*Proc. Natl. Acad. Sci. USA*
Vol. 75, No. 9, pp 4155–4159, September 1978
Biochemistry

# Mechanism of adenylate cyclase activation through the β-adrenergic receptor: Catecholamine-induced displacement of bound GDP by GTP

(turkey erythrocyte membrane/3′,5′-cyclic AMP/GTPase)

DAN CASSEL AND ZVI SELINGER

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

*Communicated by Henry Lardy, June 5, 1978*

ABSTRACT **The fate of the guanyl nucleotide bound to the regulatory site of adenylate cyclase was studied on a preparation of turkey erythrocyte membranes that was incubated with [$^3$H]GTP plus isoproterenol and subsequently washed to remove hormone and free guanyl nucleotide. Further incubation of this preparation in the presence of β-adrenergic agonists resulted in the release from the membrane of tritiated nucleotide, identified as [$^3$H]GDP. The catecholamine-induced release of [$^3$H]GDP was increased 2 to 3 times in the presence of the unlabeled guanyl nucleotides GTP, guanosine 5′-(β,γ-imino)triphosphate [Gpp(NH)p], GDP, and GMP, whereas adenine nucleotides had little effect. In the presence of Gpp(NH)p, isoproterenol induced the release of [$^3$H]GDP and the activation of adenylate cyclase, both effects following similar time courses. The findings indicate that the inactive adenylate cyclase possesses tightly bound GDP, produced by the hydrolysis of GTP at the regulatory site. The hormone stimulates adenylate cyclase activity by inducing an "opening" of the guanyl nucleotide site, resulting in dissociation of the bound GDP and binding of the activating guanosine triphosphate.**

Although the mechanism whereby hormones increase adenylate cyclase activity is not fully understood, it is well established that guanyl nucleotides are involved in this process. Thus, it has been shown that hormonal stimulation of adenylate cyclase required GTP (1, 2) and, more recently, the guanyl nucleotide binding component of the adenylate cyclase system has been partially purified (3, 4). The finding that hydrolysis-resistant analogs of GTP persistently activate the adenylate cyclase (3, 5, 6) and the demonstration of hormone-stimulated GTPase activity in turkey erythrocyte membranes (7, 8) led to the conclusion that adenylate cyclase activation is terminated upon the hydrolysis of GTP at the regulatory site. Further studies of the mechanism of hormonal stimulation of adenylate cyclase using hydrolysis resistant analogs of GTP have revealed two seemingly opposite effects of the activated hormone receptor: the hormone enhances the rate of adenylate cyclase activation by the analog guanosine 5′-(β,γ-imino)triphosphate(Gpp(NH)p) (9, 10), whereas a decrease in enzyme activity is seen when the hormone, together with GTP, is added to the Gpp(NH)p-activated preparation (5, 11, 12). Under the latter conditions, the hormone induces a release of membrane-bound Gpp(NH)p that is proportional to the decrease in enzyme activity (12). These results suggested that the hormone affects the reversal of the persistent activation of adenylate cyclase by facilitating the displacement of Gpp(NH)p by GTP.

We carried out the present study to determine whether a similar nucleotide-displacement mechanism is involved in hormonal stimulation of adenylate activity. It is shown that the inactive adenylate cyclase of turkey erythrocyte membranes contains tightly bound GDP and that the catecholamine hormone stimulates the enzyme by inducing the displacement of GDP by guanosine triphosphates.

## MATERIALS AND METHODS

[$^3$H]GTP (96% radiochemically pure as determined by chromatography on polyethyleneimine-cellulose) and [α-$^{32}$P]ATP were purchased from Radiochemical Centre, Amersham, England. App(NH)p and Gpp(NH)p were from ICN, and ATP was from Sigma (catalog no. 2383).

Preparation of turkey erythrocyte membranes (13), the adenylate cyclase assay (13), and thin-layer chromatography of tritiated guanyl nucleotides (12) were performed as described. Protein was measured by the method of Lowry *et al.* (14) using bovine serum albumin as standard.

**Measurement of Catecholamine-Induced Release of Membrane-Bound [$^3$H]GDP.** The assay of the catecholamine-induced [$^3$H]Gpp(NH)p release from turkey erythrocyte membranes (12) was adopted with minor modifications for [$^3$H]GTP. The procedure includes: (*i*) binding of [$^3$H]GTP in the presence of hormone; (*ii*) incubation with unlabeled GTP in the absence of hormone which serves to decrease the background of the assay; and (*iii*) determination of the hormone-induced release of [$^3$H]GDP as the difference between the quantity of nucleotide displaced by Gpp(NH)p in the presence and absence of hormone.

