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Irreversible Stimulation of Adenylate Cyclase Activity of Fat Cell Membranes by Phosphoramidate and Phosphonate Analogs of GTP

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Summary. The ability of 5′-guanylylimidodiphosphate (Gpp(NH)p) [1] to stimulate irreversibly the adenylate cyclase activity of fat cell membranes has been studied by preincubating the membranes with this or related analogs followed by assaying after thoroughly washing the membranes. Activation can occur in a simple Tris-HCl buffer, in the absence of added divalent cations and in the presence of EDTA. Dithiothreitol enhances the apparent degree of activation, perhaps by stabilization. The importance of utilizing optimal conditions for stabilizing enzyme activity, and of measuring the simultaneous changes in the control enzyme, is illustrated.

The organomercurial, *p*-aminophenylmercuric acetate, inhibits profoundly the activity of the native as well as the Gpp(NH)p-stimulated adenylate cyclase, but in both cases subsequent exposure to dithiothreitol restores fully the original enzyme activity. However, the mercurial-inactivated enzyme does not react with Gpp(NH)p, as evidenced by the subsequent restoration of only the control enzyme activity upon exposure to dithiothreitol. Thus, reaction with Gpp(NH)p requires intact sulfhydryl groups, but the activated state is not irreversibly destroyed by the inactivation caused by sulfhydryl blockade.

GTP and, less effectively, GDP and ATP inhibit activation by Gpp(NH)p, but interpretations are complicated by the facts that this inhibition is overcome with time and that GTP and ATP can protect potently from spontaneous inactivation. These two nucleotides can be used in the Gpp(NH)p preincubation to stabilize the enzyme.

The Gpp(NH)p-activated enzyme cannot be reversed spontaneously during prolonged incubation at 30 °C in the absence or presence of GTP, ATP, $MgCl_2$, glycine, dithiothreitol, NaF or EDTA. The strong nucleophile, neutral hydroxylamine, decreases the Gpp(NH)p-activated enzyme activity and no subsequent activation is detected upon re-exposure to the nucleotide.

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1 *Abbreviations:* Gpp(NH)p, 5′-guanylylimidodiphosphate; App(NH)p, 5′-adenylylimidodiphosphate; Gpp(CH_2)p, 5′-guanylylmethylenediphosphonate; cyclic AMP, 3′-5′-adenosine monophosphate.

Gpp(CH_2)p also results in enzyme activation detected after washing the membranes. The reaction appears to occur at the same site as Gpp(NH)p, but much higher concentrations are required and the intrinsic activity of the activated enzyme is lower than that obtained with Gpp(NH)p.

Gpp(NH)p can directly stimulate the detergent-solubilized enzyme, and this reaction is unaffected by hormones. Studies of the kinetic properties of the membrane-bound, activated enzyme indicate that it is refractory to stimulation by hormones (isoproterenol, ACTH) and by NaF. The activated enzyme demonstrates an increased V_{max} but no change in K_m for $MgCl_2$. The apparent K_m for ATP is not affected. Preincubation of membranes (followed by washing) with NaF results in substantial stimulation (increase in V_{max} and a fall in K_m for $MgCl_2$). GTP inhibits the basal and NaF-activated enzymes almost identically and much more profoundly than the Gpp(NH)p-activated enzyme.

The Gpp(NH)p-activated enzyme is not more stable than the native enzyme to spontaneous denaturation (at 4 or 24 °C) or to inactivation by a variety of chemical reagents. The affinity and quantity of ^{125}I-labeled glucagon binding to liver and fat cell membranes, and the effect of GTP in enhancing the rate of dissociation of the hormone-membrane complex, are the same in the native and Gpp(NH)p-activated (and washed) membranes. Guanosine, however, increases the rate of hormone dissociation from the Gpp(NH)p-treated membranes more effectively than from control membranes.

Preincubation of membranes with p(NH)p, followed by washing, results in marked inhibition of native as well as Gpp(NH)p-activated activity. This inhibition is not protected by GTP, ATP, or pyrophosphate, and the inhibited enzyme is not susceptible to stimulation by Gpp(NH)p.

The results are discussed in the context of a postulated covalent reaction of Gpp(NH)p and Gpp(CH_2)p with the GTP regulatory site of adenylate cyclase. It is suggested that these reactions may be models for studying the normal mechanisms by which nucleotides and hormones regulate the activity of this enzyme.

The nucleotide, GTP, is known to be required for, or to greatly enhance, stimulation of adenylate cyclase activity by a variety of hormones in many tissues (Rodbell, Birnbaumer, Pohl & Krans, 1971; Bockaert, Roy & Jard, 1972; Krishna & Harwood, 1972; Leray, Chambaut & Hanoune, 1972; Deery & Howell, 1973; Harwood, Low & Rodbell, 1973; Wolff & Cook, 1973; Bilezikian & Aurbach, 1974; Johnson, Thompson & Williams, 1974; Sato *et al.*, 1974; Siegel & Cuatrecasas, 1974). It has recently been described that although GTP has virtually no effect in the absence of hormones, the analogs Gpp(NH)p and Gpp(CH_2)p can stimulate the enzyme in the presence or absence of hormones in a variety of tissues (Swislocki, Tierney & Sonenberg, 1973; Birnbaumer, Nakahara & Yang, 1974; Landos *et al.*, 1974; Lefkowitz, 1974, 1975; Spiegel & Aurbach, 1974; Cuatrecasas, Jacobs & Bennett, 1975; Pfeuffer & Helmreich, 1975; Schramm, 1975). In the presence of these analogs, adenylate cyclase activity is stimulated to levels much higher than are observed with GTP plus hormones, or by sodium fluoride. The data indicate that Gpp(NH)p and Gpp(CH_2)p interact at the same site as GTP in the activation of adenylate cyclase, and that this site ("regulatory") is distinct from the substrate

(ATP), catalytic site of the enzyme. The GTP regulatory site is believed to be intimately involved in the hormonal regulation of enzyme activity.

Recent studies have shown that stimulation of adenylate cyclase activity by Gpp(NH)p and Gpp(CH_2)p (Cuatrecasas *et al.*, 1975; Pfeuffer & Helmreich, 1975; Schramm, 1975) persists after washing the membranes to remove the free nucleotide present in the medium. Data has been presented which strongly suggests that after initial, unproductive binding, these analogs activate adenylate cyclase by an essentially irreversible reaction, the rate of which is sensitive to hormones (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]). It has been suggested that this reaction involves specific covalent enzyme-substrate intermediates [e.g., enzyme-p(NH)p] which decay at negligible rates, thus acting to trap an active state of the enzyme. On the basis of this data, it has been proposed (Cuatrecasas *et al.*, 1975) that GTP may normally form an active but labile (e.g., pyrophosphoryl) enzyme intermediate which decays faster than it is formed, and that hormone receptors may function principally to increase the rate of formation, and thus the steady-state concentration, of this active state of the enzyme. The activation by the GTP analogs may thus help to elucidate the normal mechanisms of hormonal activation, and these analogs may, in addition, serve as active site-directed affinity labeling reagents (Cuatrecasas *et al.*, 1975).

The reaction of these analogs with adenylate cyclase is a slow, temperature-dependent process which is susceptible to interference by various nucleotides and buffer conditions. For these reasons, when the nature of the activation is studied by adding the analogs directly to the adenylate cyclase assay, as has been done in the past, interpretations may be difficult or misleading. In recent work (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]) as well as the present studies these complexities are simplified by incubating the membranes with the analogs, followed by washing, prior to assay under standard conditions. Although this approach is technically much more tedious and difficult, it permits the process of analog activation to be studied separately. The properties of the activation reaction can thus be elucidated in detail.

Materials and Methods

[α-^{32}P]ATP (10–20 Ci/mmole) was synthesized by the method of Symons (Symons, 1968; Bennett & Cuatrecases, 1975; Bennett, Mong & Cuatrecases, 1975). The nucleotide analogs

2 Jacobs, S.J., Bennett, V., Cuatrecasas, P. 1975. Kinetics of activation of rat fat cell adenylate cyclase by phosphoramidate and phosphonate analogs of GTP. *J. Biol. Chem. (submitted for publication)*.

were obtained from Boehringer, PL-Biochemicals or Miles; essentially the same results were obtained with these.

Adenylate cyclase activity was determined (Bennett & Cuatrecasas, 1975; Bennett, Mong & Cuatrecasas, 1975; Bennett, O'Keefe & Cuatrecasas, 1975) in 0.1 ml containing [α-^{32}P]ATP (0.06 to 0.2 mM, 300–800 cpm/pmole), 6 mM $MgCl_2$, 5 mM aminophylline, an ATP-regenerating system (5 mM phosphoenolpyruvate and 50 µg/ml pyruvate kinase), 50 mM Tris-HCl, pH 8.0, and 20 to 100 µg of membrane protein. After incubating for 10 to 15 min at 30 °C, the assays were stopped by boiling (1 min) and cyclic AMP was isolated from neutral alumina columns (Ramachandran, 1971; White & Zenser, 1971). The product of the stimulated enzyme was identified by paper chromatography systems as being (>95%) cyclic AMP. Although only the results of single experiments are presented, all the results cited are essentially reproducible in separate experiments. The basal enzyme activity varies among different experiments depending on whether fat pads or cells were used, the addition of ATP and regenerating system (Jacobs *et al.*, 1975[2]), variability of protein determinations, length of time elapsing before assay, buffer conditions and temperature used, and perhaps other unknown factors. Standard errors refer to the single experiment described.

