Adenylate cyclase represents a class of a complex regulatory enzyme systems that are responsible for the production of cyclic AMP, the so-called “second messenger” of hormone action. Poorly defined in physicochemical terms, the enzyme systems are present in plasma membranes and are thought to consist of regulatory units bearing hormone-specific receptors and catalytic units containing the active site of the enzyme (1). Little is known of the mechanism by which hormones increase enzyme activity, although it is generally agreed that hormones induce maximal activity without changing the affinity for the substrate, MgATP (cf. Ref. 2). This concept has been contested by de Haen in a report on the kinetic analysis of two adenylate cyclase systems in which he concludes that hormones decrease the affinity for substrate (3).

Studies of the glucagon-sensitive hepatic adenylate cyclase system revealed that a nucleotide, preferentially GTP, activates the enzyme system by a concerted or interdependent manner with glucagon (4, 5). The nucleotide also changes the binding of glucagon to its receptor from a slowly reversible to a rapidly reversible process (6). GTP appears to act on the catalytic unit at a site distinct from the active site of the enzyme (5) and inhibits the stimulatory effects of fluoride ion, a potent stimulator of adenylate cyclase systems in eukaryotic cells (7). The stimulatory effects of GTP are mimicked by ITP and ATP and have been observed with a number of adenylate cyclase systems irrespective of the nature of the receptor coupled to these systems (8-16).

We have examined the ACTH-sensitive adenylate cyclase system from rat adrenal glands to determine whether this system also exhibits a nucleotide requirement, and for comparison with other adenylate cyclase systems currently under investigation in this laboratory. The results show that GTP or, at much higher concentrations, ATP, is required for hormone action. Gpp(NH)p, an analogue of GTP that is resistant to nucleotide phosphohydrolase action, activates the enzyme system by a concerted or interdependent manner with glucagon (4, 5). The nucleotide also changes the binding of glucagon to its receptor from a slowly reversible to a rapidly reversible process (6). GTP appears to act on the catalytic unit at a site distinct from the active site of the enzyme (5) and inhibits the stimulatory effects of fluoride ion, a potent stimulator of adenylate cyclase systems in eukaryotic cells (7). The stimulatory effects of GTP are mimicked by ITP and ATP and have been observed with a number of adenylate cyclase systems irrespective of the nature of the receptor coupled to these systems (8-16).

The difficulties in describing kinetically the regulation of adenylate cyclase systems in view of the multiple actions of nucleotides and magnesium are discussed.
inhibit fluoride-stimulated activity in a noncompetitive manner. ACTH appears to enhance the adrenal adenylate cyclase activity by a process that increases the formation of states of the enzyme that are susceptible to activation by GTP, Gpp(NH)p, and at much higher concentrations, ATP. The major effects of the regulatory ligands (luminares and nucleotides) appear to be an increase in $V_{max}$, a decrease in the inhibitory effects of a species of ATP uncomplexed with Mg$^{2+}$, and an increased sensitivity to inhibition by Mg$^{2+}$.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-$^32$P]ATP, Gpp(NH)p, [α-$^32$P]App(NH)p, and App(NH)p were obtained from International Chemical and Nuclear Corp. cyclic [β$^32$P]AMP was purchased from New England Nuclear Corp. ATP, cyclic AMP, GTP, phosphocreatine, and creatine phosphokinase were purchased from Sigma Chemical Co. Synacthen was a gift of CIBA-Geigy, and hypertrophied adrenals a gift of Dr. Robert W. Bates, National Institutes of Health. Histological examination of the hypertrophied adrenals was performed by Dr. Peter Nickerson, State University of New York at Buffalo.

**Preparation**—Adrenal glands were obtained from male Sprague-Dawley rats weighing 150 to 200 g. They were cleaned of overlaying fat and homogenized at 4° with 6 strokes in a glass homogenizer in 250 mM sucrose, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.6. For some experiments adrenals were decapsulated by enucleation under gentle pressure prior to homogenization, and the capsular and decapsulated portions were processed in parallel. Homogenates were filtered through silk-screen to remove connective tissue and centrifuged at 600 x g for 10 min. The pellet was suspended in the homogenizing solution and recentrifuged; the washed pellet was suspended in 20 mM Tris-HCl-1 mM dithiothreitol, and aliquots were stored under liquid nitrogen. For some experiments the supernatant fraction of the 600 x g centrifugation was centrifuged at 10,000 x g, the pellet saved, and the supernatant centrifuged at 100,000 x g to obtain a microsomal pellet. All pellets were suspended and stored as described above. The enzyme was stable indefinitely when stored under liquid N$_2$. When in solution the enzyme was stabilized with 1 mM dithiothreitol.