(*i*). Turkey erythrocyte membranes (1–2 mg of protein per ml) were preincubated for 2 min at 30° in a medium containing 6 mM $MgCl_2$, 0.3 mM ATP, 12 mM creatine phosphate, 50 units of creatine phosphokinase per ml, 2 mM 2-mercaptoethanol, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N′*-tetracetic acid (EGTA), and 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) (pH 7.4). Unless otherwise indicated, dl-isoproterenol (20 μM) was also present. The binding of tritiated nucleotide was initiated by the addition of [$^3$H]GTP (3,000–4,000 cpm/pmol) to give a concentration of 0.25 μM, and it was terminated after a 2 min incubation at 30° by the addition of unlabeled GTP and propranolol to give concentrations of 100 μM and 20 μM, respectively. The reaction mixture was cooled, and the membranes were washed three times by centrifugation (10,000 × *g* for 10 min) and resuspension in 10 vol of cold 10 mM Mops, pH 7.4, containing 2 mM 2-mercaptoethanol.

(*ii*). The membrane pellet was suspended in the medium described in (*i*) containing, in addition, 100 μM GTP and 1 μM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; App(NH)p, adenosine 5′-(β,γ-imino)triphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

propranolol. After 10 min of incubation at 37° the membranes were washed in the cold, as described above. This treatment releases 75% of the membrane-bound tritiated nucleotide, but, due to the absence of hormone, it apparently has no effect on the nucleotide bound to the regulatory site of adenylate cyclase.

(*iii*). The catecholamine-induced release of [$^3$H]GDP was initiated by the addition of 1 vol of membrane suspension (2–3 mg/ml) to 4 vol of medium at 37° containing 6 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.2 mM EGTA, 50 mM Mops (pH 7.4), 0.1 mM Gpp(NH)p, and 50 μM dl-isoproterenol. The control system did not receive isoproterenol. After 2 min of incubation at 37°, the release was stopped by the transfer of 0.5-ml aliquots into 1.5 ml of ice cold Mops buffer and the resultant mixture was centrifuged (12,000 × *g* for 5 min). Radioactivity was measured on 1.6 ml of the supernatant. The pellet was resuspended and used to measure adenylate cyclase activity (50–100 μg of membrane protein per assay system). Nucleotide release assays were performed in duplicates. The regular deviation from the mean of the determinations was ±1–3%.

## RESULTS

**Effect of Catecholamines on the Release of Tritiated Nucleotide from [$^3$H]GTP Pretreated Membranes.** We have recently shown that turkey erythrocyte membranes contain a large excess of guanyl nucleotide binding sites unrelated to the activated adenylate cyclase. Despite this excess, the small amount of the bound GPP(NH)p that persistently activates the enzyme could be measured by preincubating the membranes with [$^3$H]Gpp(NH)p and then selectively releasing the nucleotide from the regulatory site by incubation with hormone and GTP (12). We used similar conditions in the present study to test whether [$^3$H]GTP or its metabolites are persistently bound to the regulatory site. In membrane preparations that had been briefly incubated with isoproterenol plus [$^3$H]GTP and then washed, isoproterenol was subsequently able to induce the release of about 25% of the membrane-bound tritiated nucleotide (Table 1). The apparent $K_m$ for [$^3$H]GTP was 0.1 μM, and a preincubation of 2 min was sufficient to achieve maximal catecholamine-induced release of the bound nucleotide (not shown). As shown below, the nucleotide released by the catecholamine-agonist is [$^3$H]GDP (Table 6). GDP is apparently formed by the hydrolysis of GTP bound to the regulatory site, as inferred from the fact that, in the presence of a regeneration system and ATP, the amount of [$^3$H]GDP in the preincubation medium did not exceed 10% of the added [$^3$H]GTP. The catecholamine-induced release of [$^3$H]GDP was dependent on the presence of isoproterenol during pretreatment of the membrane with [$^3$H]GTP (Table 1). A similar requirement for isoproterenol during pretreatment was found for the catecholamine-induced release of [$^3$H]Gpp(NH)p from the regulatory site (12). Conceivably, the preparation contains bound GDP at the regulatory site and the introduction of $^3$H-labeled guanyl nucleotide is dependent on a hormone-induced release of this endogenous GDP.