Fat pad "membranes" were prepared by homogenizing (Polytron) fat pads from 120–180 g Sprague-Dawley rats in 50 mM Tris-HCl, pH 8, or Krebs-Ringer's-bicarbonate, pH 7.4, followed by centrifugation (30 min, 40,000 × *g*). The use of such particulate fractions (instead of isolated fat cell membranes) facilitates greatly the recovery of membrane protein during the repeated washing and centrifugation procedures which were employed. However, with the exception of lower specific enzyme activities and less marked hormonal effects, similar results are obtained with membranes from isolated fat cells. Isolated fat cells were prepared as described by Rodbell (1964), and fat cell membranes as described by Chang, Marcus and Cuatrecasas (1974). Membrane protein was determined by the method of Lowry *et al.* (1951) after heating the samples for 40 min at 100 °C with 4 N NaOH; bovine serum albumin was used as the standard. The binding of ^{125}I-labeled glucagon to liver and fat cell membranes was determined in the presence of 1 mg/ml Bacitracin (Desbuquois & Cuatrecasas, 1972; Desbuquois, Krug & Cuatrecasas, 1974) using filtration (Millipore) or the oil flotation method (Gliemann *et al.*, 1972).

In all the studies presented, the effects of the nucleotide analogs were studied by preincubation with the membranes followed by thorough washing (at least twice, at 4 °C) of the membranes (Cuatrecasas *et al.*, 1975) before assay. Except for the experiments described by the first three Tables, the buffer used to wash the membranes was Krebs-Ringer's-bicarbonate, pH 7.4, unless indicated otherwise.

Results

Buffer Conditions for Incubating and Washing Membranes

As described earlier (Cuatrecasas *et al.*, 1975), incubation of fat cell membranes with Gpp(NH)p in a simple Tris-HCl buffer results in a substantial stimulation of adenylate cyclase activity when assayed in the thoroughly washed membranes (Table 1). Under these conditions very high concentrations of EDTA do not inhibit the stimulation, suggesting that divalent metal cations are not required for the activation. Even with suboptimal concentrations of Gpp(NH)p no inhibitory effect of EDTA is detected. Relatively high concentrations of phosphate and pyrophosphate

Table 1. Effect of EDTA and dithiothreitol on the stimulation of adenylate cyclase activity by Gpp(NH)p

Additions in preincubation	Adenylate cyclase activity [a]
None	5.3
Gpp(NH)p, 0.1 mM	35.3
+EDTA, 20 mM	46.0
+dithiothreitol, 5 mM	82.0
No additions	4.7
Gpp(NH)p, 30 μM	15.6
+EDTA, 10 mM	16.4
+sodium phosphate, 10 mM	15.4
+sodium pyrophosphate, 3 mM	16.3

Fat cell membranes were incubated for 15 min at 24 °C in Tris-HCl, pH 8, in the absence of additions or with EDTA or dithiothreitol. Gpp(NH)p was then added and the samples were incubated for an additional 10 min at 24 °C. Adenylate cyclase activity was determined after thoroughly washing (two times) the membranes with Tris-HCl, pH 8.
[a] Picomoles of cyclic AMP/min/mg of protein.

do not inhibit the stimulatory effects of Gpp(NH)p. If the incubation is performed in the presence of 5 mM dithiothreitol, a much greater apparent degree of stimulation is observed.

The apparent enhancement of the Gpp(NH)p effect by dithiothreitol (Table 1) probably reflects primarily a stabilization of enzyme activity against spontaneous inactivation. Since the loss of enzyme activity during the preincubation would make less enzyme available for stimulation by the analog, any condition which protects against inactivation would give the appearance of enhancing the effect of the analog unless proper controls are included. Indeed, it can be demonstrated (Table 2) that simply including dithiothreitol in the buffer used to wash the membranes (at 4 °C) results in higher basal as well as Gpp(NH)p-stimulated activities of adenylate cyclase. The relatively labile nature of this enzyme is also demonstrated by the fact that the basal enzyme activity can be affected simply by changing the buffer used to wash the membranes (Table 2). The optimal buffer for this procedure appears to be Krebs-Ringer's-bicarbonate, pH 7.6. Although by varying these conditions the extent of stimulation by Gpp(NH)p may appear to vary widely, the extent of stimulation parallels closely the control or unstimulated enzyme activity so that the degree of stimulation relative to the control activity is modified only slightly. These experiments illustrate the importance of determining by control experiments whether conditions which appear to potentiate the stimulation by the GTP analogs are in fact simply protecting the enzyme from spon-

Table 2. Effect of washing membranes with various buffers on the subsequent basal rate of adenylate cyclase and its stimulation by Gpp(NH)p

Buffer for washing	Adenylate cyclase activity [a]		
	Control	Gpp(NH)p	Fold-stimulation [b]
Tris-HCl, pH 8.0	1.3	2.4	1.8
Tris-HCl, pH 8.0 + 1 mM dithiothreitol	3.9	10.4	2.7
Krebs-Ringer's-bicarbonate, pH 7.6	2.5	6.3	2.5
Krebs-Ringer's-bicarbonate, pH 7.6 + 1 mM dithiothreitol	5.2	17.0	3.3

Fat cell membranes were washed two times at 4 °C in large volumes of the indicated buffer and suspended in 50 mM Tris-HCl, pH 8.0. The membranes were then incubated for 10 min at 24 °C in the presence and absence of 0.1 mM Gpp(NH)p. Adenylate cyclase activity was determined after thoroughly washing (4 °C) the membranes.

[a] Picomoles of cyclic AMP/min/mg of protein.

[b] Activity of Gpp(NH)p-treated relative to control activity.

taneous inactivation. Furthermore, since several preincubation and washing steps may need to be performed, and since some of these may require prolonged time periods or elevated temperatures, substantial inactivation may occur, thus necessitating optimal conditions for enzyme stabilization in all the incubation and washing procedures. It will be shown shortly that enzyme activity can be protected markedly by including ATP or GTP in the preincubation medium.

Possible Role of Membrane Sulfhydryl Groups

Studies were performed to explore the possibility that sulfhydryl groups may be involved in the reaction of Gpp(NH)p with adenylate cyclase (Table 3). Very mild treatment of fat pad membranes with the organomercurial, *p*-aminophenylmercuric acetate, results in marked loss of enzymatic activity. Subsequent treatment with dithiothreitol, however, leads to nearly complete restoration of activity. If the organomercurial-inactivated enzyme is treated with Gpp(NH)p and subsequently reactivated with dithiothreitol, no stimulatory effect of the nucleotide analog is detected. Virtually unimpaired stimulation is detected, however, if the inactivated enzyme is incubated with Gpp(NH)p after treatment with dithiothreitol to restore basal activity.

Although the mercurial-treated enzyme is incapable of reacting with Gpp(NH)p, as evidenced upon reactivation with dithiothreitol, the

Table 3. Effect of organomercurial and role of protein sulfhydryl groups on the stimulation of adenylate cyclase activity by Gpp(NH)p

Incubation			Adenylate cyclase activity [a]
First	Second	Third	
Experiment I			
No additions	No additions	No additions [b]	5.7
No additions	No additions	Gpp(NH)p [b]	44.2
Organomercurial	No additions	No additions [b]	0.2
Organomercurial	No additions	Dithiothreitol [b]	4.7
Organomercurial	Gpp(NH)p	No additions [b]	0.2
Organomercurial	Gpp(NH)p	Dithiothreitol [b]	0.3
Organomercurial	Dithiothreitol	Gpp(NH)p [b]	41.1
Experiment II			
No additions	No additions	No additions	1.5
Gpp(NH)p	No additions	No additions	61.8
No additions	Organomercurial	No additions	0.2
No additions	Organomercurial	Dithiothreitol	1.3
Gpp(NH)p	Organomercurial	No additions	0.1
Gpp(NH)p	Organomercurial	Dithiothreitol	54.6

Fat pad membranes were incubated for 5 min at 24 °C (experiment I) or 20 min at 30 °C (experiment II) in Krebs-Ringer's-bicarbonate buffer, pH 7.4, containing the indicated compounds (first incubation). The membranes were washed (4 °C) and incubated for 20 min at 30 °C (experiment I) or 5 min at 24 °C (experiment II) with the indicated additions (second incubation). After washing, the membranes were incubated for 10 min at 30 °C as indicated (third incubation). The membranes were washed before assay. The concentrations of *p*-aminophenylmercuric acetate (organomercurial), Gpp(NH)p and dithiothreitol were 0.5, 0.2 and 20 mM, respectively. Dithiothreitol was used at very high concentrations in order to accelerate the rate of reactivation.

[a] pmoles of cyclic AMP/min/mg.

