20% glycerol, 10 mM ATP and different inhibitors at concentrations shown in the table. The contents of tubes were mixed and incubated for 1 h at 4 °C. Following this, 0.5 ml Dextran-coated charcoal suspension was added to each tube to remove free nucleotides and inhibitors. Following centrifugation at 1000 × *g* for 5 min, portions (0.7 ml) of supernatant were used to measure the extent of receptor activation by using ATP-Sepharose batch assays

| Inhibitor | Concentration | [$^3$H]R5020-receptor complex bound to ATP-Sepharose |
|---|---|---|
| | mM (μg/ml) | % |
| Control | – | 100 |
| Pyridoxal 5′-phosphate | 5 | 84 |
| *o*-Phenanthroline | 3 | 83 |
| Sodium levamisole | 10 | 84 |
| Sodium tungstate | 10 | 59 |
| Sodium molybdate | 10 | 19 |
| Rifamycin AF/013 | (175) | 11 |
| Heparin | (300) | 0 |
| Aurin tricarboxylic acid | 0.02 | 0 |

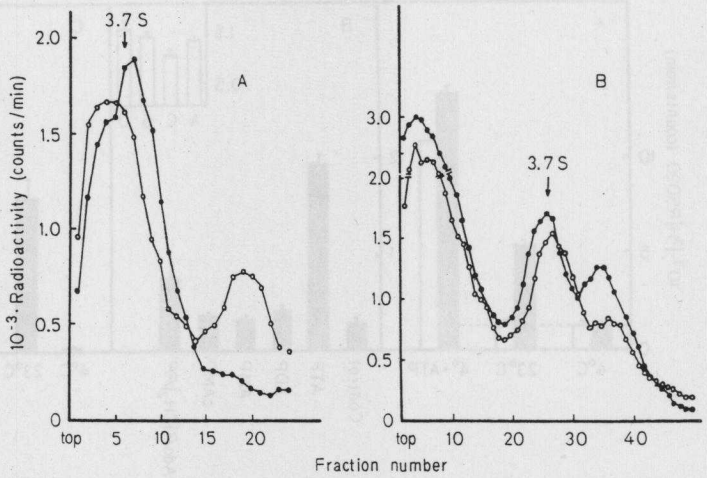


Fig. 4. *Effect of ATP on the sedimentation profiles of progesterone-receptor complex.* (A) Samples (0.2 ml) containing [$^3$H]R5020-receptor complex were treated with 10 mM ATP or buffer C for 1 h at 4 °C in a total volume of 0.3 ml. Aliquots (0.2 ml), were then layered on 5–20% linear sucrose gradients containing 0.01 M KCl. [$^{14}$C]ovalbumin was centrifuged on a separate gradient as a sedimentation standard (3.7 S). (○——○) Control; (●——●) + 10 mM ATP. (B) Aliquots (0.2 ml) containing [$^3$H]estradiol-receptor complexes from rat uterus cytosol were mixed with 0.1 ml buffer C or equal volume of ATP (10 mM, final concentration) and allowed to stand at 4 °C for 1 h. Samples (0.2 ml) were layered onto 5–20% sucrose gradients containing 0.3 M KCl and centrifuged on a Beckman L5-75 ultracentrifuge for 16 h in an SW 50.1 rotor. [$^{14}$C]ovalbumin (3.7 S) was centrifuged in a separate gradient. The 0.1 ml fractions were collected by piercing the bottom of each gradient tube. (○——○) Control; (●—— _ ●) ATP-treated

tricarboxylic acid completely inactivated the receptor's ability to bind to nuclei or ATP-Sepharose following incubation with 10 mM ATP. These inhibitors are known to act by different mechanisms and it is not clear as to how they block ATP-activation. They may be interacting with the ATP-binding sites of the receptor or with sites which are exposed following the action of ATP and are involved in the binding to nuclei or ATP-Sepharose.

### *Effects of ATP on the Sedimentation Properties of Progesterone-Receptor Complex*

Fig. 4A illustrates the sedimentation profile of progesterone-receptor complex on 5–20% sucrose gradients under low-salt conditions. The non-activated receptor-complex (○——○) migrated as an aggregate in the region of 7–8 S in addition to its sedimentation as a entity of 3.5–4 S. However, after activation at 23 °C (not shown) or at 4 °C with 5–10 mM ATP (●——●), the receptor changed to a form of 4–5 S under low ionic conditions. These results suggest that sedimentation behavior of non-activated progesterone receptor in low-salt gradients is altered upon activation and is consistent with the similar findings of Nishigori and Toft [28] and Schrader et al. [9]. Thus, ATP activation appears to be comparable to other procedures used to induce receptor transformation.