The enhancement of [$^3$H]GDP release by catecholamines exhibited the same stereospecific requirements as the activation of adenylate cyclase through the β-adrenergic receptor, and this enhancement was inhibited by the β-adrenergic blocker propranolol (Table 2). In contrast to catecholamines, $F^-$ did not enhance the release of tritiated guanyl nucleotide, nor did it affect the release of [$^3$H]GDP by isoproterenol plus Gpp(NH)p (Table 2). This finding indicates that stimulation of adenylate cyclase by $F^-$ does not involve a release of GDP from the regulatory site.

Because it has been shown that GTP and its analogs activate the adenylate cyclase whereas GDP acts as an inhibitor (15, 16), it is expected that the displacement of bound GDP by free guanosine triphosphate would result in the activation of adenylate cyclase. The correlation between displacement of GDP by guanosine triphosphate and activation of the turkey erythrocyte adenylate cyclase was studied by introducing a tritiated guanyl nucleotide to the regulatory site through treatment with [$^3$H]GTP and isoproterenol, and then incubating the preparation for different amounts of time with Gpp(NH)p in the presence or absence of isoproterenol. As shown in Fig. 1, both the catecholamine-induced [$^3$H]GDP release and the persistent activation of adenylate cyclase in the presence of isoproterenol followed a similar time course and leveled off after 2 min of incubation. It should be noted that a tritiated nucleotide was released at an appreciable rate also in the absence of hormone, while the activation of adenylate cyclase by Gpp(NH)p under these conditions was very slow. This suggests that the majority of sites that release the guanyl nucleotide in the absence of hormone during the incubation period are not related to the activation of adenylate cyclase.

**Nucleotide Requirement for the Catecholamine-Induced [$^3$H]GDP Release.** The isoproterenol-induced [$^3$H]GDP release was augmented in the presence of various unlabeled nucleotides. When present at a concentration of 10 μM, GDP, GTP, and Gpp(NH)p gave maximal effects (Table 3), whereas ATP, GMP, and AMP had no effect (not shown). At 1 mM GMP enhanced the release, whereas AMP and ATP had little effect. A similar nucleotide requirement was reported for the catecholamine-induced release of Gpp(NH)p from turkey erythrocyte

Table 1. Isoproterenol-induced release of [$^3$H]nucleotide from turkey erythrocyte membranes pretreated with [$^3$H]GTP

| | [$^3$H]Nucleotide, pmol/mg protein | | | |
|---|---|---|---|---|
| | | Released in presence of: | | Increment released |
| Pretreatment | Membrane-bound | No addition | Isoproterenol | by isoproterenol |
| [$^3$H]GTP | 2.02 | 0.25 | 0.28 | 0.03 |
| [$^3$H]GTP + isoproterenol | 2.77 | 0.28 | 1.03 | 0.75 |

Membranes pretreated with 0.25 μM[$^3$H]GTP in the presence or absence of 20 μM isoproterenol were washed and assayed for [$^3$H]-nucleotide release in the presence of $Gpp(NH)_p$ (see *Materials and Methods*).

Table 2. β-Adrenergic specificity of the catecholamine-induced [$^3$H]GDP release from turkey erythrocyte membranes

| | [$^3$H]Nucleotide released, pmol/mg protein | |
|---|---|---|
| Addition | Total | Increment |
| No addition | 0.40 | — |
| dl-Isoproterenol | 1.26 | 0.86 |
| l-Norepinephrine | 1.26 | 0.86 |
| l-Epinephrine | 1.19 | 0.79 |
| l-Epinephrine + dl-propranolol | 0.48 | 0.08 |
| d-Epinephrine | 0.50 | 0.10 |
| NaF | 0.40 | 0.00 |
| NaF + isoproterenol | 1.19 | 0.79 |

Membranes were treated with [$^3$H]GTP and isoproterenol and then washed and assayed for [$^3$H]nucleotide release in the presence of $Gpp(NH)_p$ and the indicated additions (catecholamines = 50 μM, propranolol = 5 μM, NaF = 10 mM).