[b] These incubations also contained 1 mM ATP, which was used to stabilize the enzyme against spontaneous inactivation.

Gpp(NH)p-activated enzyme can be completely (even more than the control) but reversibly (by dithiothreitol) inhibited by the organomercurial (Table 3). These studies show that adenylate cyclase which is inactivated by blocking sulfhydryl groups cannot react with Gpp(NH)p, but that once the native enzyme has reacted with Gpp(NH)p the inactivation elicited by sulfhydryl blockade does not result in the reversal of the intrinsically and permanently stimulated state of the enzyme. The results are consistent with the observation (Cuatrecasas *et al.*, 1975) that detergent-solubilized, Gpp(NH)p-stimulated adenylate cyclase activity is quantitatively adsorbed to an organomercurial-agarose affinity column, and that the enzyme can be eluted from such a column in an activated state with

dithiothreitol. Although sulfhydryl groups appear to be essential for enzymatic activity and reactivity with Gpp(NH)p, there is no evidence to suggest that this nucleotide is reacting directly with sulfhydryl groups on the enzyme.

Effect of Nucleoside Phosphate Analogs

The reaction of adenylate cyclase with Gpp(NH)p is inhibited by GTP and, less effectively, by GDP (Table 4). This is consistent with the view that Gpp(NH)p is reacting at the GTP regulatory site of the enzyme. High concentrations of ATP and its analog $Ap(CH_2)pp$ also result in some inhibition. The inhibition observed with ATP and its analog is probably more marked than the data suggests since, as will be described shortly, these nucleotides effectively stabilize the enzyme against spontaneous inactivation. These results suggest that high concentrations of ATP can bind to the GTP regulatory site of the enzyme, in agreement with the observation (Rodbell, Birnbaumer *et al.*, 1971) that ATP at high concentrations can replace GTP in the GTP-dependent activation of adenylate cyclase by hormones.

Table 4. Effect of various nucleoside phosphate analogs on the stimulation of adenylate cyclase activity by Gpp(NH)p

Addition		Adenylate cyclase activity [a]
No additions		5.1 ± 0.2
Gpp(NH)p,	30 μM	15.9 ± 0.6
Gpp(NH)p,	30 μM +	
ATP,	3 mM	15.4 ± 0.6
	5 mM	14.1 ± 0.5
$Ap(CH_2)pp$,	2 mM	13.2 ± 0.3
GTP,	10 μM	12.7 ± 0.3
	50 μM	11.1 ± 0.2
	200 μM	8.0 ± 0.2
	1 mM	5.8 ± 0.3
GDP,	0.2 mM	15.1 ± 0.5
	1 mM	6.7 ± 0.4
GMP,	5 mM	15.7 ± 0.6

Fat pad membranes were incubated for 10 min at 24 °C in Krebs-Ringer's-bicarbonate buffer, 1 mM dithiothreitol, containing the indicated compounds. The membranes were washed (4 °C) twice and assayed for adenylate cyclase activity.

[a] pmoles of cyclic AMP/min/mg protein, mean value ± standard error of triplicate replications.

Stabilization of Adenylate Cyclase Activity by ATP and GTP

Incubation of fat pad membranes with ATP or GTP results in substantial stabilization of adenylate cyclase activity (Table 5). This effect is even more apparent if the washed membranes are subsequently stimulated with Gpp(NH)p. Although the stimulated activity is much greater in the GTP- or ATP-treated membranes, the degree of stimulation relative to the control activity is the same (about 10-fold) in all instances. It has been reported previously (Cuatrecasas *et al.*, 1975) that the Gpp(NH)p-stimulated enzyme activity is also protected from inactivation by the addition of GTP or ATP. Furthermore, the ATP- or GTP-incubated (and washed) membranes still respond very well to hormones whereas the Gpp(NH)p-treated membranes are refractory to hormones and to further treatment with Gpp(NH)p (to be described). These data point again to the marked differences in the permanency of the effects obtained with GTP and Gpp(NH)p.

These results, especially the inability of GTP to reverse the stimulated state, are consistent with the irreversible nature of the Gpp(NH)p reaction. In addition, the data suggest that in certain types of preincubation procedures the addition of ATP simultaneously with Gpp(NH)p may be helpful in stabilizing the basal as well as stimulated activity, thus providing more nearly optimal conditions for the reaction. Since the potential inhibition

Table 5. Stabilization against spontaneous inactivation of adenylate cyclase activity by GTP and ATP

Incubation		Adenylate cyclase activity [a]	Fold stimulation
First	Second		
No additions	No additions	2.4	
GTP	No additions	4.5	
ATP	No additions	5.9	
App(NH)p	No additions	5.7	
No additions	Gpp(NH)p	25.3	10
GTP	Gpp(NH)p	47.7	10
ATP	Gpp(NH)p	56.5	9.6
App(NH)p	Gpp(NH)p	57.3	10

Fat pad membranes were incubated for 20 min at 30 °C in Krebs-Ringer's-bicarbonate, 1 mM dithiothreitol, containing 5 mM GTP, 5 mM ATP or 2.5 mM App(NH)p as indicated. The membranes were washed (4 °C) twice and incubated for 20 min at 30 °C in the same buffer with and without 0.1 mM Gpp(NH)p. Adenylate cyclase activity was determined after washing the membranes.

[a] pmoles of cyclic AMP/min/mg of protein.

by ATP or GTP is very weak, and since it is readily overcome with time (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]) because of the irreversibility of the Gpp(NH)p reaction, the interference caused by this inhibition may be very minimal, especially with ATP.

Irreversibility of Gpp(NH)p-Stimulated State of Adenylate Cyclase

The stimulation of enzyme activity achieved by treatment with Gpp(NH)p is not lost by incubating the thoroughly washed membranes for as long as 40 min at 30 °C (Table 6). In addition, the activity of such prestimulated, washed and incubated membranes is refractory to further stimulation by Gpp(NH)p under conditions where, judging from control experiments (Table 6), reversal of the stimulated state should have been detectable by susceptibility to restimulation. It has previously been shown (Cuatrecasas *et al.*, 1975) that GTP 5 mM) does not induce reversal of the Gpp(NH)p-stimulated state, despite the fact that these two nucleotides are apparently binding to the same site.

Table 6. Lack of spontaneous reversal of Gpp(NH)p-stimulated adenylate cyclase activity and refractoriness to further stimulation

Incubation				Adenylate cyclase activity[a]
First	Second		Third	
30 °C, 20 min	4 °C, 40 min	30 °C, 40 min	30 °C, 20 min	
No additions	ATP		ATP	3.2 ± 0.1
No additions	ATP		Gpp(NH)p	10.4 ± 0.6
Gpp(NH)p	ATP		ATP	19.1 ± 0.9
Gpp(NH)p	ATP		Gpp(NH)p	18.4 ± 0.8
Gpp(NH)p	Gpp(NH)p		ATP	19.5 ± 0.6
No additions		ATP	ATP	2.1 ± 0.2
No additions		ATP	Gpp(NH)p	7.4 ± 0.4
Gpp(NH)p		ATP	ATP	16.8 ± 0.7
Gpp(NH)p		ATP	Gpp(NH)p	15.9 ± 0.5

Fat pad membranes were incubated for 20 min at 30 °C in Krebs-Ringer's-bicarbonate buffer, pH 7.4, 1 mM dithiothreitol, without additions or with 0.1 mM Gpp(NH)p. The membranes were washed (4 °C), suspended in the same buffer, incubated for 40 min at 4 or at 30 °C in the presence of 4 mM ATP or 0.1 mM Gpp(NH)p, washed again, resuspended in the same buffer, and incubated for another 20 min at 30 °C with 5 mM ATP or 0.1 mM Gpp(NH)p. Adenylate cyclase was assayed after washing and resuspending in the same buffer.

[a] pmoles of cyclic AMP/min/mg protein, average ± standard error of triplicate replications.

Various compounds were tested for their ability to selectively reverse the Gpp(NH)p-stimulated state of adenylate cyclase (Table 7). Glycine, $MgCl_2$, dithiothreitol, sodium fluoride and, notably, EDTA do not cause reversal. In some cases the activity is enhanced, but this is probably simply a reflection of the stabilization of the unstimulated activity. Although certain other chemical treatments can be shown to decrease the stimulated activity (to be presented shortly), it has not yet been possible to demonstrate suppression which is reversible by Gpp(NH)p treatment or selective loss of activity of the stimulated relative to the control enzyme.