Receptor activation is considered to involve structural changes in the receptor molecule which enable it to interact with sites on nuclear chromatin or polyanionic resins in general [2]. It has also been suggested that the steroid-receptor complexes may be present under two different conformations which possibly differ by the presence (activated form) or the absence (non-activated form) of positively charged groups at the surface of the molecule, thus changing its affinity for polyanions [2]. The actual molecular events in receptor activation or the specific conformational changes that occur in the receptor molecule remain uncertain. The most dependable conformational change, however, has been reported in the mammalian estrogen receptor which appears to dimerize upon activation, and this change can be detected by sedimentation analysis [37]. Fig. 4B illustrates our results on the sedimentation of [$^3$H]estradiol-receptor complex from rat uterus on 5–20% linear sucrose gradients containing high-salt (0.3 M KCl). A majority of the cytosol receptor sedimented in the 4-S region (○——○) with a shoulder in the 5-S region representing some partial activation of receptor. Upon activation by ATP treatment (●——●), the receptor developed a distinct peak at 5 S characteristic of an activated estradiol receptor. Similar peaks at 5 S could be generated by activating the receptor by warming the cytosol receptor complexes at 23 °C. This ATP or heat-induced alteration in the rate of sedimentation of estradiol-receptor complex was reproducible in five separate experiments. Presence of 10 mM sodium molybdate before or during receptor activation blocked the conversion of the 4-S form to the 5-S form. Both 4-S and 5-S receptor peaks in Fig. 4 appear elevated, suggesting a possible stabilizing effect of ATP. That the results in Fig. 4 represented an activation or transformation phenomenon was confirmed by further testing estrogen receptor binding to isolated nuclei and DNA-cellulose as a measure of receptor activation (not shown). Therefore, ATP presence alters the non-activated form of steroid receptors in a fashion which is similar to changes induced by conventional procedures of receptor activation.

### *Effects of Divalent Cations and EDTA on Receptor Activation*

Divalent cations have been reported to be effective in causing changes in steroid-receptor properties [3, 38, 39]. In the present studies the effects of $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ (0.1–5 mM) were investigated on the process of receptor activation using ATP-Sepharose batch assay measurements. These cations were ineffective in causing receptor activation by themselves or influencing the ATP-caused activation (not shown). Only calcium appeared to increase the extent of receptor activation by about 15%. Although endogenous ions may be influencing the activation of cytosol receptor. the exogen-

ous amounts appeared to have no effect when added alone or along with ATP. Presence of EDTA has been observed to have adverse effects on the process of receptor activation. Jensen et al. [29] had noted previously that EDTA decreases the extent of activation of rat uterine estradiol receptor, while Nishigori et al. [34] have reported that EDTA can reverse the inhibitory effects of vanadate. Since the divalent cations were ineffective in influencing the process of receptor activation *in vitro* by ATP, the possibility was considered that addition of a chelator, like EDTA, might increase the effectiveness of the cations. In our studies (not shown) EDTA (> 5 mM) presence caused 35–40% decrease in the extent of ATP-activation of progesterone receptor. The pattern of divalent cation effect observed (not shown) did not alter in the presence of EDTA. Because of its inhibitory effects, EDTA was not included in the buffers used in activation studies.

### *Effect of ATP on the Resolution of Progesterone Receptor Forms A and B*

The chick oviduct has been reported to contain two distinct forms of progresterone receptor which differ significantly in physico-chemical characteristics [40] and can be separated by chromatography over DEAE-cellulose. We attempted to investigate whether ATP treatment of the cytosol would alter either the proportion of receptor forms A to B or convert one to the other. Fig. 5 illustrates results of chromatography of cytosol progesterone-receptor complexes incubated with and without 10 mM ATP. The preparations were treated with Dextran-coated charcoal prior to chromatography over the ion-exchanger to remove excess or unbound ATP. The nucleotide treatment caused a significant reduction in the A peak eluted with 0.15 M KCl with only a minor loss of binding in the peak B. Since in an ion-exchange chromatography, the net charge on the protein molecules is of immense importance. it is possible that incubation with nucleotide induced a conformational change in the receptor molecule causing the positively charged groups to move out [2] resulting in a lower binding of the receptor in the first peak. The decreased receptor binding in the first peak was compensated by a greater receptor content in the flow-through (not shown). On further quantification of total receptor content in DEAE-Sephacel flow-through, 0.15 M and 0.3 M KCl fractions, equal amounts of progesterone-receptor complexes were found to be present in control and ATP-treated samples. This argument is further strengthened by the reports that peak A represents a DNA binding form [40] and that the DNA binding is a property of an activated receptor. The interpretation of the results from the above experiment is difficult and arbitrary and the exact mechanism is not clear.