With the exception of the experiments described in Table I, the washed 600 x g pellet obtained from homogenates of whole adrenals served as the enzyme source for studies depicted in the tables and figures.

Hypertrophied adrenals (17) were prepared by excising and discarding the reddish pulpy core which contained the medulla and zona reticularis; the remaining outer brownish section, comprised mostly of zona fasciculata (>95%) and small amounts of zona glomerulosa, was prepared as described above for normal adrenals. All ACTH-stimulated adenylate cyclase activity was in the outer section.

Protein was measured according to the method of Lowry et al. (18) with the use of bovine serum albumin as standard.

**Adenylate Cyclase Assay**—Adenylate cyclase was assayed by measuring the formation of cyclic [β$^32$P]AMP from either [α-$^32$P]ATP or [α-$^32$P]App(NH)p. Unless otherwise indicated, the assay medium contained 5 mM MgCl$_2$, 2 to 5 x 10$^6$ cpm of $^32$P as [α-$^32$P]App(NH)p or [α-$^32$P]ATP, 1 mM cyclic AMP, 1 mM dithiothreitol, 0.2% bovine serum albumin, 25 mM Tris-HCl, pH 7.6, and a nucleotide-regenerating system consisting of 5 mM phosphocreatine and 50 units/ml of creatine phosphokinase. Concentrations of ATP and App(NH)p are indicated in the legends to the tables and figures. Total assay volume was 0.1 ml, and the reaction was initiated with the addition of adenylate membranes; membrane protein concentration ranged from 40 to 200 µg/ml. Reaction time was 5 min at 30°. NaF concentration was 20 mM, and ACTH was 2 x 10$^{-5}$ M. Basal activity is that in the absence of guanine nucleotide and ACTH. The reaction was stopped with the addition of 0.1 ml of stopping solution (19), and cyclic [β$^32$P]AMP isolated with the use of a recently developed method (19).

All experiments were repeated at least twice with different membrane preparations. Values shown represent the means of duplicate determinations in which individual values fell within the mean ± 10%.

**RESULTS**

Mammalian cells are known to contain GTP in concentrations up to 10% of that of ATP (20-22), and the latter is thought to be in the millimolar range (21, 23). Since GTP in the micromolar range produces maximal effects on adenylate cyclase activity (see below), low levels of contaminating endogenous nucleotide may be expected to obscure the effects of added nucleotide. An example of this phenomenon is seen in Table I, which shows the distribution of adenylate cyclase activity in particulate fraction of adrenal glands divided into capsular and decapsulated portions. Assays were performed with 0.5 mM ATP, a substrate level which permitted assessment of the effects of both GTP and ACTH (see Fig. 2). While the hormone enhanced activity in all fractions, the response to GTP was variable; the nucleotide considerably enhanced both basal and ACTH-stimulated activities in the 600 x g fraction of the capsular gland, but had no effect on activity in the 10,000 x g fraction of the decapsulated gland. In order to test whether this lack of a GTP effect may have resulted from contamination by endogenous nucleotide, the 10,000 x g fraction of the decapsulated gland was dialyzed against 300 volumes of 20 mM Tris-HCl (pH 7.0)-1 mM dithiothreitol, at 0° for 4 hours. Following such treatment, the dialyzed fraction exhibited a sensitivity to GTP comparable to that observed with the 600 x g fraction of the capsular gland which served as the control and was unaffected by dialysis. Thus, contaminating endogenous GTP (or other activating nucleotides) may affect the apparent distribution of enzyme activity among the various particulate fractions, as well as obscure the effects of added nucleotide.

Since we wished to investigate the role of GTP in the actions of ACTH on the adrenal system, it was obvious that the 10,000 x g fraction was unsuitable for such purposes. Accordingly, the 600 x g fraction, which displayed marked sensitivity to nucleotides and the highest specific activity of the various fractions investigated was used in all studies. Since preliminary studies revealed no qualitative differences between the 600 x g fractions of capsular and decapsulated glands, experiments were conducted with 600 x g fractions derived from homogenates of whole adrenal glands.