Attempts to Reverse Stimulation with Hydroxylamine

Because of the possibility that the reaction of Gpp(NH)p may involve ester or other bonds which may be selectively cleaved by strong nucleophiles, neutral hydroxylamine was used to try to chemically reverse the Gpp(NH)p-stimulated state of adenylate cyclase. Hydroxylamine decreases profoundly the activity of the stimulated enzyme, although the activity of the control enzyme is also decreased (Table 8). However, no further stimulation by Gpp(NH)p is seen subsequently on the stimulated (and reversed) enzyme while the residual activity of the control can be

Table 7. Possible reversibility of Gpp(NH)p-stimulated adenylate cyclase activity by various compounds

Membrane	Addition	Adenylate cyclase activity [a]
Control	None	4.6
	$MgCl_2$, 10 mM	5.6
	Glycine, 0.1 M	6.6
	Dithiothreitol, 5 mM	6.4
	NaF, 10 mM	8.4
	EDTA, 10 mM	5.9
Gpp(NH)p-treated	None	58.6
	$MgCl_2$, 10 mM	69.2
	Glycine, 0.1 M	62.7
	Dithiothreitol, 5 mM	63.5
	NaF, 10 mM	51.0
	EDTA, 10 mM	55.1

Fat pad membranes were incubated for 20 min at 30 °C in 50 mM Tris-HCl, pH 8.0, with and without 0.1 mM Gpp(NH)p. The membranes were washed (4 °C), resuspended in the same buffer and incubated for 10 min at 30 °C in the presence of the indicated compound. The membranes were washed, suspended in the same buffer and assayed for adenylate cyclase activity.

[a] pmoles of cyclic AMP/min/mg protein.

Table 8. Effect of hydroxylamine on the adenylate cyclase activity of fat pad membranes pretreated with Gpp(NH)p

Incubation			Adenylate cyclase activity [a]
I	II	III	
ATP	ATP	ATP	4.7±0.1
ATP	ATP	Gpp(NH)p	11.9±0.2
ATP	HA	ATP	0.8±0.05
ATP	HA	Gpp(NH)p	3.6±0.1
Gpp(NH)p	ATP	ATP	30.2±0.4
Gpp(NH)p	ATP	Gpp(NH)p	27.6±0.3
Gpp(NH)p	HA	ATP	7.1±0.1
Gpp(NH)p	HA	Gpp(NH)p	6.3±0.1

Rat fat pad membranes suspended in Krebs-Ringer's-bicarbonate buffer, 1 mM dithiothreitol, were incubated sequentially for three periods (20 min at 30 °C) with 2 mM ATP 0.1 mM Gpp(NH)p, or 0.1 M neutral (pH 8) hydroxylamine hydrochloride (HA), as indicated. After each incubation the membranes were washed and suspended in the same buffer, except for the final suspension which was in 50 mM Tris-HCl, pH 7.4.

[a] pmoles cyclic AMP produced/min/mg of protein, mean ± standard error.

stimulated fivefold. Here, as in other experiments (e.g., Table 6), no spontaneous reversal of the Gpp(NH)p effect can be detected by subsequent restimulation. Hydroxylamine clearly cannot be acting by cleaving a specific chemical bond in a reversible manner, as would be expected if a hydroxamate of the leaving group were formed. However, the possibility of a hydroxylamine-induced reversal with formation of a specific, inactivated enzyme-hydroxamate product cannot be excluded.

Reaction with Gpp(CH_2)p

Earlier studies (Cuatrecasas *et al.*, 1975) have shown that the stimulation of adenylate cyclase activity by Gpp(CH_2)p, like that of Gpp(NH)p, persists after thoroughly washing the membranes. Reaction of fat cell membranes with very high, saturating (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]) concentrations of Gpp(CH_2)p or Gpp(NH)p for 20 min at 4 °C does not result in stimulation of enzyme activity. As observed for the phosphonate analog, the stimulatory effects of Gpp(CH_2)p are also inhibited by GTP (not shown), and isoproterenol increases the rate at which the analog stimulates the enzyme (Jacobs *et al.*, 1975[2]). Despite these similarities in the action of the two analogs, some interesting differences exist. Much higher concentrations of Gpp(CH_2)p are required com-

pared to Gpp(NH)p (Fig. 1 and Cuatrecasas *et al.*, 1975), and the intrinsic activity of the Gpp(CH_2)p-activated enzyme is lower (Fig. 1).

In attempts to further ascertain whether these two analogs are activating by the same mechanism and by reaction at the same site, membranes were treated sequentially with Gpp(CH_2)p and Gpp(NH)p. Membranes treated with maximal concentrations of Gpp(NH)p are not stimulated further by subsequent reaction with Gpp(CH_2)p (data not shown). In contrast, the Gpp(CH_2)p-stimulated enzyme is stimulated further by treatment with Gpp(NH)p (Table 9). However, the fold-stimulation observed does not suggest that Gpp(NH)p is acting to enhance the Gpp(CH_2)p-stimulated enzyme. It is rather more likely that stimulation by Gpp(CH_2)p is incomplete and that the high concentrations of Gpp(CH_2)p used results in protection against inactivation, thus making more of the native enzyme available for subsequent stimulation by Gpp(NH)p. In support of this explanation is the observation that simultaneous addition of both analogs in varying concentrations results in the inhibition of Gpp(NH)p stimulation by Gpp(CH_2)p (Table 10). With the highest concentrations of the

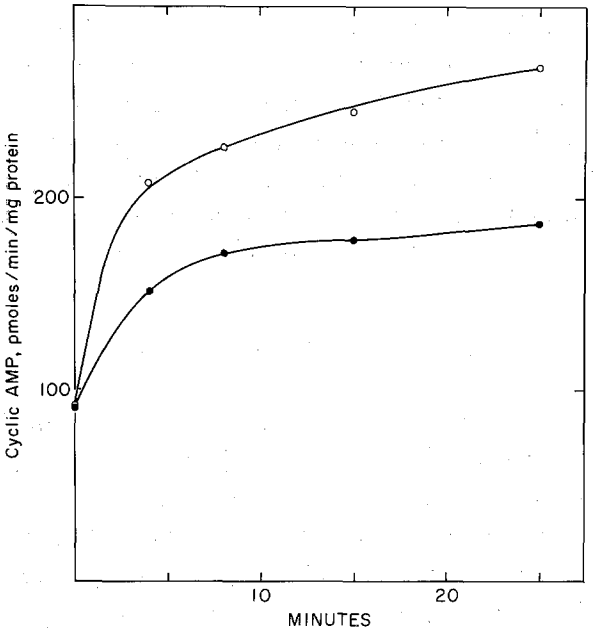

Fig. 1. Effect of time of preincubation on the stimulation of adenylate by Gpp(NH)p and Gpp(CH_2)p. Membranes from isolated fat cells were incubated with 0.1 mM Gpp(NH)p (○—○) or 2 mM Gpp(CH_2)p (●—●) in 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, 0.25 mM ATP, 5 mM $MgCl_2$, 5 mM phospho(enol) pyruvate, and 50 μg/ml of pyruvate kinase for the indicated times at 30 °C. The membranes were washed twice and assayed for enzyme activity

Table 9. Effect of Gpp(NH)p on adenylate cyclase activity of membranes previously treated with $Gpp(CH_2)p$

Incubation		Adenylate cyclase activity [a]
First	Second	
No addition	No addition	0.4
No addition	Gpp(NH)p, 20 μM	2.7
No addition	Gpp(NH)p, 0.2 mM	4.5
$Gpp(CH_2)p$, 0.5 mM	No addition	6.5
$Gpp(CH_2)p$, 0.5 mM	Gpp(NH)p, 20 μM	8.5
$Gpp(CH_2)p$, 0.5 mM	Gpp(NH)p, 0.2 mM	12.4
$Gpp(CH_2)p$, 2 mM	No addition	21.1
$Gpp(CH_2)p$, 2 mM	Gpp(NH)p, 20 μM	33.0
$Gpp(CH_2)p$, 2 mM	Gpp(NH)p, 0.2 mM	40.6

Fat pad membranes were incubated for 15 min at 35 °C in 50 mM Tris-HCl, pH 8, containing the indicated concentration of $Gpp(CH_2)p$. The membranes were washed and incubated again for 5 min at 30 °C with Gpp(NH)p. Adenylate cyclase activity was determined after washing the membranes. Higher temperatures (35 °C) were used in these experiments to improve the stimulation of $Gpp(CH_2)p$; under these conditions, substantial inactivation of enzyme activity occurs.

[a] pmoles of cyclic AMP/min/mg of protein.

latter, inhibition of the Gpp(NH)p effect is complete and the stimulatory effect observed results entirely from the reaction of the less effective activator. These results also suggest that the intrinsic activity of the $Gpp(CH_2)p$-activated enzyme is lower than that induced by Gpp(NH)p. Thus, the two nucleotides appear to react at the same site but the reaction

Table 10. Inhibition by $Gpp(CH_2)p$ of Gpp(NH)p stimulation of adenylate cyclase activity

Addition		Adenylate cyclase activity [a]
No additions		6.4
Gpp(NH)p,	40 μM	68.5
	0.1 mM	94.2
$Gpp(CH_2)p$,	80 μM	22.3
	2 mM	39.0
Gpp(NH)p,	40 μM	
+$Gpp(CH_2)p$,	80 μM	87.7
+$Gpp(CH_2)p$,	0.4 mM	76.1
+$Gpp(CH_2)p$,	2 mM	38.4

Fat pad membranes were incubated for 5 min at 30 °C in 50 mM Tris-HCl, pH 8, in the presence of the indicated nucleotide analogs. The membranes were washed twice and assayed for adenylate cyclase activity.