### *Specificity of Steroid Binding and Scatchard Analysis*

Although an increase in binding of progesterone-receptor complex to affinity resins or nuclei could result from an increase in the steroid binding by receptor or from a stabilizing effect of ATP on the progesterone receptor complex; our results suggest otherwise. The steroid binding by the progesterone receptor from cytosol is not altered in the presence of upto 10 mM ATP whether the nucleotide is added before or after the formation of steroid-receptor complex (not shown). Also, ATP treatment neither alters the kinetic properties of progesterone receptor as analyzed by Scatchard analysis nor changes its steroid binding specificity (Fig. 6). The steroid-binding specificity and equilibrium constant ($K_d$)

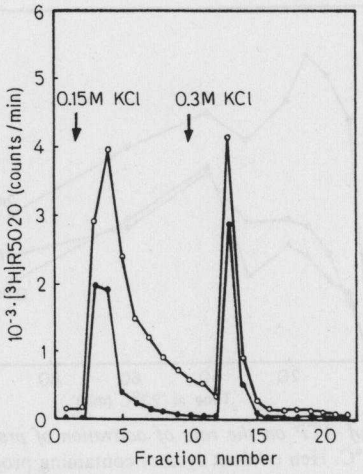


Fig. 5. *Effect of ATP-treatment on the resolution of progesterone-receptor over DEAE-Sephacel.* Hen oviduct cytosol containing progesterone-receptor complex was divided into two groups. Group A was incubated with buffer C for 1 h at 4 °C while group B received 10 mM ATP. The total volume of the sample was adjusted to 1 ml. Both groups were separately chromatographed over 5-ml columns of DEAE-Sephacel. The columns were washed with 30 ml buffer A and 12 2-ml fractions were then collected with buffer C containing 20% glycerol and either 0.15 M KCl or 0.3 M KCl. (○——○) Control; (●——●) + 10 mM ATP

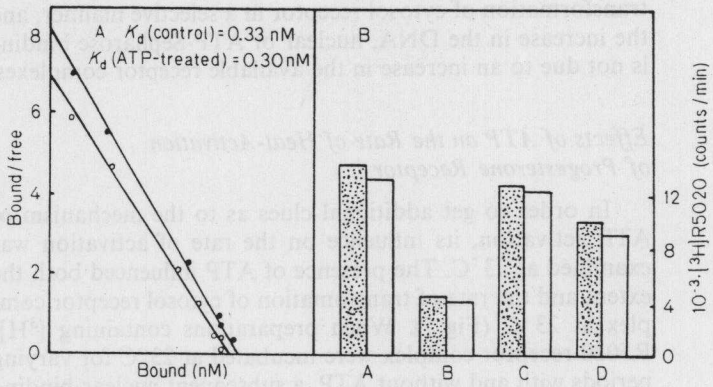


Fig. 6. *Effect of ATP on the steroid-binding properties of progesterone-receptor.* (A) Comparison of control and ATP-treated receptor by Scatchard analysis. Steroid binding was measured by preparing two series of incubation tubes (in duplicate) containing 0.1 ml cytosol, 0.2–31 nM [$^3$H]R5020 in a total volume of 0.5 ml. The incubation mixture contained 20% glycerol. One series of tubes (●) contained 10 mM ATP. (○) Control. Parallel tubes containing 1 μM unlabeled R5020 were used for background determination. After 2 h at 4 °C, the binding was measured by charcoal adsorption assay. (B) Effect of ATP on the steroid-binding specificity of progesterone-receptor. Aliquots (0.1 ml) of hen oviduct cytosol were incubated in two series of tubes containing 10 nM [$^3$H]R5020, 20% glycerol and excess (1 μM) unlabeled steroids (B–D) in a total volume of 0.5 ml. One series contained 10 mM ATP (□). After 2 h at 4 °C, the steroid binding was measured by charcoal binding assays. (A) Control; (B) + R5020; (C) + cortisol; (D) + estradiol

were essentially unchanged by the nucleotide presence. ATP has been shown to stabilize the steroid receptor proteins [18, 41, 42]. We observed that the avian progesterone receptor behaves quite differently as no stabilization effect of the nucleotide was observed when the receptor complexes were incubated at elevated temperature. As noted earlier, ATP does not appear to either increase the steroid-binding capacity of progesterone receptor or influence the dissociation or

