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Irreversible Activation of Adenylate Cyclase of Toad Erythrocyte Plasma Membrane by 5′-Guanylylimidodiphosphate

Vann Bennett* and Pedro Cuatrecasas**

Department of Pharmacology and Experimental Therapeutics and Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Summary. The irreversible activation of adenylate cyclase by 5′guanylylimidodiphosphate, a phosphoramidate analog of 5′GTP, has been examined in toad (*Bufus marinus*) plasma membranes using the technique of preincubating the membranes with the nucleotide under various controlled conditions followed by washing and subsequent assay of enzyme activity. Activation of adenylate cyclase by Gpp(NH)p, but not GTP, is essentially permanent and persists following extensive washing, prolonged incubation at 30 °C in the absence of the nucleotide, and after dissolution of the membranes with Lubrol PX. (−)-Isoproterenol increases the activation observed with maximal concentrations of Gpp(NH)p from eight- to 10-fold (in the absence of hormone) to 50- to 100-fold; final activities as high as 10–15 nmoles of cyclic AMP per min per mg protein are achieved. The activated state obtained with isoproterenol and Gpp(NH)p is also permanent and is not inhibited by propranolol. The synergism between Gpp(NH)p and hormone requires the simultaneous presence of these compounds, and the time-dependent enhancement of activation with (−)-isoproterenol may be interrupted by addition of propranolol.

The stimulation is slow, and may proceed for as long as 45 min at 30 °C in the presence of maximal concentrations of Gpp(NH)p and (−)-isoproterenol. Very little activation occurs at 0 °C. The time course of activation at 30 °C exhibits an accelerating phase lasting from 5 to 30 min when Gpp(NH)p is added directly during assay of cyclase activity or when the membranes are preincubated for various times and washed prior to assay for a fixed time. The lag period occurs in the presence and absence of (−)-isoproterenol, although the rate of increase in velocity is greater with hormone. The length of the accelerating phase decreases with increasing concentrations of Gpp(NH)p, although it is still evident with maximal levels of Gpp(NH)p and hormone. However, prewarming the membranes at 30 °C for 10 min in the absence of Gpp(NH)p or (−)-isoproterenol results in an immediate onset of linear activation at a rate which is achieved in untreated membranes only after about 10 min. The events occurring during prewarming at 30 °C are readily reversible since chilling the warmed mem-

* *Present Address:* Biological Laboratories, Harvard University, Cambridge, Massachusetts 02146.

** *Present Address:* Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, N.C. 27709, to whom *reprint requests* should be addressed.

branes to 0 °C results in a time course of activation identical to that of membranes maintained at 0 °C until addition of Gpp(NH)p.

Activation is proportional to the concentration of Gpp(NH)p within the range of 10^{-8} to 10^{-4} mM. The apparent affinity for Gpp(NH)p increases with increasing time of incubation. The primary effect of increasing the concentration of Gpp(NH)p is to decrease the time required to obtain a maximal rate of activation.

The possible relevance of these findings to the mechanism of action of Gpp(NH)p, adenylate cyclase and hormones is discussed within the context of current views of biological membranes which recognize the lateral mobility of membrane molecules.