[a] pmoles of cyclic AMP/min/mg of protein.

of Gpp(CH_2)p is of lower apparent affinity and it results in a less effectively stimulated enzyme.

Stimulation of Detergent-Solubilized Enzyme by Gpp(NH)p

It has recently been shown (Cuatrecasas *et al.*, 1975) that the Gpp(NH)p-stimulated state of adenylate cyclase of fat pad membranes is retained upon solubilization with nonionic detergents. It can also be demonstrated that addition of Gpp(NH)p to the unstimulated, detergent-solubilized enzyme results in direct activation of enzyme activity (Fig. 2). However, unlike the effects observed with intact membranes (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]), the hormones, (−)-isoproterenol and glucagon, do not affect the rate or extent of this activation (Fig. 2). Swislocki *et al.* (1973) have previously shown that Gpp(CH_2)p can activate detergent-solubilized rat liver adenylate cyclase.

Properties of Gpp(NH)p-Stimulated Adenylate Cyclase

Effect of hormones and sodium fluoride. The Gpp(NH)p-activated enzyme from isolated fat cell membranes is refractory to stimulation by sodium fluoride, (−)-isoproterenol and ACTH under conditions in which

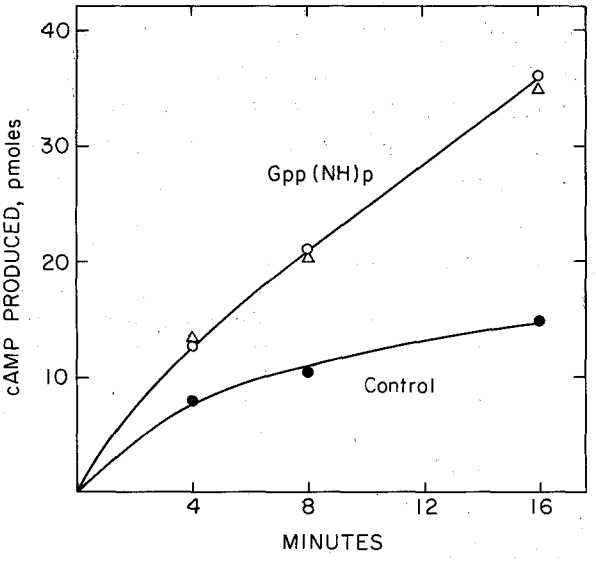

Fig. 2. Stimulation of detergent-solubilized adenylate cyclase by Gpp(NH)p. Fat pad membranes were suspended in Tris-HCl, pH 8, containing 1% Lubrol-PX and sonicated three times for 1 sec. The sample was adjusted with $MgCl_2$ (8 mM) and albumin (0.015%) and centrifuged for 40 min at 40,000 × *g*. The clear supernatant was assayed at 30 °C for adenylate activity in the absence (●) or presence (○, △) of 10 μM Gpp(NH)p and 10^{-6} M (−)-isoproterenol (△). The latter hormone also had no effect on the control enzyme activity. Glucagon (10^{-6} M) also has no effect on either process (not shown)

substantial effects are observed on the control (preincubated and washed) membranes (Table 11).[3]

Effect of ATP and $MgCl_2$ concentrations on adenylate cyclase preactivated by Gpp(NH)p and NaF. Under conditions of high $MgCl_2$ concentration, the Gpp(NH)p-activated enzyme is stimulated at all concentrations of the substrate ATP (Fig. 3). Although the principal effect appears to be on V_{max}, an effect (increase) in the apparent K_m for ATP is also observed. Lefkowitz (1974), studying the effects of Gpp(NH)p added directly to the adenylate cyclase (cardiac) assay, has reported that the effect is primarily on V_{max}.

As described previously for adenylate cyclase of brain (Perkins & Moore, 1971), parotid gland (Schramm & Naim, 1970), adrenal (Kelley

Table 11. Response of adenylate cyclase of fat cell membranes to NaF and hormones following preincubation in the presence and absence of Gpp(NH)p

Additions	Adenylate cyclase activity [a]	
	Control	Gpp(NH)p-treated
NaF		
No additions	28	144
2 mM	58	142
5 mM	112	156
10 mM	90	146
(−)-Isoproterenol		
No additions	36	371
0.5 μM	81	334
5 μM	129	354
50 μM	174	344
ACTH		
No additions	46	201
2 μM	62	200

Fat cell membranes were incubated (20 min at 24 °C) in the absence and presence of Gpp(NH)p (5 μM) in a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phospho(enol) pyruvate (5 mM), ATP (0.2 mM), $MgCl_2$ (6 mM), pyruvate kinase (60 μg/ml) and 1 to 2 mg per ml of membrane protein, and were then diluted 20-fold in ice-cold Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM). The suspensions were centrifuged (30 min at 35,000 × *g*) and the resulting membrane pellets assayed for adenylate cyclase activity (15 min at 30 °C) with additions as described below. The influence of each stimulant was determined in a separate experiment. The specific activity of adenylate cyclase is higher in these experiments because of the inclusion of dithiothreitol, ATP and an ATP-regenerating system (Jacobs *et al.*, 1975[2]).

[a] pmoles of cyclic AMP/min/mg protein.

3 When membranes from fat pads rather than isolated cells are used, the Gpp(NH)p-activated enzyme still shows some sensitivity to NaF (but not hormones). The reason for this difference is not understood.

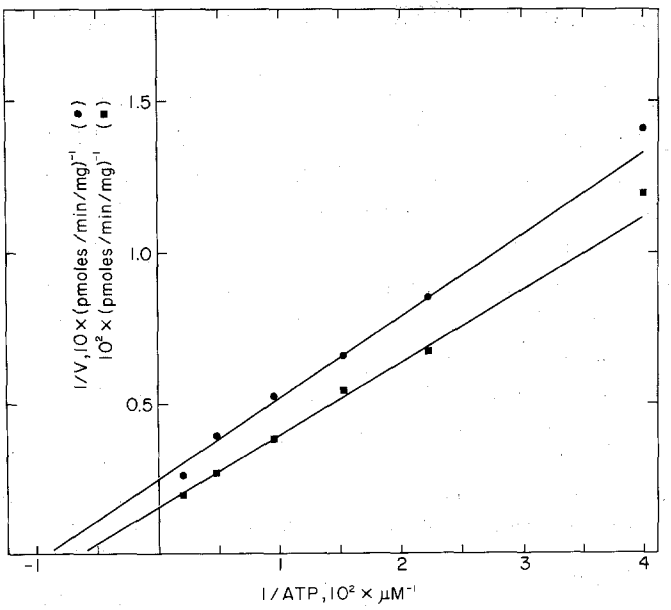

Fig. 3. Influence of increasing concentrations of ATP on the adenylate cyclase activity of control (●) and Gpp(NH)p-stimulated (■) fat pad membranes. Rat fat pad membranes (*see* Materials and Methods) were suspended in Tris-HCl (50 mM, pH 7.8) containing dithiothreitol (1 mM) at a concentration of about 1 mg/ml of membrane protein, and incubated for 10 min at 30 °C either with no addition (●), or in the presence of 200 μM Gpp(NH)p (■). The suspensions were then diluted 20-fold with ice-cold Tris-HCl buffer containing 1 mM dithiothreitol, pelleted (30 min at 35,000 × *g*) and washed once more with the same buffer. The final membrane pellets were suspended in the same Tris-HCl buffer and assayed for adenylate cyclase activity (10 min at 30 °C) (*see* Materials and Methods) in the presence of aminophylline (5 mM), phospho(enol) pyruvate (5 mM), $MgCl_2$ (6.2 mM), [α-^{32}P]ATP (5 μM, 10 Ci/mmole), pyruvate kinase (60 μg/ml), 22 μg of membrane protein, and various concentrations of ATP. The data are expressed as a double-reciprocal plot of activity versus ATP concentration; the values were determined in triplicate

& Koritz, 1971), heart (Severson, Drummond & Sulakhe, 1972) and peritoneal granulocytes (Constantopoulos & Najjar, 1973; Najjar & Constantopoulos, 1973), treatment with NaF results in persistently activated (remaining after thoroughly washing the membranes) adenylate cyclase activity in membranes from isolated fat cells (Fig. 4). In the latter, the stimulation by NaF is as great or greater than that seen with Gpp(NH)p. Examination of the $MgCl_2$ dependence of activity reveals no change in the apparent K_m for $MgCl_2$ for the Gpp(NH)p-activated enzyme (Fig. 4). In contrast, the NaF-activated (preactivated and washed) enzyme displays a change in V_{max} as well as a fall in the apparent K_m for $MgCl_2$ (note differences in vertical axis, Fig. 4, right). Birnbaumer, Pohl and Rodbell (1969) have previously described that in fat cell membranes NaF (examined