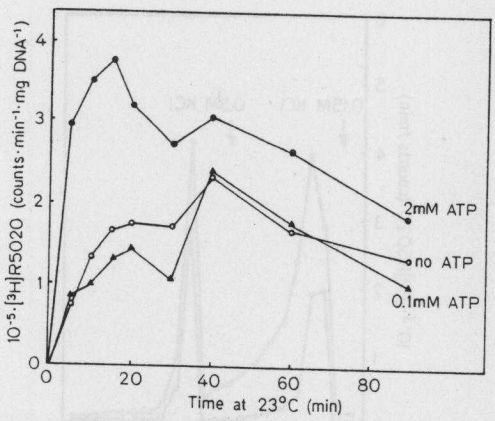


Fig. 7. *Effect of ATP on the rate of activation of progesterone-receptor complex at 23 °C.* Hen oviduct cytosol containing progesterone-receptor complexes formed at 4 °C, was divided into three sets consisting of a series of tubes (in duplicate). One set received buffer C (control, ○——○) while others received either 0.1 mM ATP (▲——▲) or 2 mM ATP (●——●). All three sets were then incubated immediately at 23 °C for time periods shown in the figure. The extent of receptor activation was then measured by nuclear binding assays

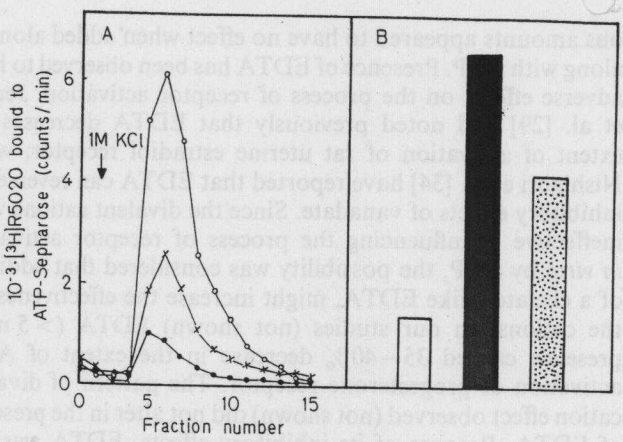


Fig. 8. *Activation of progesterone-receptor fractionated by poly(ethyleneglycol).* The freshly prepared cytosol was fractionated with 15% saturation of poly(ethyleneglycol) 6000 (Sigma). The precipitate was stored at −80 °C and dissolved in 1/10 cytosol volume of buffer C just prior to use. The redissolved preparation was incubated with 12 nM [$^3$H]-R5020 for 2 h at 4 °C. Following this, aliquots containing receptor complexes were activated by either incubation with 10 mM ATP for 1 h at 4 °C or by warming at 23 °C for 1 h. Control samples received buffer C only and were kept at 4 °C. The extent of receptor activation was determined by measuring the binding of receptor complexes to ATP-Sepharose using either column chromatography (A) or batch assay (B). (A): (●——●) control, 4 °C; (×——×) 23 °C, 1 h; (○——○) 10 mM ATP, 1 h at 4 °C. (B): (□), control, 4 °C; (■) 10 mM ATP, 1 h at 4 °C; (▦) 23 °C, 1 h

stability of the receptor complexes at 4 °C or at 37 °C (not shown). Therefore, the nucleotide must be influencing the transformation of cytosol receptor in a selective manner, and the increase in the DNA, nuclear or ATP-Sepharose binding is not due to an increase in the available receptor complexes.