As with any tissue the possibility exists that the adrenal contains more than one adenylate cyclase system. A problem in interpretation of data would result if, in the absence of hormone, the activity measured were that of an enzyme different from the ACTH-stimulated enzyme. We could not determine whether enzyme from different cell types contributed to activity measured in 600 x g fractions from normal adrenals. However, the hypertrophied adrenals did permit studies of the enzyme in fractions derived primarily, if not exclusively,
nucleotides or on the differential effects of Mg"\(^{2+}\) and ATP in the system, 10 nM 5'-GMP stimulated both basal and ACTH-

Effects of GTP and Gpp(NH)p—The effects of GTP on several parameters of the adrenal adenylate cyclase system when tested at low substrate concentration (0.1 mM ATP) are shown in Fig. 1A. Over the same concentration range, the nucleotide enhanced both basal and hormone-stimulated activities; inhibition of fluoride-stimulated activity is described below. Note the minimal activation by ACTH in the absence of guanine nucleotides. The lowest concentration of GTP that produced an observable effect was 10 nM, and half-maximal activation of basal and ACTH-stimulated activities occurred with 0.2 \(\mu\)M GTP. At concentrations above 10 \(\mu\)M the nucleotide inhibited activity in the absence (see also Table IV) and presence of ACTH.

As the data in Fig. 1A show, the hormone and GTP acted synergistically to increase cyclic AMP production, i.e., with maximal ACTH and GTP, activity was twice the predicted sum of the activities observed with each agent alone.

While the 3-fold stimulation of activity by GTP (Fig. 1A) may have resulted from interaction of the nucleotide with membranes that bore residual native ACTH, studies with membranes from animals bearing pituitary tumor transplants indicate otherwise. The blood levels of ACTH in the tumor-bearing animals may be 1000 times normal (17), and pairs of adrenals from these animals weighed over 500 mg, which is 10 times normal. However, when compared directly, both normal and hypertrophied adrenal preparations exhibited equivalent sensitivity to ACTH; half-maximal activity for both was achieved with 80 nM ACTH. If retention of ACTH on the membranes was a problem, one would have expected an apparent decrease in sensitivity to added ACTH with membranes from the hypertrophied adrenals.

Evidence in support of the notion that GTP acts as a regulatory ligand and not as a phosphorylating agent has been reported for the glucagon-sensitive hepatic adenylate cyclase system, where it was shown that the nonphosphorylating analog, Gpp(CH\(_2\))p, mimicked the actions of GTP (5). A comparison of the effects of GTP and another analog, Gpp(NH)p, on the adrenal system is seen in Fig. 1. As with GTP, this compound acted synergistically with the hormone; the Gpp(NH)p concentration required for half-maximal activation was approximately 4-fold higher than that for GTP. A striking effect of Gpp(NH)p was the elevation of activity in the absence of ACTH to a level nearly twice that produced by a maximal concentration of GTP.

Gpp(NH)p, as has been reported for App(NH)p, the imidodiphosphate analog of ATP, probably does not act as a phosphorylating agent. App(NH)p has been shown not to substitute for ATP in phosphorylation reactions and to resist breakdown by nucleotide phosphohydrolases (24). It is therefore likely that the effects of Gpp(NH)p resulted from the interaction of the nucleotide at a regulatory site on the enzyme system.

Other Guanine Nucleotides—In order to test the effects of GDP on the adrenal adenylate cyclase system, assays were conducted in the absence of the nucleotide-regenerating system and with the use of App(NH)p as substrate. This was necessary since it has been shown that the regenerating system under conditions employed in these experiments efficiently converts GDP to GTP. The data in Table II show that GDP, at 10 \(\mu\)M, inhibited basal activity by approximately 40%, and, in contrast to GTP and Gpp(NH)p, permitted only minimal activation by ACTH. However, with the addition of the regenerating system no differences were seen between the effects of GDP and GTP, reflecting the rapid conversion of GDP to GTP. These data, showing that the regulatory site of the adrenal enzyme system exhibits a requirement for the \(\gamma\)-phosphate of the activating nucleotide, are in contrast to information published with nearly all other adenylate cyclase systems (see “Discussion”).

In Table II it is seen also that the addition of the regenerating system resulted in considerable inhibition under all conditions. Further studies have revealed that this inhibition is due primarily to creatine phosphate, as has been reported for the fat cell enzyme (13). With the use of ATP as substrate inhibition is less than with App(NH)p. However, even with ATP and 20 mM creatine phosphate, a concentration commonly used in adenylate cyclase studies, inhibition was approximately 50%. The nature of the inhibition is unknown, but we have determined that creatine phosphate does not compete with substrate.