Stimulation of adenylate cyclase (ATP-pyrophosphate-lyase [cyclizing] EC 4.6.1) of eukaryotic cells by hormones (Rodbell, Birnbaumer, Pohl & Krans, 1971; Bockhaert, Roy & Jard, 1972; Krishna, Harwood, Barber & Jamieson, 1972; Leray, Chambaut & Hanoune, 1972; Deery & Howell, 1973; Harwood, Low & Rodbell, 1973; Wolff & Cook, 1973; Bilezikian & Aurbach, 1974; Johnson, Thompson & Williams, 1974; Rodbell, Lin & Salomon, 1974; Siegel & Cuatrecasas, 1974) and by cholera toxin (Bennett & Cuatrecasas, 1975; Bennett, Mong & Cuatrecasas, 1975) converts this enzyme to a purine nucleotide-sensitive state such that it is activated by 5′-GTP, 5′ITP, and, to a much lesser extent, by 5′ATP. It is thought that these compounds interact with the enzyme in an allosteric manner at a regulatory site (or subunit) which is distinct from the catalytic site of the enzyme and that this regulatory site is essential for hormonal control of enzyme activity. Adenylate cyclase is also stimulated by synthetic analogs of GTP containing a modified γ-phosphate such as 5′guanylylimidodiphosphate (Gpp(NH)p)[1], 5′guanylylmethylenediphosphate (Gpp(CH_2)p)[1] and guanosine 5′-O-(3 thiotriphosphate) (GTPS)[1] (Harwood *et al.*, 1973; Lefkowitz, 1974; Londos *et al.*, 1974; Spiegel & Aurbach, 1974; Cuatrecasas, Jacobs & Bennett, 1975*a*, *b*; Jacobs *et al.*, 1975[2]; Pfeuffer & Helmreich, 1975; Schramm & Rodbell, 1975). These compounds appear to act at the same site as GTP, although they activate adenylate cyclase in the absence of hormone to levels much higher than those achieved with GTP or GTP plus hormone. The rate of activation of adenylate cyclase by these analogs is slow, although once attained is essentially irreversible and persists after extensive washing of membranes and even following

1 Abbreviations used: Gpp(NH)p, 5′-guanylylimidodiphosphate; Gpp(CH_2)p, 5′-guanylylmethylene diphosphate; GTPS, guanosine 5′-O-(3 thiotriphosphate); cyclic AMP, 3′-5′-adenosine monophosphate.

2 Jacobs, S., Bennett, V., Cuatrecasas, P. 1975. Kinetics of irreversible activation of adenylate cyclase of fat cell membranes by phosphomium and phosphoramidate analogs of GTP. *J. Cycl. Nuc. Res., submitted for publication.*

solubilization of membranes with nonionic detergents (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975; Lefkowitz & Caron, 1975; Pfeuffer & Helmreich, 1975; Salomon, Lin, Londos, Rendell & Rodbell, 1975).

In most previous studies the possible involvement of a phosphorylation reaction in the stimulation by these compounds has been excluded on the basis that the β-γ phosphate linkage of Gpp(NH)p and Gpp(CH_2)p is thought to be relatively resistant to enzymatic hydrolysis. The α-β phosphodiester bonds of the analogs, however, are, in principle, not different from those of GTP, and extensive hydrolysis has recently been demonstrated in biological tissues (Jacobs *et al.*, 1975). It has been proposed (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975), on the basis of the irreversible nature of the activation and the slow rate of the process, that the synthetic nucleotides activate adenylate cyclase as the result of formation of stable, covalent, S-P-O-P-, p(NH)P- or P(CH_2)P-enzyme complexes and that GTP normally stimulates the enzyme by forming a labile pyrophosphoryl-enzyme intermediate. Hormones, according to this view, may function by increasing the rate of pyrophosphorylation.

The activation of adenylate cyclase by these GTP analogs may thus be useful in understanding the normal mechanisms of action of GTP as well as hormones. The present report examines some features of the kinetics and concentration dependence of the irreversible stimulation of the adenylate cyclase of toad erythrocyte plasma membranes by Gpp(NH)p, with special emphasis on a novel accelerating phase during the initial time course. The process of activation is examined by preincubation of membranes with Gpp(NH)p, followed by washing to remove free nucleotide prior to assay of adenylate cyclase activity, an approach which has been introduced recently (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975).