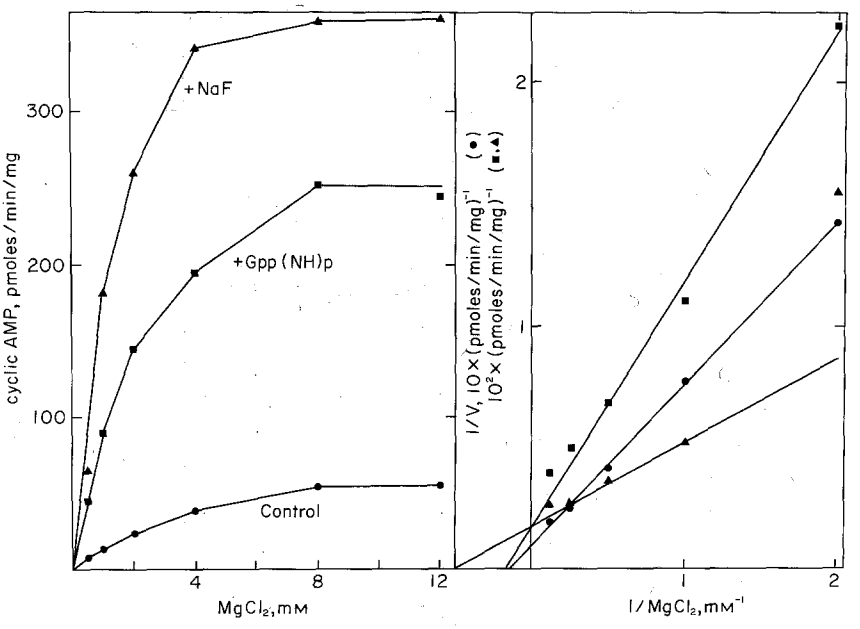

Fig. 4. The influence of free Mg^{2+} concentration on adenylate cyclase activity of fat cell membranes preincubated with Gpp(NH)p (■), NaF (▲), or with no additions (●). Fat cell membranes were suspended in a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phospho(enol) pyruvate (5 mM), ATP (0.2 mM), $MgCl_2$ (6.2 mM), pyruvate kinase (60 μg/ml), and about 1 mg/ml of membrane protein, and incubated (15 min at 30 °C) in the presence of either 5 μM Gpp(NH)p (■), 10 mM NaF (▲), or with no additions (●). The suspensions were then diluted 20-fold with ice-cold Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), and centrifuged (30 min at 35,000 × *g*). The membrane pellets were assayed for adenylate cyclase activity (15 min at 30 °C) in the presence of aminophylline (5 mM), phospho(enol) pyruvate (5 mM), [α-^{32}P]ATP (0.12 mM, 226 cpm per pmole), $MgCl_2$ (0.12 mM), pyruvate kinase (60 μg/ml), Tris-HCl (50 mM, pH 8), 60 μg of membrane protein, and various concentrations of $MgCl_2$. The data are expressed as magnesium-stimulated activity (stimulated activity minus basal activity), where the activities in the absence of free Mg^{2+} were 10, 19, and 1 pmole/min/mg for the Gpp(NH)p-treated, NaF-treated and control membranes, respectively. The values were determined in triplicate

in the assay) affects primarily the K_m for $MgCl_2$. In heart microsomal adenylate cyclase, however, NaF (in the assay) alters the V_{max} without affecting the K_m for $MgCl_2$ (Drummond & Duncan, 1970; Drummond, Severson & Duncan, 1971; Severson *et al.*, 1972).

Effect of GTP. Since it is proposed that Gpp(NH)p reacts at the GTP of adenylate cyclase, it is of interest to know whether GTP has any effects on the Gpp(NH)p-activated enzyme. As described previously (Cryer, Jarrett & Kipnis, 1969; Harwood *et al.*, 1973; Lefkowitz, 1974), GTP inhibits the basal adenylate cyclase of fat cell membranes (Fig. 5). The magnitude of inhibition and the GTP concentration dependence are nearly identical

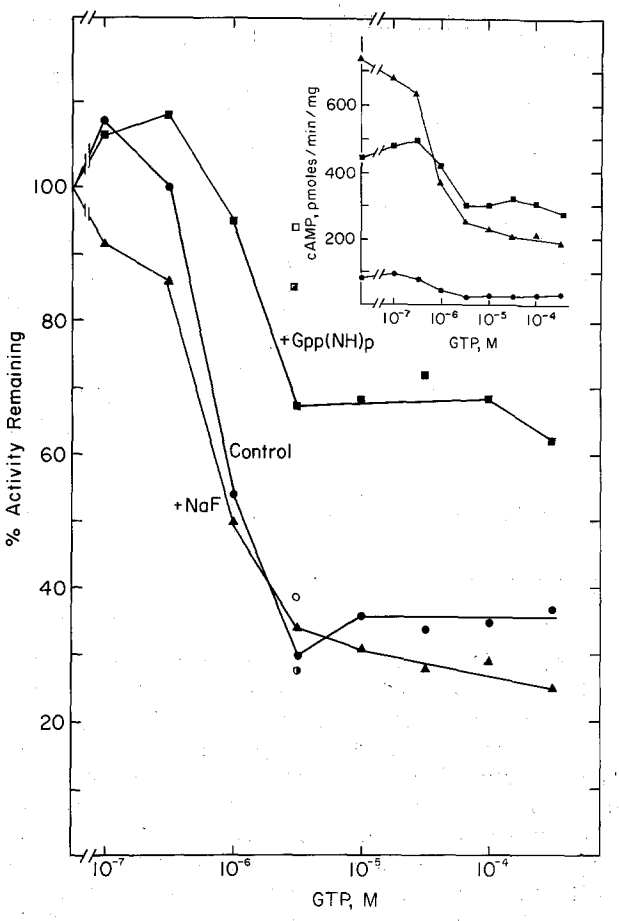

Fig. 5. The influence of increasing concentrations of GTP on the adenylate cyclase activity of fat cell membranes preincubated with Gpp(NH)p (■, □, ◪), NaF (▲), or with no additions (●, ○, ◑). Fat cell membranes were suspended in a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), ATP (0.2 mM), phospho(enol) pyruvate (5 mM), $MgCl_2$ (6.2 mM), pyruvate kinase (60 μg/ml), and about 1 mg/ml of membrane protein, and incubated (15 min at 30 °C) either alone (●, ○, ◑), with 10 mM NaF (▲), or with 5 μM Gpp(NH)p (■, □, ◪). The suspensions were then diluted 20-fold with Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), centrifuged (30 min at 35,000 × *g*), and the resulting membrane pellets assayed for adenylate cyclase activity (15 min at 30 °C) in the presence of aminophylline (5 mM), phospho(enol) pyruvate (5 mM), $MgCl_2$ (6.2 mM), [α-^{32}P]ATP (0.25 mM, 120 cpm/pmole), pyruvate kinase (60 μg/ml), Tris-HCl (50 mM, pH 8), 55 μg of membrane protein, and various concentrations of GTP. The symbols □, ◪, ○, ◑ indicate the extent of inhibition observed with GTP in other experiments. The values were determined in triplicate

for the control and NaF-treated (and washed) membranes (Fig. 5). In the case of the Gpp(NH)p-activated enzyme, however, substantially less inhibition is observed, and greater (about fivefold) concentrations of GTP are required to achieve detectable inhibition. In many experiments, con-

centrations of GTP as high as 5×10^{-5} M have virtually no effect on the Gpp(NH)p-activated enzyme while the control enzyme is inhibited by more than 50%. These results suggest that significant differences exist between the NaF- and Gpp(NH)p-activated enzymes with respect to this property.

Stability to Inactivation of the Gpp(NH)p-Stimulated Adenylate Cyclase

The Gpp(NH)p-stimulated enzyme activity is not more stable than the control activity to spontaneous inactivation, whether measured at 4 or 24 °C, and whether measured in the particulate or Lubrol PX-solubilized state. For example, in carefully controlled experiments the stimulated (eightfold) particulate activity falls by 12 and 29%, and the control falls by 5 and 35%, after storage at 4 °C (Tris-HCl, 1 mM dithiothreitol) for 90 min and 16 hr, respectively. At 24 °C the activities fall by 74 and 76% at 90 min, and by 91 and 96% at 16 hr, for the control and activated enzymes, respectively. If these same samples are solubilized with Lubrol PX, the activated enzyme falls by 4 and 49% (4 °C), or by 82 and 97% (24 °C), at 90 min and 16 hr, respectively. The control, solubilized enzyme falls by 5 and 61% (4 °C), or by 80 and 98% (24 °C), at 90 min and 16 hr, respectively.

Furthermore, a number of nonspecific and specific nucleophiles and oximes used to try to selectively reverse the activity of the activated enzyme nearly always result in the same degree of inactivation of the activated and control enzymes. In addition to hydroxylamine (Table 8), hydrazine inhibits both enzymes equally (not shown). Approximately the same degree of inactivation is observed for both enzymes with the following reagents used at varying concentrations and at various time periods at 24 °C: 2-(hydroxyiminomethyl)-1-methylpyridinium iodide (2-PAM); 1,1′-trimethylene *bis* (4-hydroxyiminoimethyl pyridinium) dibromide (TMB_4); monoisonitrosoacetone (MINA); diacetyl monoxime (DAM) (data not shown).