Cyclic GMP and guanosine over the concentration range of 1 nM to 10 \(\mu\)M had no effect on the enzyme. With App(NH)p as substrate, 5'-GMP was also without effect. On the other hand, with ATP as substrate, and in the presence of the regenerating system, 10 \(\mu\)M 5'-GMP stimulated both basal and ACTH-

- Y. Salomon, unpublished observations.
stimulated activity to the extent seen with a half-maximal level of GTP. These effects of 5'-GMP may be explained, however, by the presence of contaminating enzymes in the creatine phosphokinase preparation which convert GMP to GDP in the presence of ATP, and the subsequent conversion of GDP to GTP.

Effects of Combinations of Different Guanine Nucleotides—It was of interest to determine the effects of combinations of the different guanine nucleotides (GDP, GTP, Gpp(NH)p) on the adrenal enzyme system. The effects of GDP on activation by GTP could not be determined since in the presence of the regenerating system GDP is converted to GTP. However, the effects of GDP on stimulation by Gpp(NH)p were studied with App(NH)p as substrate (no regenerating system, Table III), as were the effects of GTP on stimulation by Gpp(NH)p with ATP as substrate (with regenerating system, Table IV). The data in Table IV show that submaximal concentrations of GTP inhibited the effects of Gpp(NH)p (10 μM), and that when the two nucleotides were present at equimolar concentration the Gpp(NH)p action was completely inhibited; activity was equivalent to that seen with GTP in the absence of Gpp(NH)p. The data in Table III show that GDP, while inhibiting basal activity, inhibited Gpp(NH)p-stimulated activity to a greater extent, i.e., inhibition by GDP was proportionally greater with Gpp(NH)p than in the absence of the stimulating nucleotide. These results suggest that the three guanine nucleotides act at the same site on the enzyme system, and that the γ-phosphate of the nucleotide is required for but need not be metabolized in the process of enzyme activation.

Other Nucleotides—A comparison of the effects of GTP and of several other nucleoside triphosphates on basal and ACTH-stimulated activities is shown in Table V. The two pyrimidine nucleotides tested, CTP and UTP, had little or no effect at 10 μM. ITP, a purine nucleotide, was stimulatory, but to a lesser extent than was GTP. As is shown below (Fig. 2), ATP in the millimolar range appeared also to act at the regulatory site of the enzyme system. These findings are consistent with other studies on adenylate cyclase systems that show, in general, that purine nucleotides are considerably more effective than are pyrimidine nucleotides (5, 12, 16).

Actions of Fluoride—In the presence of 5 mM MgCl₂, half-maximal activation was seen with approximately 5 mM NaF. If, however, MgCl₂ and NaF were combined 4 hours prior to the initiation of the reaction with membranes, fluoride was inhibitory at concentrations above 5 mM. Such inhibition may be accounted for by the removal of Mg²⁺ by fluoride; the solubility product constant of these two ions is 10⁻²² (25).

As is seen in Fig. 1, both GTP and Gpp(NH)p inhibited fluoride-stimulated activity. From studies on the hepatic enzyme it was concluded that GTP and fluoride acted noncompetitively since increasing the fluoride concentration did not overcome inhibition by GTP (5). We have tested the ability of GTP to inhibit activity in the presence of varying amounts of fluoride and found that, in general, inhibition was more prominent at high concentration of fluoride (20 mM) than at concentrations below 10 mM. Moreover, with a submaximal fluoride concentration (5 mM), GTP actually enhanced fluoride-stimulated activity. These data support the notion that fluoride and GTP act noncompetitively, and show that the concentration range of fluoride required to activate the enzyme is unrelated to the fluoride concentration required to elicit inhibition by GTP. High levels of fluoride may increase

### Table III

Effects of GDP on basal and Gpp(NH)p-stimulated activities

Substrate was 0.1 mM App(NH)p, and membrane protein was 10 μg per assay. The nucleotide-regenerating system was omitted.

| GDP added | No additions | 10 μM Gpp(NH)p |
|---|---|---|
| None | 14.2 | 55.6 |
| 1 μM | 13.8 | 48.8 |
| 10 μM | 10.1 | 23.4 |
| 50 μM | 7.5 | 15.4 |