Materials and Methods

[α^{32}P]-ATP (10–20 Ci per mmole) was synthesized according to the method of Symons (1968) (*see* Bennett & Cuatrecasas, 1975*a*, *b*). 5'ATP, 5'GTP, neutral alumina, (−)-isoproterenol bitartrate, propranolol-HCl, phosphoenolpyruvate, Tris base, and aminophylline were purchased from Sigma; 2'3', isopropylidine adenosine was from Aldrich; pyruvate kinase and myokinase were from Boehringer; and dithiothreitol and Gpp(NH)p were obtained from P-L Biochemicals. [^{3}H]cyclic AMP and [^{32}P]-orthophosphoric acid (carrier-free), in 0.02 N HCl, were from New England Nuclear. Staphylococcal nuclease was from Worthington; Female toads (*Bufus marinus*) weighing 150–300 g were purchased from the National Reagent Company, Bridgeport, Conn.

Adenylate cyclase activity of toad erythrocyte membranes was determined as described previously (Bennett & Cuatrecasas, 1975*a*) in a 0.1 ml volume containing Tris-HCl (50 mM, pH 8), aminophylline (5 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (6.2 mM), pyruvate kinase

(60 μg/ml added as an ammonium sulfate suspension), [α^{32}P]5′ATP (0.6 mM, 50–150 cpm per picomole), and 20–100 μg of membrane protein. All additions were made at 0 °C, and the reaction initiated by transfer of the samples to a 30 °C bath. The assays were terminated after 15–20 min by boiling the tubes for 1–2 min, and cyclic AMP isolated in 75–80% yield (determined for each sample) from neutral alumina columns (Ramachandran, 1971; White & Zenser, 1971). Assay blanks (boiled membranes) were usually less than 50 cpm per 10^6 cpm of total radioactivity; activities were at least 500 cpm above the blank values. The product obtained from the alumina columns co-migrated with [^{3}H]cyclic AMP in several TLC and ion-exchange column chromatographic systems. The production of the cyclic AMP was linear with respect to protein up to 100 μg per tube, and with respect to time up to 20 min (with the exceptions discussed below). The concentration of substrate (0.6 mM) utilized in this study is less than the K_m (1.8 mM) (Bennett & Cuatrecasas, 1975*a*), and was adopted because of the improved specific activity of [^{32}P]-ATP. Much higher activities (up to 15,000 pmoles cyclic AMP formed per min per mg) can be obtained with 3 mM ATP, although the actual cpm of the product is lower.

Toad erythrocyte plasma membranes were prepared essentially as described previously (Bennett & Cuatrecasas, 1975*a*). Briefly, serum and buffy coat were removed from heparinized whole blood (obtained daily) by 5–6 washes with a buffer containing $MgCl_2$ (1 mM), $CaCl_2$ (1.2 mM), KCl (1.9 mM), $NaHCO_3$ (2.4 mM), NaCl (120 mM), at a pH of 7.5 (amphibian Ringer's). The washed, packed cells were lysed by the rapid introduction of 40 volumes of an ice-cold hypotonic solution containing 5 mM Tris-HCl, 0.2 mM $CaCl_2$, pH 8, and highly purified staphylococcal nuclease (1 μg per ml). After 10–15 min at 0 °C, the ghosts were enucleated by vigorous shaking for 10–20 sec and the free nuclei and remaining nucleated ghosts were pelleted by centrifugation at 2,000 × g for 10 min at 0 °C. The supernatant, containing the plasma membranes, was made 1 mM in $MgCl_2$ and 50 mM in KCl and centrifuged at 35,000 × g for 30 min at 0 °C. The resulting membrane pellets were resuspended and treated as described in the Figure legends. Phase-contrast microscopy of these preparations revealed essentially no contamination with nuclei or with other cell types.

Membrane protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), after heating the samples for 30 min at 90 °C with 4 N NaOH. Bovine serum albumin was used as the standard.