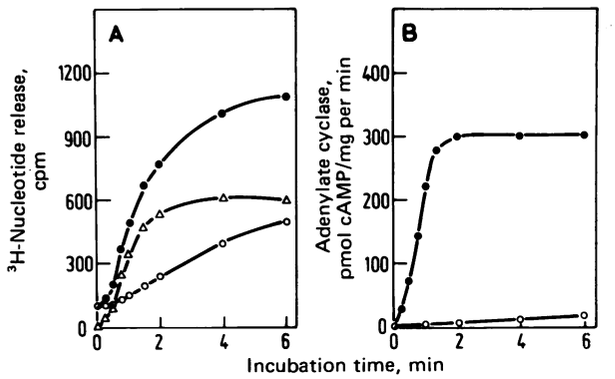


FIG. 1. Time courses of isoproterenol-induced [$^3$H]GDP release and adenylate cyclase activation in the presence of Gpp(NH)p. Membranes were pretreated for 2 min with 20 $\mu$M isoproterenol plus 0.25 $\mu$M [$^3$H]GTP and then washed. Release of [$^3$H]GDP (*A*) was assayed by incubation of the pretreated membranes at 37° in the presence of 0.1 mM Gpp(NH)p with (●) or without (○) 50 $\mu$M isoproterenol. At the times indicated, the release was stopped by cooling and centrifugation. Each point represents the counts in the supernatant of 0.2 mg of membrane protein. The difference (△) between ● and ○ indicates the isoproterenol-induced release of [$^3$H]GDP. The membrane pellets were resuspended and assayed for adenylate cyclase activity (*B*). The assay systems contained 50 $\mu$M propranolol and 0.2 mM GTP which served to stop activation of adenylate cyclase by Gpp(NH)p.

adenylate cyclase (11, 12). Apparently, the nucleotide released by the hormone is concurrently replaced by the guanyl nucleotide that is present in the medium. However, since the [$^3$H]-GDP released from the membrane yields a very low concentration of free tritiated nucleotide (~1 nM), it is unlikely that the unlabeled nucleotide enhances the release solely by preventing the rebinding of the tritiated nucleotide. It therefore appears that the free nucleotide may act through an additional guanyl nucleotide binding site (17).

Table 3. Effect of nucleotides on the isoproterenol-induced [$^3$H]GDP release

| Unlabeled nucleotide addition | [$^3$H]Nucleotide released, pmol/mg membrane protein | | |
|---|---|---|---|
| | No addition | Isoproterenol | Increase by isoproterenol |
| None | 0.31 | 0.65 | 0.34 |
| 1 mM GMP | 0.48 | 1.21 | 0.73 |
| 1 mM AMP | 0.36 | 0.78 | 0.42 |
| 10 $\mu$M GDP | 0.83 | 1.69 | 0.86 |
| 10 $\mu$M Gpp(NH)p | 0.73 | 1.58 | 0.85 |
| Regeneration system | 0.59 | 0.94 | 0.35 |
| Regeneration system + 1 mM ATP* | 0.89 | 1.46 | 0.57 |
| Regeneration system + 10 $\mu$M GTP | 0.85 | 1.70 | 0.85 |

Pretreatment with [$^3$H]GTP plus isoproterenol was as described in *Materials and Methods* (stage *i*). The treatment that serves to decrease the background of the assay (stage *ii*) was carried out in the absence of unlabeled GTP, because its addition in the standard procedure obscured the nucleotide requirement for the isoproterenol-induced [$^3$H]GDP release. This modification of the assay resulted in an increase of the hormone-independent [$^3$H]nucleotide release in the presence of unlabeled nucleotide (first column), whereas the isoproterenol-induced release of [$^3$H]GDP in the presence of Gpp(NH)p was not affected. Regeneration system: 5 mM phospho*enol*pyruvate and pyruvate kinase at 50 $\mu$g/ml.

* Purified ATP prepared enzymatically from AMP.

**Properties of GMP-Isoproterenol Pretreated Adenylate Cyclase.** The activation of turkey erythrocyte adenylate cyclase by Gpp(NH)p is very slow in the absence of hormone (see Fig. 1). However, after treatment of the membranes with GMP plus isoproterenol, the activation by Gpp(NH)p became hormone independent and was not inhibited by the hormone antagonist propranolol (Table 4). Similar results were previously reported for detergent-solubilized adenylate cyclase from pigeon erythrocyte membranes (3, 4). This hormone-independent state of adenylate cyclase progressively decayed upon incubation at 25°. The presence of GMP during the incubation prevented the loss of the response to Gpp(NH)p, whereas GDP and GTP enhanced it. The decay was not due to the inactivation of the catalytic enzyme, because in the presence of isoproterenol, Gpp(NH)p gave undiminished activation of adenylate cyclase (Table 4). These results can be attributed to the presence of GMP, introduced to the regulatory site through a hormone-induced displacement of bound GDP (Table 3). Apparently, GMP binds much more loosely than GDP and can thus be released or displaced by free nucleotide, even in the absence of hormone. The displacement of GMP by Gpp(NH)p gives a persistently active adenylate cyclase, whereas treatment with GDP or GTP restores a tightly bound GDP to the regulatory site, thereby abolishing the ability of Gpp(NH)p to activate the enzyme in the absence of hormone.