### Table IV

Inhibition of Gpp(NH)p effect by GTP

Substrate was 0.1 mM ATP, and the nucleotide-regenerating system was present. Membrane protein was 12 μg per assay.

| GTP added | No additions | 10 μM Gpp(NH)p |
|---|---|---|
| None | 19 | 124 |
| 0.1 μM | 31 | 111 |
| 1.0 μM | 53 | 88 |
| 10 μM | 66 | 73 |
| 50 μM | 82 | 40 |

Fig. 2. Effects of varying ATP concentration on basal and ACTH-stimulated activities in the presence and absence of GTP. Mg²⁺ concentration was 10 mM, and GTP was 10 μM. Membrane protein concentration was 17 μg per assay.
susceptibility to inhibition by the nucleotide by forming a complex with Mg\(^{2+}\), and, thus, changing considerably the assay medium composition (see "Discussion").

**Effects of GTP on Substrate Utilization**—In order to determine whether GTP affects interaction of enzyme with substrate, basal and hormone-stimulated activities were tested with and without the nucleotide over the ATP concentration range of 0.025 to 5.0 mM in the presence of 10 mM Mg\(^{2+}\) (Fig. 2). This Mg\(^{2+}\) concentration was employed to minimize levels of uncomplexed ATP. In addition to serving as substrate, ATP appeared to fulfill a second role in this system, in that, like GTP, it permitted activation by the hormone when present in sufficient concentrations; at the lowest ATP concentrations tested activation by ACTH was minimal in the absence of GTP, but at the higher ATP concentrations activation by the hormone no longer required GTP. Such results probably reflect interaction of ATP (or contaminating GTP, Ref. 4) at the nucleotide-sensitive regulatory site on the enzyme system, a phenomenon observed also with the hepatic adenylate cyclase system (5).

Lineweaver-Burk plots of the data from Fig. 2 show that in the presence of GTP, basal and ACTH-stimulated activities exhibited no difference in their affinity for substrate (K\(_s\) = 0.16 mM), and that the primary action of ACTH was to increase V\(_\text{max}\) (Fig. 3). Curves derived from experiments conducted in the absence of GTP were nonlinear, being concave downward, and did not permit calculation of a K\(_s\) value. A possible explanation for the nonlinear curves would be activation by substrate, and, as noted, ATP did appear to serve as a regulator as well as substrate.

In addition to its action as substrate and as possible regulatory ligand, a third effect of ATP may be observed in Fig. 2; at the highest ATP concentration tested (5 mM), activity in the absence and presence of GTP (no hormone) was inhibited, while ACTH-stimulated activity seemed not to be affected. The differential effects of excess ATP on activity in the presence and absence of ACTH are discussed below.

**Differential Effects of ATP on Basal and ACTH-stimulated Activities**—de Haen has reported recently (3) that ATP uncomplexed with Mg\(^{2+}\) serves as a competitive inhibitor of the binding of the productive form of substrate, MgATP\(^{2+}\), to the active site of the fat cell and ventricular adenylate cyclase systems. He concluded also that the hormone-stimulated states of the enzyme were less susceptible to inhibition by uncomplexed ATP than the basal states of these enzyme systems. Since Mg\(^{2+}\), at a fixed concentration of substrate, alters the concentration of uncomplexed ATP, it should follow from de Haen's findings that decreasing the concentration of Mg\(^{2+}\) from 10 mM, as in Fig. 2, to 2 mM should increase the concentration of uncomplexed ATP and should cause greater inhibition of basal activity than of ACTH-stimulated activity over a comparable range of substrate concentrations. As shown in Fig. 4, activities obtained in the presence of ACTH (with or without GTP) did not differ substantially with 2 mM Mg\(^{2+}\) compared with 10 mM Mg\(^{2+}\) (Fig. 2) over the range of 0.1 to 1.0 mM ATP. By contrast, basal activity with 2 mM Mg\(^{2+}\) was reduced substantially compared to that observed with 10 mM Mg\(^{2+}\) over the same range of substrate concentrations. It should be noted also that the inhibitory effects of decreasing Mg\(^{2+}\) concentration cannot be related simply to the complexing of Mg\(^{2+}\) by ATP since, as shown in Fig. 2, inhibition of activity was evident even when Mg\(^{2+}\) was present in 5 mM excess over ATP. These data provide, therefore, qualitative support for the hypothesis put forth by de Haen; namely, that hormonal activation of adenylate cyclase results in the formation of an enzyme state that is less susceptible to inhibition by uncomplexed ATP.