### *Effects of ATP on the Rate of Heat-Activation of Progesterone Receptor*

In order to get additional clues as to the mechanism of ATP activation, its influence on the rate of activation was examined at 23 °C. The presence of ATP influenced both the extent and the rate of transfomation of cytosol receptor complex at 23 °C (Fig. 7). When preparations containing [$^3$H]-R5020-receptor complex were incubated at 23 °C for varying periods with and without ATP, a subsequent nuclear-binding assay revealed a 2−3-fold increase in the nuclear uptake of complexes pretreated with higher concentrations of ATP (> 1 mM). However, lower nucleotide concentrations (< 1 mM) had no significant effect on the rate of activation of progesterone-receptor complex. The findings are consistant with those of Toft et al. [43] who observed similar effects of the nucleotide on the rate of heat-transformation of avian progesterone receptor.

### *Activation of Poly(ethyleneglycol)-Fractionated Receptor*

The preparation of fresh cytosol for each experiment on the process of receptor activation offers an inconvenient and cumbersome procedure. Attempts were made to store the receptor preparations in a precipitate form which still retained the receptor in a non-activated state. Ammonium sulfate fractionation yields a receptor which binds to isolated nuclei and DNA-cellulose [7] and hence is in an activated state. We fractionated the hen oviduct cytosol with 15% polyethylene glycol and stored the precipitated receptor pellets at −80 °C. The preparations could be redissolved, labeled with steroid, and the resulting progesterone-receptor complexes could be activated by an incubation with ATP at 4 °C. Fig. 8 illustrates an ATP-activation of poly(ethyleneglycol)-fractionated receptor as measured by its binding to ATP-Sepharose using either columns (Fig. 8A) or batch assays (Fig. 8B). Even in these preparations ATP was quantitatively more effective in activating the receptor at 4 °C than the incubation of receptor preparations at 23 °C. Fractionation with poly(ethyleneglycol) should, therefore, serve as a useful procedure to concentrate the receptor protein in a non-activated form which can be stored at low temperature until needed.

## DISCUSSION

We have presented evidence that suggests that the progesterone-receptor complex from hen oviduct can be activated by a treatment with ATP at 0−4 °C. This phenomenon appears to be of general occurrence as rat uterine estradiol receptor (Fig. 4B) and hepatic glucocorticoid receptor [16, 17] also exhibit the same responses. The activation achieved by incubation of receptor with ATP is comparable to the transformation of receptor reported by other conventional procedures. ATP activation allows a 10−15-fold increase in binding of receptor to nuclei, DNA cellulose or ATP-Sepharose, and is a better quantitative procedure of receptor activation. ATP has also been reported to influence a variety of receptor properties including stabilization of glucocorticoid receptor [30] and rate of heat-activation of progesterone receptor [43]. The exact mechanism by which ATP brings its effects on steroid-receptors has remained unlcear.

Binding to ATP-Sepharose requires an activated form of receptors [10, 15, 16, 43]. Cytosol progesterone receptor is in non-activated form and transforms into an activated form in presence of free ATP. This would mean that ATP affects both non-activated and activated receptor forms. Activated receptor appears to have a binding site for ATP as revealed by affinity chromatography studies [10, 13, 14, 16, 44]. The

ATP effects seen on the non-activated receptor could be indirect. The other possibility is that ATP binds to a non-activated receptor and shifts the equilibirium in favor of an activated receptor form which then binds to polyanionic resins or ATP-Sepharose more readily. The inability of ATP analogs, adenosine 5′-[$\beta,\gamma$-imido]triphosphate and adenosine 5′-[$\alpha,\beta$-methylene]triphosphate, to influence receptor activation suggests that an enzymatic process possibly involving phosphorylation-dephosphorylation may be involved in the process of receptor activation [30,40–42,45]. At 4 °C, involvement of an enzyme reaction for receptor activation may appear unlikely but can not be ruled out. The role of molybdate is unclear at this point. Receptor activation has been shown to be blocked by molybdate [28,30–32] and it has been proposed [45] that a dephosphorylation step may be necessary for translocation of cytosol receptor onto nuclear sites [45]. The results of present studies would argue that phosphorylation, instead of dephosphorylation, may be involved in receptor activation and that the inhibitory action of molybdate seen on receptor activation may be due to a direct interaction between molybdate and the receptor.

Finally, ATP-activation of receptor offers another technique for studying *in vitro* the activation of steroid-receptors in general. The procedure is convenient, quantitative, less cumbersome and is carried out at mild assay conditions which would minimize the structural alterations and metabolism of the cytosol components. In addition to its advantages in studying the receptor activation, the method should help reveal information on the receptor site(s) involved in the process of activation. Since ATP enhances the rate of heat-activation, the nucleotide may be a biologically important modifier of progesterone receptor activation.

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