Results

Features of the Activation of Adenylate Cyclase by Gpp(NH)p

Irreversibility of Activated State. The Gpp(NH)p stimulation of toad erythrocyte membrane adenylate cyclase is extraordinarily persistent, as has been reported for other cell types (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975; Lefkowitz & Caron, 1975; Salomon *et al.*, 1975; Schramm & Rodbell, 1975). Following incubation with Gpp(NH)p, erythrocyte membranes retain fully their stimulated adenylate cyclase activity after extensive washing, incubation up to 45 min at 30 °C, sonication, and even solubilization by Lubrol PX (data not shown). Furthermore, the enhanced activity obtained by incubating membranes in the presence of (−)-isoproterenol and Gpp(NH)p (Table 1) exhibits an identically high stability, and also persists following incubation of the thoroughly washed membranes with propranolol as described by Schramm and Rodbell

Table 1. Requirement for simultaneous presence of (−)-isoproterenol and Gpp(NH)p for enhanced activation of adenylate cyclase during preincubation of membranes with Gpp(NH)p

Membrane treatment[a]		Adenylate cyclase activity (pmoles/min/mg)
1st Incubation	2nd Incubation	
No addition	no addition	18±0.5
GTP[c]	no addition	17±0.6
GTP and (−)-isoproterenol[c]	no addition	19±1.1
(−)-Isoproterenol	no addition	19±0.8
Gpp(NH)p[c]	no addition	173±7
Gpp(NH)p[c]	(−)-isoproterenol	166±8
(−)-Isoproterenol[c]	Gpp(NH)p	200±9
(−)-Isoproterenol and Gpp(NH)p	no addition	935±34

[a] Erythrocyte plasma membranes (about 1 mg/ml of membrane protein) were suspended (0 °C) in a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (6 mM), ATP (0.6 mM), and pyruvate kinase (60 μg/ml) and divided into aliquots (1 ml). Additions were made as described above and the samples warmed to 30 °C. After 10 min, 40 ml of a solution (0 °C) containing Tris-HCl (50 mM, pH 8), KCl (50 mM), dithiothreitol (1 mM), and $MgCl_2$ (1 mM) was added and the samples centrifuged (35,000 × g, 30 min at 0 °C). The membrane pellets were resuspended in 1 ml of the original buffer and the samples warmed to 30 °C. After 10 min, the samples were diluted and centrifuged in the same manner as before, and the final membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (15 min at 30 °C). Activities of control samples (not shown) treated with no additions followed by either (−)-isoproterenol or Gpp(NH)p, or (−)-isoproterenol and Gpp(NH)p together, were essentially the same as activities of membranes treated in the reverse order.

[b] Expressed as the mean of triplicate determinations ± one standard deviation.

[c] 5 μM.

(1975) (data not shown). In contrast, membranes incubated with either GTP or GTP plus (−)-isoproterenol demonstrate no increase in adenylate cyclase activity following washing (Table 1).

Synergism between (−)-Isoproterenol and Gpp(NH)p. Membranes incubated with Gpp(NH)p alone (and subsequently washed) demonstrate an eight- to 10-fold stimulation of adenylate cyclase activity while those treated with Gpp(NH)p and (−)-isoproterenol together exhibit at least a 50-fold activation (Table 1). (−)-Isoproterenol alone results in no permanent stimulation (Table 1), and only a 1.5- to twofold activation when added directly to the assay (Bennett & Cuatrecasas, 1975*a*, *b*). Membranes treated with (−)-isoproterenol followed by washing and subsequent exposure to Gpp(NH)p, and those treated in the reverse order, exhibit the same activity as membranes incubated only with Gpp(NH)p (Table 1). Furthermore, the augmented activation of cyclase with Gpp(NH)p

and (−)-isoproterenol can be interrupted at any time by the addition of propranolol (to be described in Fig. 3), although this agent is incapable of reversing stimulation once it has occurred. Enhancement of stimulation by (−)-isoproterenol thus requires the simultaneous presence of Gpp(NH)p and the hormone. Essentially identical findings have been reported for frog erythrocyte membranes (Schramm & Rodbell, 1975). Propranolol was used routinely in the remaining studies to rapidly quench the activation obtained in the presence of Gpp(NH)p and isoproterenol.