Also seen in Fig. 4 is the inhibition of all activities when the ATP concentration approached, but not necessarily exceeded, that of magnesium. However, even under conditions of excess ATP, inhibition of basal or GTP-stimulated activity was proportionally greater than that seen in the presence of ACTH.

Another interesting aspect of the experiments depicted in Fig. 4 was the wide variation, dependent upon ATP concentration, of the ratios of ACTH-stimulated to basal activities. For example, at 0.1, 1.0, and 2.0 mM ATP, the ratios of hormone-stimulated to basal activities were, respectively, 3, 7, and 11 in
the presence of GTP, and 1.5, 7, and 15 in the absence of the guanine nucleotide. Such data indicate that ratios of hormone to basal activities do not serve as reliable indicators of receptor function, or of the ability of a system to respond to hormone. In most of the experiments presented in this report, activities with ACTH were usually only 2- to 4-fold greater than basal activities. However, experiments were conducted under conditions of relatively high Mg**+ATP ratios, and under such conditions basal activities may be relatively high due to relief from ATP inhibition.

Effects of Mg**+.—The above data indicate that basic differences exist between basal and hormone-activated states of the adrenal enzyme with respect to inhibition by a form of ATP uncomplexed with Mg**+. Studies on the adenylate cyclase system from liver (26) and thyroid (27) indicate that when activated by hormones these enzymes may be more sensitive to inhibition by high Mg**+ concentrations than they are in the basal state. The adrenal adenylate cyclase system may be affected similarly by Mg**+. Fig. 5 shows the effects of varying the Mg**+ concentration from 1 to 40 mM on ACTH-stimulated activity when tested at three ATP concentrations in the presence and absence of GTP. Under conditions where a minimal nucleotide effect was manifested (no GTP, 0.1 mM ATP), varying the Mg**+ concentration from 1 to 40 mM resulted in a small elevation in activity; activity under these conditions was essentially basal activity despite the fact that ACTH was included in the assay medium. Increasing ATP to 0.5 mM partially fulfilled the nucleotide requirement for ACTH action (see Fig. 2), and under these conditions increasing the Mg**+ concentration produced a noticeable inhibition (Fig. 5). At 2.5 mM ATP, a level sufficient to satisfy the nucleotide requirement, inhibition by Mg**+ was marked. In Panel B of Fig. 5 it is seen that in the presence of GTP, Mg**+ concentrations above 10 mM were inhibitory at all concentrations of ATP tested.

One explanation for these data would hold that nucleotide (ATP or GTP) uncomplexed with Mg**+ is required by the regulatory site, and that inhibition by Mg**+ was the result of a lowering of the concentration of the unbound nucleotide. However, in further experiments we found the apparent \( K_r \) for Mg**+ not to vary over a wide range of GTP concentrations. Further studies have revealed that the ATP- or Gpp(NH)p-activated enzyme, even in the absence of ACTH, is more susceptible to inhibition than is the basal enzyme (data not shown). As is discussed below, such inhibitory effects of the nucleotide must be taken into consideration in any formal description of the adrenal adenylate cyclase system.

**DISCUSSION**

Our results show that the \( \gamma \)-phosphate of the guanine nucleotide is important for activation of the adrenal adenylate cyclase system. These data are in contrast to those published for several other adenylate cyclase systems showing that nucleotide mono- and diphosphates, as well as the triphosphates, serve as enzyme activators (5, 12, 14–16). However, such studies were done in the presence of both ATP and a nucleotide-regenerating system, conditions which permit conversion of the mono- and diphosphate forms to the nucleotide triphosphates. In studies done in the absence of a nucleotide-regenerating system, and with App(NH)p as substrate, the hepatic glucagon-sensitive enzyme was reported to be activated by GDP and GTP (5). More recent studies, however, have revealed that the liver enzyme is inhibited by GDP in the absence of glucagon and only weakly activated by GTP in the presence of hormone. Moreover, as is shown herein for the adrenal enzyme, the actions of Gpp(NH)p on the hepatic enzyme are blocked by GDP.* While the fat cell adenylate cyclase appears to discriminate somewhat between GDP and GTP, a direct comparison with the adrenal enzyme is difficult given the multiple effects of guanine nucleotides on the fat cell enzyme (13).