Effect of Gpp(NH)p Treatment of Liver and Fat Cell Membranes on the Binding of ^{125}I-Labeled Glucagon

The binding of ^{125}I-labeled glucagon to liver membranes is altered markedly by guanyl nucleotides (Rodbell, Krans, Pohl & Birnbaumer,

1971; Birnbaumer & Pohl, 1973; Rodbell, Lin & Salomon, 1974).[4] GTP, for example, increases the rate of dissociation of the bound hormone and decreases the steady-state amount of bound hormone. Because addition of Gpp(NH)p to the assay mimics these effects, albeit at 100-times greater concentrations than GTP (Rodbell, Krans *et al.*, 1971), the effects of preincubating freshly prepared liver and fat cell membranes with Gpp(NH)p on the subsequent glucagon binding properties of the washed membranes was studied in detail. Under a variety of conditions studied, the binding of ^{125}I-labeled glucagon is similar in the control and Gpp(NH)p-treated, washed membranes (data not shown). In numerous experiments and under varying conditions the apparent affinity of binding, whether measured by competition-displacement curves using native glucagon or by saturation kinetics, the quantity of glucagon bound at varying concentrations of ^{125}I-labeled glucagon, and the rate of spontaneous dissociation (at various temperatures) of the bound hormone, are the same (not shown). Furthermore, GTP enhances the rate of dissociation of bound hormone equally well in the control and Gpp(NH)p-treated membranes (Table 12). Although an apparent lack of correlation exists between the effects of GTP and of preactivation by Gpp(NH)p on glucagon binding, it is important that free guanosine can enhance the rate of glucagon dissociation from the Gpp(NH)p-treated membranes much better than from the control membranes (Table 12).

Irreversible Inhibition of Adenylate Cyclase Activity by p(NH)p

In the course of performing control experiments it was discovered that the nonnucleotide analog of pyrophosphate, p(NH)p, could profoundly inhibit adenylate cyclase activity of fat cell membranes. This inhibitory effect persists after thoroughly washing the p(NH)p-treated membranes (Table 13). As in the previously described experiments, GTP and ATP can be shown to protect against spontaneous inactivation, but these nucleotides protect very minimally, if at all, against inactivation by p(NH)p. The latter compound can also inhibit irreversibly the Gpp(NH)p-stimulated activity (not shown). Furthermore, Gpp(NH)p can stimulate the activity of membranes previously treated with p(NH)p, but the degree of stimulation is strictly proportional to the residual activity.

4 It is also of interest that stimulation of adenylate cyclase of liver membranes by cholera toxin results in an increase in the apparent affinity of glucagon for enzyme activation and for binding, and it decreases the rate of dissociation of the membrane-bound hormone (Bennett, O'Keefe & Cuatrecasas, 1975).

Table 12. Effect of guanine nucleotides on the rate of dissociation of ^{125}I-labeled glucagon from control and Gpp(NH)p-treated membranes

Addition		^{125}I-Labeled glucagon bound (cpm)
Control membranes		
No additions		21,700
GTP,	50 μM	14,900
	0.5 mM	16,700
GMP,	50 μM	19,600
	0.5 mM	17,700
Guanosine,	1 mM	17,700
	10 mM	17,800
Gpp(NH)p-treated membranes		
No additions		22,800
GTP,	50 μM	14,400
GTP,	0.5 mM	16,500
GMP,	50 μM	19,300
	0.5 mM	18,100
Guanosine,	1 mM	15,400
	10 mM	10,200

Light liver membranes (Chang, Bennett & Cuatrecasas, 1975), were incubated in the absence (control) and presence of 0.2 mM Gpp(NH)p plus 10^{-5} M (−)-isoproterenol for 20 min at 30 °C in Krebs-Ringer's-bicarbonate buffer, pH 7.4, 2 mM dithiothreitol. The membranes were washed twice (4 °C) by centrifugation with the same buffer (no additions) and suspended in 50 mM Tris-HCl, pH 7.4, 0.1% albumin, 1 mM dithiothreitol and 0.1% bacitracin (Desbuquois & Cuatrecasas, 1972). ^{125}I-labeled glucagon (3×10^5 cpm/ml, 150μCi/μg) was added and the samples were incubated at 24 °C for 10 min. The membranes were washed twice and suspended (4 °C) in the same Tris-HCl buffer. The indicated compounds were added to 0.2 ml samples of the suspension, and these samples were incubated for 15 min at 30 °C. Binding was determined by the oil flotation method using a Beckman microfuge (Chang & Cuatrecasas, 1974; Chang *et al.*, 1975).

Thus, the irreversible inhibition caused by this compound appears to be unrelated to the GTP regulatory site of the enzyme. Notably, this effect is not observed with pyrophosphate or phosphate (Table 1).

Discussion

The present studies describe approaches for studying the activation of adenylate cyclase by Gpp(NH)p and Gpp(CH_2)p, and some properties of this process are presented. Unlike previously used methods, the activation process is studied separately from the measurement of enzyme activity (assay) during a controlled period of preincubation (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]).

Table 13. Inhibition of adenylate cyclase activity by prior incubation of membranes with inorganic imidodiphosphate (p(NH)p)

Addition	Adenylate cyclase activity[a]
No additions	7.9±0.2
GTP, 3 mM	20.9±0.6
ATP, 3 mM	26.5±0.5
p(NH)p, 2 mM[b]	3.2±0.1
+GTP, 3 mM	6.8±0.1
+ATP, 3 mM	9.6±0.2
+App(NH)p, 0.5 mM	3.7±0.1

Fat cell membranes were incubated for 20 min at 30 °C in Krebs-Ringer's-bicarbonate, 1 mM DTT, containing the indicated compounds. The membranes were washed (4 °C) twice before determining adenylate cyclase activity.

[a] pmoles of cyclic AMP/min/mg protein, mean value±standard error of triplicate replications.

[b] Gpp(NH)p can stimulate the activity of membranes previously treated with p(NH)p, and the degree of stimulation is proportional to the residual activity.

On the basis of this and related (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]) studies, it is suggested that after initial unproductive binding of the analog, activation results from an irreversible, covalent reaction with the GTP regulatory site of the enzyme, and that this reaction is analogous to (but more stable than) that which normally occurs when GTP and hormones activate adenylate cyclase. From chemical considerations it has been proposed (Cuatrecasas *et al.*, 1975) that the active intermediate may be enzyme-p(NH)p or enzyme-p(CH_2)p which derives its permanent state of activation by being relatively resistant to hydrolysis compared to the proposed normal active intermediate, enzyme-pp. Since hormones appear to require GTP (or analogous nucleotides) for activation, and since the rate but not the extent of Gpp(NH)p and Gpp(CH_2)p activation is affected by hormones (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]), it has been suggested that hormones may stimulate principally by increasing the rate of formation, and thus the steady-state concentration, of an active, pyrophosphorylated enzyme intermediate.

There are compelling reasons for postulating that activation by these analogs is essentially irreversible. The activation persists after the nucleotide analog free in the medium is removed by thorough washing of the membranes, and no reversal of the stimulated state can be observed by further, prolonged incubations (even at 30 °C), even in the presence of high concentrations of GTP, which presumably binds to the same site. The activated state of the enzyme is maintained upon solubilization with

nonionic detergents (Cuatrecasas *et al.*, 1975), and this active enzyme can be quantitatively adsorbed to, washed and eluted from an organomercurial-agarose column (Cuatrecasas *et al.*, 1975). The activated state is also maintained during chromatography on relatively large Sepharose 6B columns (*unpublished*). In addition, the washed, activated membrane enzyme which is inactivated with an organomercurial reagent can be completely reconstituted by subsequent treatment with dithiothreitol (Table 3). Furthermore, although GTP inhibits the activation by Gpp(NH)p, this inhibition is overcome with longer periods of activation, and the apparent "K_m" for GTP (inhibition) or for Gpp(NH)p (activation) varies with the length of the preincubation (Jacobs *et al.*, 1975[2]). Despite the apparent irreversibility of the activation, virtually no activation occurs at 4 °C (Fig. 2 and Cuatrecasas *et al.*, 1975), and even at 24 or 30 °C the rate of activation is relatively slow despite the utilization of concentrations of the analog beyond which no further effects on the rate or extent of the activation are detectable (Jacobs *et al.*, 1975[2]). In addition to the slow and temperature-dependent nature of the reaction, the concentrations of the analog (especially of Gpp(CH_2)p) required to achieve a maximal *rate* of activation are extremely high relative to the protracted character of the activation which is elicited. Thus, although the persistent nature of the activation could in principle be compatible with a simple, potentially reversible binding process of extraordinarily high affinity, the kinetics, temperature and concentration dependencies and the GTP effects are most difficult to reconcile with such a mechanism.