**Properties of Adenylate Cyclase with a Vacant Guanyl Nucleotide Site.** We have recently found that turkey erythrocyte adenylate cyclase persistently activated by Gpp(NH)p or GTP$\gamma$S, readily reverted to the inactive state upon incubation with hormone and (ethylenedinitrilo)tetraacetic acid (EDTA) (unpublished observations). Since binding of the GTP analog to the regulatory site requires the presence of $Mg^{++}$ (18), it is conceivable that EDTA facilitates a hormone-induced release of the bound guanyl nucleotide by decreasing its affinity for the regulatory site. The release of the bound nucleotide in the presence of EDTA does not require the presence of free nucleotide, and therefore it is likely that treatment with hormone and EDTA results in a vacant regulatory site. It was of interest

Table 4. Properties of turkey erythrocyte adenylate cyclase pretreated with GMP and isoproterenol

| Preincubation with | Adenylate cyclase activity* | |
|---|---|---|
| | Propranolol | Propranolol + Gpp(NH)p |
| Buffer | 3 | 8 |
| Isoproterenol | 3 | 16 |
| GMP | 4 | 42 |
| GMP + isoproterenol | 13 | 211 |
| → Buffer | | 104 |
| → GMP | | 199 |
| → GDP | | 35 |
| → GTP | | 13 |

Membranes were suspended at 2 mg/ml in a medium containing 6 mM $MgCl_2$, 0.2 mM EGTA, 2 mM 2-mercaptoethanol, and 50 mM Mops, pH 7.4. Isoproterenol (50 $\mu$M) and GMP (1 mM) were added where indicated. After a 2 min incubation at 37° the membranes were washed in the cold and assayed for adenylate cyclase activity in the presence of 10 $\mu$M propranolol, with or without 0.1 mM Gpp(NH)p. In the latter four experiments further incubation for 5 min at 25° was performed in the additional presence of GMP (1 mM), GDP (0.1 mM), or GTP (0.1 mM), followed by washing and determination of adenylate cyclase activity. The system with GTP contained also phospho*enol*pyruvate (5 mM) and pyruvate kinase (50 $\mu$g/ml).

* Adenylate cyclase activity, pmol/mg per min, in the presence of 0.1 mM Gpp(NH)p and 50 $\mu$M isoproterenol was very similar in all the systems (282–309 pmol/mg per min).

to release the bound GDP by treatment with hormone and EDTA and to study the properties of the resulting preparation. The effect of treatment with isoproterenol and EDTA on [$^3$H]GDP bound to the regulatory site was not determined directly, but rather as the decrease in the amount of [$^3$H]GDP released from the preparation upon subsequent incubation under the conditions required for the activation of adenylate cyclase, i.e., in the presence of $Mg^{++}$, Gpp(NH)p and isoproterenol. As shown in Table 5, pretreatment with EDTA together with isoproterenol completely abolished the catecholamine-induced release of [$^3$H]GDP, whereas pretreatment with EDTA alone had little effect. In contrast to the preparation that had been treated with isoproterenol and GMP, the adenylate cyclase pretreated with isoproterenol and EDTA could not be activated by Gpp(NH)p unless a catecholamine was also present (Table 5). These experiments suggest that the removal of bound GDP is not sufficient for the introduction of the activating guanosine triphosphate to the regulatory site.