Although the \( \gamma \)-phosphate of the guanine nucleotide is important for enzyme activation, the evidence does not indicate that a phosphorylation reaction occurs. This is supported by the finding that Gpp(NH)p is more active than GTP and by previous studies with the hepatic adenylate cyclase system showing that Gpp(CH)p substitutes for GTP (5). The possibility that GDP inhibits by interacting with the nucleotide regulatory site suggests a possible explanation for the differences between levels of activation achieved with GTP and Gpp(NH)p. If the regulatory site were to possess phosphohydrolase activity, the action of GTP, as opposed to that of Gpp(NH)p, would be limited by its hydrolysis to GDP. Studies on the mode of GTP interaction must await separation of the enzyme from nucleotide phosphohydrolases present in plasma membranes.

In providing a kinetic analysis of two adenylate cyclase systems de Haen has contributed significantly to the understanding of adenylate cyclase regulation (3). Our data support qualitatively his hypothesis that hormones render these enzymes less susceptible to inhibition by a species of uncomplexed ATP. de Haen concluded also that hormones decrease the affinity for substrate, MgATP; in the case of the fat cell enzyme ACTH shifted the \( K_r \) upward by greater than 5-fold. We were able to calculate the \( K_r \) for the adrenal enzyme by adding GTP to satisfy the nucleotide requirement at the regulatory site, and by performing experiments in the presence of a relatively high magnesium concentration (10 mM) to overcome inhibitory effects of uncomplexed ATP (Fig. 3). These experiments produced no evidence for an ACTH-induced decrease in the affinity for substrate. However, in the absence of GTP, ATP in excess of 3 mM was required to satisfy the nucleotide requirement of the adrenal enzyme; the calculated increase in \( K_r \) of the ACTH-activated fat cell enzyme may reflect the need for higher ATP to fulfill the regulatory requirements.

---

*Y. Salomon, M. C. Lin, C. Londos, M. Rendell, and M. Rodbell, *J. Biol. Chem.*, in press.
function. de Haen noted systematic deviations between the theoretical curves and experimental points in his report (3). It would appear that the failure to consider the regulatory action of ATP contributed to these deviations.

In formulating a kinetic model it is essential, of course, to take into account all possible effects of Mg\(^{2+}\). de Haen has considered two actions: formation of substrate (MgATP) and alteration of the level of uncomplexed ATP, an inhibitor. Our data reveal another effect of magnesium, inhibition by the cation per se. The latter effect was particularly evident when the enzyme was activated by hormone. It is likely that yet another effect of magnesium requires attention, that is, the effect of the cation on the level of activating nucleotide. We have shown recently that it is the free, and not the magnesium-complexed form of the nucleotide, that activates the glucagon-sensitive hepatic adenylate cyclase system. Thus, by decreasing the level of uncomplexed nucleotide, increasing the Mg\(^{2+}\) concentration removes a factor (free nucleotide) which is both an inhibitor and an activator. However, with the adrenal enzyme the inhibition resulting from the removal of activating nucleotide is obscured by the inhibition of the cation per se; the latter occurred even in the presence of an excess of GTP.

The current literature provides little opportunity for comparing data on substrate utilization by the adrenal enzyme with other adenylate cyclase systems. In none of the published reports on the kinetics of substrate utilization were experiments performed in the presence of nucleotides other than ATP (cf. Refs. 2 and 3). As we have shown, only in the presence of GTP does the adrenal enzyme exhibit typical Michaelis-Menten kinetics. Since it may be assumed that most, if not all, eukaryotic adenylate cyclase systems are subject to regulation by purine nucleotides (5, 8-16, 28), the question arises as to how assessments of \(K_m\) were made in view of the likelihood that ATP served both as substrate and activator. One possible explanation is provided by our finding that as little as 0.2 mg/ml of a 10,000 \(\times \) g adrenal fraction appeared to contain a sufficient level of contaminating nucleotides to satisfy the regulatory site. It is reasonable to suggest that some studies have been conducted, unknowingly, in the presence of activating nucleotides, and that the nucleotide source was the enzyme fraction itself. For instance, in studies on the renal medullary vasopressin-sensitive enzyme, Neer found no evidence for a regulatory role of ATP (2). However, those studies were performed with a membrane fraction enriched in both plasma membranes and mitochondria. The latter may have been a nucleotide source in Neer’s studies, as well as in our studies on the 10,000 \(\times \) g adrenal fraction.