In the simplest rationalization of the available data, it is visualized that the analogs first form an inactive, reversible Michaelis complex of low (probably lower than GTP)[5] affinity with the GTP regulatory site of the enzyme. Subsequently a relatively slow, temperature-dependent covalent reaction occurs at that site which yields the permanently stimulated form of the enzyme. Since hormones affect the rate (but not extent) of the reaction even at saturating concentrations of the analog, it is likely that it is this second reaction that is facilitated by hormones. By analogy it has been postulated (Cuatrecasas *et al.*, 1975) that GTP normally participates by a similar covalent interaction but that the rate of decay of the intermediate is more rapid than the rate of its formation. In this scheme hormones would stimulate adenylate cyclase activity by increasing the

5 At early times, the concentration dependence of these analogs for permanent stimulation is very high (Jacobs *et al.*, 1975[2]); compared to GTP, much higher concentrations are required when present in the assay (Rodbell, Krans *et al.*, 1971), and GTP can inhibit activation at concentrations lower than Gpp(NH)p (Lefkowitz, 1974).

rate of covalent bond formation so that it would exceed the rate of decay. The analogs, by forming enzyme derivatives which are not readily susceptible to the normal mechanisms of decay (e.g., by pyrophosphatase or phosphatase hydrolysis), serve as powerful active site-directed affinity labeling reagents.

Pfeuffer and Helmreich (1975) recently proposed that a normally inhibitory Gpp(NH)p-binding protein dissociates from adenylate cyclase upon binding of the analog, thus leaving an activated enzyme subunit in a manner analogous to that occurring with cyclic AMP-dependent protein kinases. This mechanism, however, does not readily explain the inability of GTP to induce a permanently activated enzyme or to prevent stimulation by Gpp(NH)p, and the need to involve a special irreversible mechanism still exists on the basis of the considerations discussed above. Furthermore, since as discussed above simple binding alone cannot readily explain the data, the critical activating step must still depend on a subsequent complex and unique (probably covalent) reaction. Thus, the need to postulate dissociation of an inhibitory component, although possible in principle and not incompatible with the data and views suggested here, *need not* be postulated to explain the existing data. In addition, the fact that the intrinsic activity of the Gpp(CH_2)p-activated enzyme is less than that induced by Gpp(NH)p speaks against activation resulting from the dissociation of an inhibitory nucleotide-binding component of the enzyme since in such a case no differences in the activity of the uninhibited enzyme would be predicted.

Although binding studies of [^{3}H]Gpp(NH)p to erythrocyte ghosts indicate that the binding observed is reversible (Lefkowitz, 1974; Spiegel & Aurbach, 1974), extensive studies correlating the binding with the activation reaction indicate that the gross, overall binding observed with this compound is totally unrelated to the process of adenylate cyclase activation in erythrocyte ghosts (*manuscript in preparation*). It is pertinent that the mechanisms postulated here and elsewhere (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]) would predict that the activated enzyme could be labeled with β- or γ-^{32}P-labeled, but not α-^{32}P or ^{3}H-labeled Gpp(NH)p. Furthermore, if stoichiometric labeling of adenylate cyclase occurs, only very small quantities of radioactivity can be expected to be incorporated as a result of the reactions in question.

It is known that addition of GTP or Gpp(NH)p to liver membranes causes marked changes in the binding of ^{125}I-labeled glucagon (Rodbell, Krans *et al.*, 1971; Birnbaumer & Pohl, 1973; Bennett *et al.*, 1975). The observation that the binding of ^{125}I-labeled glucagon to Gpp(NH)p-

treated and washed membranes is the same as that observed with untreated membranes, and that GTP affects the binding equally in both membrane preparations (Table 12) may appear to contradict the hypothesis that this analog is reacting with the GTP site of the enzyme. Explanations might include the possibility that in the preincubation experiments the reaction of Gpp(NH)p is incomplete, that the effect of GTP on glucagon binding is unrelated to hormonal activation of the enzyme (Birnbaumer & Pohl, 1973), or that the effect of GTP on glucagon binding is caused by the initial, reversible binding of the nucleotide rather than by its subsequent covalent reaction. The latter explanation may be the most likely in view of the fact that both GTP and Gpp(NH)p produce similar effects on glucagon binding at 4 °C (Rodbell, Krans *et al.*, 1971; and *unpublished*) whereas no permanent activation is observed at this temperature with Gpp(NH)p (Cuatrecasas *et al.*, 1975). In addition, the ability of Gpp(NH)p (like that of GTP) to enhance dissociation of the membrane-bound hormone occurs immediately and does not display a lag or accelerating phase (Rodbell, Krans *et al.*, 1971; Cuatrecasas *et al.*, 1975), in contrast to the activation of enzyme activity at even the highest concentrations of the analog. Furthermore, 100-times greater concentrations of Gpp(NH)p compared to GTP are required for altering glucagon binding (Rodbell, Krans *et al.*, 1971), consistent with the lower affinity of Gpp(NH)p for the initial binding which precedes the irreversible step. These observations strongly suggest that the modification of glucagon binding by GTP and its analogs is related only to the initial phase of reversible binding. Since it has been suggested (Cuatrecasas *et al.*, 1975) that the activated enzyme is an enzyme-p(NH)p (or enzyme-pp) complex, it follows that the base or sugar moieties of the nucleotide may be the components most necessary to induce the effects on glucagon binding. The fact that the free nucleoside, guanosine, can modify glucagon binding in the Gpp(NH)p-treated membranes much better than in the control membranes (Table 12) is consistent with this hypothesis. These considerations, in light of the postulated overall mechanisms for hormonal activation (Cuatrecasas *et al.*, 1975), may help in interpreting the known facts and paradoxes in the interrelationships between the effects of GTP and the changes in glucagon binding and activation.

In the context of the interpretations presented here and elsewhere (Cuatrecasas *et al.*, 1975; Jacobs *et al.*, 1975[2]) GTP or a similar nucleotide[6]

6 It is possible that in certain tissues nucleoside triphosphates other than GTP may participate, perhaps by the same mechanism. Other nucleotides, such as ITP, can activate adenylate cyclase (Rodbell, Birnbaumer *et al.*, 1971; Wolff & Cook, 1973; Bilezikian & Aurbach, 1974; Birnbaumer *et al.*, 1974) and they can also inhibit (although weakly) the activation by Gpp(NH)p (Lefkowitz, 1974).

is believed to play a key role in the activation of adenylate cyclase by hormones. In the absence of hormones, the rate of covalent bond formation is presumably too slow (relative to the rate of decay) to permit a high degree of activation by GTP alone. However, the views presented here predict that under certain conditions (or in certain tissues) where the rate of this covalent step may be increased, GTP should exhibit stimulation in the absence of hormones. Indeed, in most tissues GTP in the incubation medium has been reported to stimulate, albeit slightly, the basal adenylate cyclase activity. In fat cell membranes, which are exceptional in that GTP inhibits basal activity (Cryer *et al.*, 1969; Harwood *et al.*, 1973; Lefkowitz, 1974; Fig. 5), solubilization with detergents converts the enzyme to a form which is readily stimulated rather than inhibited by GTP (*unpublished*).

There is considerable evidence that Gpp(NH)p reacts at the GTP regulatory site of the enzyme (Spiegel & Aurbach, 1974; Lefkowitz, 1974; 1975; Jacobs *et al.*, 1975[2]). The fact that GTP in the assay can still cause some inhibition of the Gpp(NH)p-stimulated (and washed) fat cell enzyme (Fig. 5) may appear to contradict the speculation that the GTP-regulatory site has been "permanently" modified by covalent reaction with a portion of the analog molecule. However, since the inhibition by GTP in this tissue is unusual and its basis is not understood, it may be that this inhibition is mediated by a mechanism different from that which normally mediates stimulation. Alternatively, since the postulated enzyme intermediate would be enzyme-p(NH)p, it is possible, as discussed above for glucagon binding, that the *nucleoside* moiety of GTP may not be excluded from interacting with and thus modifying the normal GTP binding site. In this respect it is pertinent that the inhibitory effect of GTP is substantially less marked, and requires much higher concentrations of GTP, for the Gpp(NH)p-activated compared to the control or NaF-activated enzymes (Fig. 5). In addition, at least some of the observed inhibition may actually reflect the contribution of some residual, non-Gpp(NH)p-activated enzyme, especially since in some experiments the GTP inhibitory effect on the preactivated enzyme is very minimal or insignificant.

It is interesting that treatment of membranes with NaF also results in an activation which is detected after thoroughly washing the membranes (Fig. 5; Schramm & Naim, 1970; Kelley & Koritz, 1971; Perkins & Moore, 1971; Severson *et al.*, 1972; Najjar & Constantopoulos, 1973). Although the differences in the responses of the F-activated and Gpp(NH)p-activated enzymes to GTP (Fig. 5), as well as the nature of the changes in the kinetic properties of $MgCl_2$ dependence (Fig. 4), suggest different mechanisms of activation, the possible interrelationships between these

two methods of activation must await further study. The interesting suggestion has been made that fluoride acts primarily to stimulate a phosphatase that removes phosphate from an inhibited form of adenylate cyclase, thus converting the enzyme to an active, dephospho form (Constantopoulos & Najjar, 1973; Layne *et al.*, 1973; Najjar & Constantopoulos, 1973).

At present it is not possible to explain the interesting inhibitory effect observed by treating membranes with p(NH)p (Table 13). This compound, however, does not appear to alter activity by reacting with the GTP regulatory site of the enzyme.

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