**Identification of the Nucleotide Released by Isoproterenol.** Analysis of the tritiated nucleotides released from the membrane is complicated by the presence in the turkey erythrocyte membrane of nucleotide metabolizing enzymes that hydrolyze GTP, convert GDP to GMP and GTP, and degrade GDP to GMP. In the presence of 1 mM unlabeled GDP, however, only a small fraction of the nucleotide was metabolized, and the addition of inorganic pyrophosphate inhibited the nucleoside diphosphatase activity even further. Under these conditions, the nucleotide released from the membrane was mostly [$^3$H]GDP, and the increase in nucleotide release in the presence of isoproterenol was exclusively in GDP (Table 6). Since the addition of App(NH)p to the assay system ensured an almost complete inhibition of GTP hydrolysis by nonspecific nucleoside triphosphatases, it is unlikely that the nucleotide released by isoproterenol was GTP which was later hydrolyzed to GDP. The identification of GDP as the nucleotide released by the hormone is further supported by the finding that membranes pretreated with [$\gamma$-$^{32}$P]GTP and isoproterenol showed no catecholamine-induced release of $^{32}$P-labeled nucleotide, although the hydrolysis of [$\gamma$-$^{32}$P]GTP during pretreatment was less than 10% (not shown). It should be noted that the bound tritiated nucleotide in membranes preincubated with [$^3$H]GTP was mainly GDP (75%), whereas the amount of bound GMP was very low (2%) (Table 6).

Table 5. Effect of treatment with isoproterenol and EDTA on the catecholamine-induced [$^3$H]GDP release and on adenylate cyclase activation by Gpp(NH)p

| Treatment | Incubation with Gpp(NH)p and: | [$^3$H]Nucleotide, pmol/mg protein: Released | [$^3$H]Nucleotide, pmol/mg protein: Increment released by IP | Adenylate cyclase activity, pmol/mg per min |
|---|---|---|---|---|
| None | Propranolol | 0.43 | — | 6 |
| | Isoproterenol | 1.28 | 0.85 | 296 |
| EDTA | Propranolol | 0.29 | | 6 |
| | Isoproterenol | 1.01 | 0.72 | 243 |
| EDTA + isoproterenol | Propranolol | 0.28 | — | 9 |
| | Isoproterenol | 0.32 | 0.04 | 255 |

Membranes pretreated with [$^3$H]GTP plus isoproterenol were incubated for 5 min at 37° in 10 mM Mops, pH 7.4, containing 2 mM 2-mercaptoethanol and 1 mM EDTA, with or without 50 $\mu$M isoproterenol. After four centrifugal washes in the cold, the membrane preparations were assayed for catecholamine-induced release of tritiated nucleotide by incubation for 2 min with Gpp(NH)p, as described in *Materials and Methods* (stage iii), except that the control systems that did not receive isoproterenol received 1 $\mu$M propranolol. Adenylate cyclase activity was determined in the presence of 10 $\mu$M propranolol and 0.1 mM Gpp(NH)p. IP = isoproterenol.

Table 6. Composition of the guanyl nucleotides released from turkey erythrocyte membranes pretreated with isoproterenol and [$^3$H]GTP

| Nucleotide | Membrane bound | Released at 37°: −Isoproterenol | Released at 37°: + Isoproterenol | Increased by isoproterenol |
|---|---|---|---|---|
| GTP | 650 | 70 | 75 | 5 |
| GDP | 2250 | 340 | 1040 | 700 |
| GMP | 60 | 55 | 85 | 30 |
| Total | 2960 | 465 | 1200 | 735 |

Incubation with isoproterenol plus [$^3$H]GTP and the ensuing washes were performed according to *Materials and Methods*. Membrane-bound tritiated nucleotides were analyzed by chromatography on polyethyleneimine-cellulose after treatment of 0.3 mg of membranes with 5% trichloroacetic acid and subsequent removal of trichloroacetic acid by extraction with ether. The assay of catecholamine-induced release of tritiated nucleotide was performed by incubation of 0.3 mg of [$^3$H]GTP pretreated membranes for 2 min at 37° in the presence of 6 mM $MgCl_2$, 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 25 mM Mops (pH 7.4), 0.5 mM adenosine 5′-($\beta$,$\gamma$-imino)triphosphate [App(NH)p], 1 mM GDP, and 2 mM $NaPP_i$. Isoproterenol was added where indicated. After cooling and centrifugation, the supernatant was concentrated and chromatographed. No significant counts were found in areas of the chromatogram other than those of the marker nucleotides (GTP, GDP, and GMP). Values are cpm.