Time Course of Gpp(NH)p-Activation of Adenylate Cyclase

Addition of Gpp(NH)p during the Assay. The activation of adenylate cyclase after addition of Gpp(NH)p directly to the assay medium demonstrates an accelerating time course (Fig. 1), as has been reported in other cell systems (Londos *et al.*, 1974; Lefkowitz & Caron, 1975; Salomon *et al.*, 1975; Schramm & Rodbell, 1975). The activity in the presence of Gpp(NH)p and isoproterenol initially rises with a doubling time of about 2 min, and achieves a constant rate of increase after about 9 min. In contrast, membranes preincubated with Gpp(NH)p and hormone, and subsequently washed and assayed for adenylate cyclase activity, exhibit zero-order kinetics (Fig. 1). These findings could, in principle, be explained if Gpp(NH)p stimulated the enzyme slowly (relative to the period of assay) with either a linear or accelerating time course, or following an absolute delay. The fact that preactivated membranes exhibit linear kinetics indicates only that the stimulated state, once attained, is stable in the absence of Gpp(NH)p. Thus, activity measured in pretreated membranes reflects the instantaneous extent of activation at the point in time that the incubation with Gpp(NH)p was terminated, and conveys no information as to the rate of the process.

In order to examine the kinetics of Gpp(NH)p stimulation, as well as to obtain meaningful concentration-response relationships, it is necessary to preincubate membranes with the analog and to wash them prior to assay of cyclase activity. Such studies are feasible, in principle, since the activation is essentially permanent, and since the process is readily terminated by removing Gpp(NH)p or hormone (Figs. 1 and 3). The well-recognized lability of adenylate cyclase offers a significant technical problem in experiments requiring prolonged incubations at elevated temperatures and multiple washes. This difficulty has been partially overcome in fat cell membranes by inclusion of ATP, dithiothreitol and magnesium in the preincubation buffer (Jacobs *et al.*, 1975). Under these conditions,