The kinetic model of de Haen (3) is, as he states, a useful first approximation in describing the behavior of adenylate cyclase. However, it is clear that the multiple effects of magnesium and ATP, described above, as well as the effects of guanine nucleotides, constitute the minimal elements that should be incorporated into any further kinetic models for the enzyme.

Experiments designed to provide data for kinetic analysis of membrane-bound adenylate cyclase systems with the use of ATP and GTP are complicated by the need to add a nucleotide-regenerating system, as membrane bound nucleotide phosphohydrolases would otherwise rapidly deplete the nucleotides. As has been shown for the fat cell (13) and adrenal enzymes, the creatine phosphate, a constituent of the regenerating system, is an inhibitor. Thus, further studies would require a determination of the inhibitory species of creatine phosphate (free or magnesium-complexed). The availability of App(NH)p and Gpp(NH)p, nucleotide phosphohydrolase-resistant nucleotides which serve as substrate and regulatory nucleotide, respectively, permit studies in the absence of a regenerating system. An analysis of the adrenal enzyme with the use of these compounds will be forthcoming.

Acknowledgments—We gratefully thank our colleagues Drs. M. C. Lin, B. R. Martin, M. Schramm, and Y. Salomon for their participation in the discussions of this study and for their many helpful suggestions.

REFERENCES

1. Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1967) Ann. N.Y. Acad. Sci. 139, 703
2. Neer, E. J. (1973) J. Biol. Chem. 248, 4775-4781
3. de Haen, C. (1974) J. Biol. Chem. 249, 2756-2762
4. Rodbell, M., Lin, M. C., and Salomon, Y. (1974) J. Biol. Chem. 249, 59-66
5. Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. J. (1971) J. Biol. Chem. 246, 1877-1882
6. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1872-1876
7. Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1971) in Cyclic AMP: Academic Press, New York
8. Goldfine, I. D., Roth, J., and Birnbaumer, L. (1972) J. Biol. Chem. 247, 1211-1218
9. Krishna, G., Harwood, J. P., Barber, A. J., and Jamieson, G. A. (1972) J. Biol. Chem. 247, 2253-2254
10. Bockaert, J., Roy, C., and Jard, S. (1972) J. Biol. Chem. 247, 7073-7081
11. Tarray, F., Chamhaut, A., and Hanoune, J. (1972) Biochem. Biophys. Res. Commun. 48, 1386-1390
12. Wolff, J., and Cook, G. H. (1973) J. Biol. Chem. 248, 350-355
13. Harwood, J. P., Low, H., and Rodbell, M. (1973) J. Biol. Chem. 248, 6239-6245
14. Kuo, W. N., Hodgins, D. S., and Kuo, J. F. (1973) J. Biol. Chem. 248, 2703-2711
15. Sat0, S., Yamada, T., Furihata, R., and Makuichi, M. (1974) Biochem. Biophys. Acta 332, 166-174
16. Kelmekian, J. P., and Aurbach, G. D. (1974) J. Biol. Chem. 249, 157-161
17. Bates, R. W., Milskov, S., and Garrison, M. M. (1962) Endocrinology 71, 943-948
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
19. Salomon, Y., Londos, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541-548
20. Mandel, P., Winterth, M., Klein-Pete, N., and Mandel, L. (1963) Nature 198, 1000-1001
21. Colby, C., and Edlin, G. (1970) Biochemistry 9, 917-920
22. Bartlett, G. (1970) in Adv. Exp. Med. Biol. 6, 245-256
23. Mandel, P. (1984) Prog. Nucleic Acid Res. Mol. Biol. 3, 299-334
24. Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971) in Biochemistry 10, 2484-2489
25. Sillen, L. G., and Martell, A. E. (1964) in Stability Constants, Special Publication 17 of The Chemical Society, Burlington House, London
26. Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1971) J. Biol. Chem. 246, 1849-1856
27. Wolff, J., and Jones, A. B. (1971) J. Biol. Chem. 246, 3939-3947
28. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J., and Rodbell, M. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3087-3090
Multiple inhibitory and activating effects of nucleotides and magnesium on adrenal adenylate cyclase.
C Londos and M Rodbell

J. Biol. Chem. 1975, 250:3459-3465.

Access the most updated version of this article at http://www.jbc.org/content/250/9/3459

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/9/3459.full.html#ref-list-1