## DISCUSSION

Our previous studies have suggested that the activity of turkey erythrocyte adenylate cyclase is regulated by means of two reactions: a hormone-induced formation of an active adenylate cyclase–GTP complex and the hydrolysis of the bound GTP that terminates the activation (13, 19). This mechanism is consistent with the hormone-stimulated GTPase activity (7, 8), accounts for the maximal activation of adenylate cyclase by hydrolysis-resistant analogs of GTP, and is further corroborated by the finding that cholera toxin enhances adenylate cyclase activity by causing an inhibition of the GTPase reaction (13, 20, 21). The present study extends these observations and shows that the activation of adenylate cyclase through the action of hormone and a guanosine triphosphate coincides with a release of membrane-bound GDP. These results indicate that the GTPase reaction at the regulatory site generates an inactive adenylate cyclase possessing tightly bound GDP, and that the hormone increases adenylate cyclase activity by inducing a repetitive introduction of the activator GTP to replace the hydrolyzed nucleotide. The elevated GTPase activity in the presence of hormone (7, 8) results from the enhanced displacement of GDP by the substrate GTP (Fig. 2).

The present findings are in accord with a previous suggestion by Blume and Foster (16) that the lag in the activation of adenylate cyclase by Gpp(NH)p is due to the slow release of tightly bound endogenous GDP, and that the hormone increases the rate of the activation by decreasing the affinity of GDP to the regulatory site. According to the model of Blume and Foster, the hormone is required solely for the release of GDP, whereas the subsequent binding of the guanosine triphosphate is hormone independent. In the turkey erythrocyte system, however,

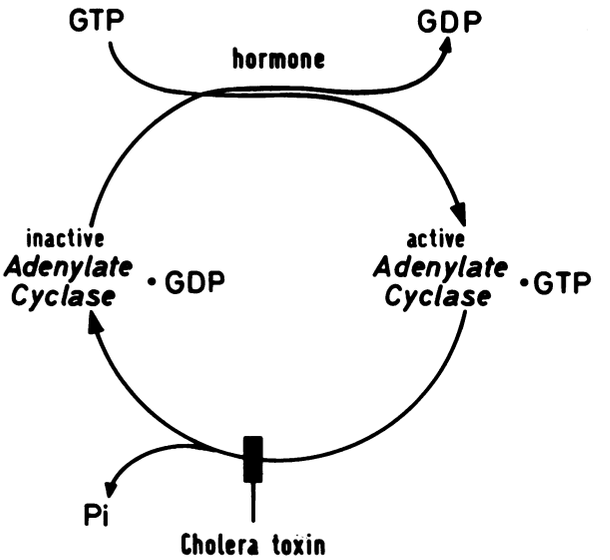


FIG. 2. The regulatory GTPase cycle of turkey erythrocyte adenylate cyclase. The inactive adenylate cyclase contains tightly bound GDP at the regulatory site. Activation is achieved upon the displacement of bound GDP by free GTP. The hormone stimulates the enzyme by facilitating the nucleotide displacement reaction. The subsequent hydrolysis of GTP at the regulatory site reverts the system to the inactive enzyme-GDP complex. Cholera toxin causes an inhibition of GTP hydrolysis, thereby increasing adenylate cyclase activity. Because the hormone facilitates the binding of GTP to the regulatory site and the bound nucleotide is subsequently hydrolyzed, the regulatory cycle results in a hormone-stimulated GTPase activity. (For detailed analysis see ref. 19.)

incubation of adenylate cyclase in the presence of hormone and EDTA brought on the release of GDP from the regulatory site but did not result in a preparation that could be activated by Gpp(NH)p in the absence of hormone (Table 5). This indicates that even when the regulatory site is vacant, it does not bind the activating guanyl nucleotide unless the hormone is also present.

It should be pointed out that the displacement of bound guanyl nucleotide by free nucleotide is not confined to a regulatory site occupied by GDP. In the Gpp(NH)p-activated adenylate cyclase of turkey erythrocytes the addition of hormone and GTP brought on the release of the Gpp(NH)p bound to the regulatory site and the concomitant decrease in enzyme activity (11, 12). Unlike the turkey erythrocyte enzyme, persistent activation of pigeon erythrocyte (3) and rat liver (22) adenylate cyclase systems could be reversed by incubation in the presence of GTP but in the absence of hormone. The latter adenylate cyclase systems differ from the turkey erythrocyte preparation also in their considerable extent of basal adenylate cyclase activity in the presence of GTP or its hydrolysis-resistant analogs. These properties of the pigeon erythrocyte and rat liver adenylate cyclase can be ascribed to a displacement of the nucleotide bound to the regulatory site by free guanyl nucleotides, which occurs to an appreciable extent also in the absence of hormone. Thus, the persistent active state is reversed in these systems due to a hormone-independent displacement of the hydrolysis resistant analog by GTP, while basal adenylate cyclase activity probably reflects the rate of displacement of the bound GDP by guanosine triphosphate in the absence of hormone.

Upon treatment of turkey erythrocyte adenylate cyclase with hormone and GMP, activation of the enzyme by Gpp(NH)p became hormone independent (Tables 3 and 4). This is apparently due to the replacement of GDP by GMP at the regulatory site. Conceivably, when nucleotides with high affinity such as GDP and Gpp(NH)p occupy the regulatory site of the turkey erythrocyte system, their displacement by free nucleotide completely depends on activation of the hormone receptor, whereas GMP is loosely bound and can thus be released and replaced by Gpp(NH)p to give the persistently active enzyme, even in the absence of hormone.

The data, when taken together, indicate that the guanyl nucleotide site exists in a dynamic equilibrium between an "open" conformation that readily binds and releases various guanyl nucleotides and a "closed" conformation in which both the association of nucleotides and its reversal are rather slow. The activated hormone receptor shifts the equilibrium to the "open" conformation and, thus, it enhances the displacement of the bound nucleotide by free nucleotide. Under physiological conditions, in which free GTP is present, an enhancement of the displacement of GDP by GTP is probably the mechanism whereby the hormone increases adenylate cyclase activity.

This work was supported by Grant 753 from the Unites States–Israel Binational Science Foundation.

1. Rodbell, M., Birenbaumer, L., Pohl, S. L. & Krans, H. M. J. (1971) *J. Biol. Chem.* **246,** 1877–1882.
2. Rodbell, M., Lin, M. C. & Salomon, Y. (1974) *J. Biol. Chem.* **249,** 59–65.
3. Pfeuffer, T. & Helmreich, E. J. M. (1975) *J. Biol. Chem.* **250,** 867–876.
4. Pfeuffer, T. (1977) *J. Biol. Chem.* **252,** 7224–7234.
5. Schramm, M. & Rodbell, M. (1975) *J. Biol. Chem.* **250,** 2232–2237.
6. Helmreich, E. J. M., Zenner, H. P., Pfeuffer, T. & Cori, C. F. (1976) in *Current Topics in Cellular Regulation,* eds. Horecker, B. L. & Stadtman, E. R. (Academic, New York), pp. 41–87.
7. Cassel, D. & Selinger, Z. (1976) *Biochim. Biophys. Acta* **452,** 538–551.
8. Cassel, D. & Selinger, Z. (1977) *Biochem. Biophys. Res. Commun.* **77,** 868–873.
9. Salomon, Y., Lin, M. C., Londos, C., Rendell, M. & Rodbell, M. (1975) *J. Biol. Chem.* **250,** 4239–4245.
10. Sevilla, N., Steer, M. L. & Levitzki, A. (1976) *Biochemistry* **15,** 3494–3499.
11. Sevilla, N. & Levitzki, A. (1977) *FEBS Lett.* **76,** 129–134.
12. Cassel, D. & Selinger, Z. (1977) *J. Cyclic Nucleotide Res.* **3,** 11–22.
13. Cassel, D. & Selinger, Z. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3307–3311.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
15. Salomon, Y., Lin, M. C., Londos, C., Rendell, M. & Rodbell, M. (1975) *J. Biol. Chem.* **250,** 4239–4245.
16. Blume, A. J. & Foster, C. J. (1976) *J. Biol. Chem.* **251,** 3399–3404.
17. Pramod, M. L., Welton, A. F. & Rodbell, M. (1977) *J. Biol. Chem.* **252,** 5942–5946.
18. Spiegel, A. M., Brown, E. M., Fedak, S. A., Woodward, C. J. & Aurbach, G. D. (1976) *J. Cyclic Nucleotides Res.* **2,** 47–56.
19. Cassel, D. & Selinger, Z. (1977) *J. Cyclic Nucleotide Res.* **3,** 393–406.
20. Levinson, S. L. & Blume, A. J. (1977) *J. Biol. Chem.* **252,** 3766–3774.
21. Johnson, G. L. & Bourne, H. R. (1977) *Biochem. Biophys. Res. Commun.* **78,** 792–798.
22. Londos, C., Lin, M. C., Welton, A. F., Lad, P. M. & Rodbell, M. (1977) *J. Biol. Chem.* **252,** 5180–5182.