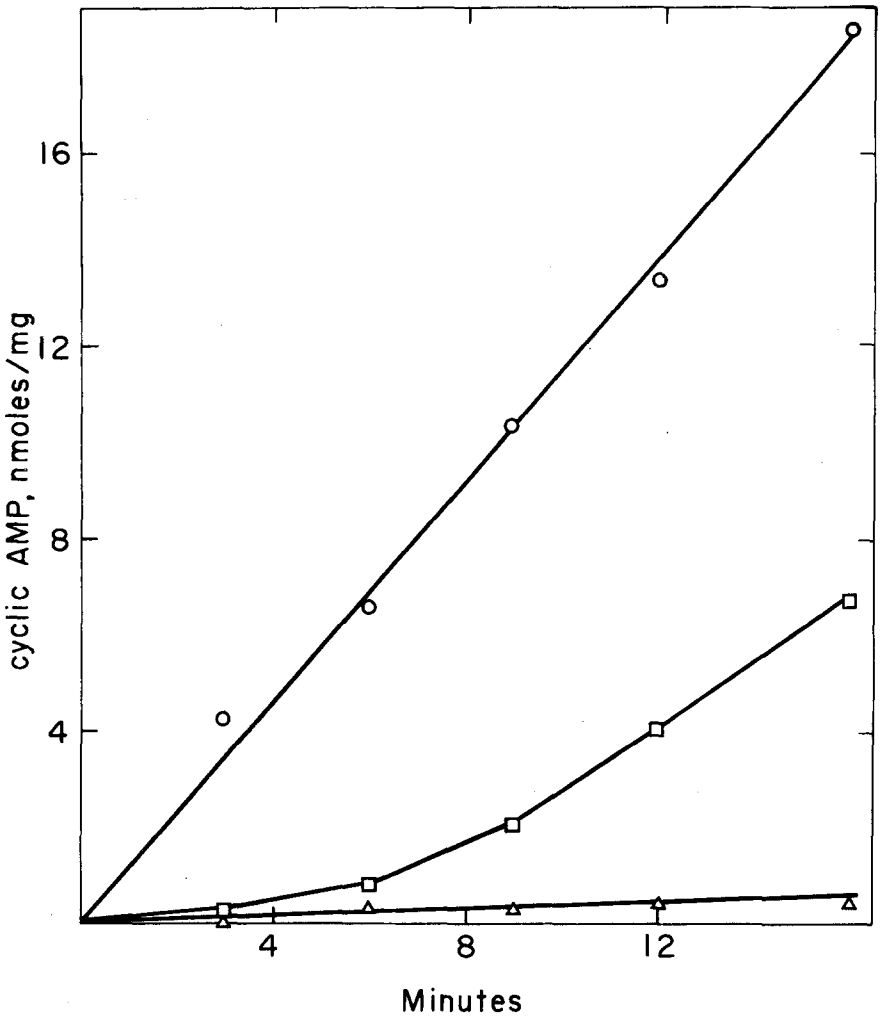

Fig. 1. Time course of cyclic AMP production by toad erythrocyte plasma membranes assayed in the presence of (−)-isoproterenol (△) or (−)-isoproterenol and Gpp(NH)p (□), or pre-incubated with (−)-isoproterenol and Gpp(NH)p and washed prior to assay (○). Toad erythrocytes (3 ml packed cells) were washed and lysed (*see* Materials and Methods), and the plasma membranes suspended in a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (6.7 mM), ATP (0.67 mM), pyruvate kinase (70 μg/ml), at a concentration of 1 mg/ml membrane protein, and divided into two portions. One of these was made 5 μM in Gpp(NH)p and 5 μM in (−)-isoproterenol, and both aliquots were incubated for 15 min at 30°. The membranes were then diluted 20-fold with a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), KCl (50 mM), $MgCl_2$ (1 mM), and centrifuged 30 min at 35,000 × *g* (0 °C). The pellets were resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (*see* Materials and Methods) at various times. The membranes which received no additions during the first incubation were assayed either in the presence of (−)-isoproterenol (5 μM) (△), or (−)-isoproterenol (5 μM) and Gpp(NH)p (5 μM) (□), while those treated with (−)-isoproterenol and Gpp(NH)p initially (○) were assayed with no additions. The data are expressed as the mean of triplicate determinations

toad erythrocyte membranes retain up to 80% of the adenylate cyclase activity after a 20–40 min period of incubation at 30 °C (not shown).

Preincubation of Membranes with Gpp(NH)p Prior to Assay. When the process of Gpp(NH)p activation is examined separately from the adenylate cyclase assay (Fig. 2), it is evident that the stimulation is relatively slow; it is incomplete even after 35 min of incubation at 30 °C. In some experiments (e.g., Fig. 4), activation continues up to 45 min, at which point the lability of the cyclase may become a limiting factor. The activation is saturable with respect to Gpp(NH)p concentration at 20 min of incubation (to be described in Fig. 7), at which time the process is continuing at a nearly maximal rate (Fig. 4). The process is highly temperature dependent, and occurs very slowly, if at all, at 0 °C (not shown).

The accelerating time course of activation observed when Gpp(NH)p is added to the assay (Fig. 1) is also clearly present during the preincubation period (Figs. 2–6). During the first 6–8 min the activation in the presence of Gpp(NH)p and (−)-isoproterenol increases with a doubling time of approximately 0.5 min, and then attains a constant rate which in some experiments begins to fall after about 15 min (Fig. 2). The stimulation obtained with Gpp(NH)p in the absence of hormone occurs at a much slower rate, although it follows a similar accelerating pattern with a doubling time of about 3 min during the first 15 min of incubation. It is possible that the principal effect of (−)-isoproterenol is on the rate of increase in activation, and that the stimulation achieved in the presence and absence of hormone would be equal if the incubation could be continued long enough. Such experiments were not feasible because of the instability of adenylate cyclase. However, it is pertinent that hormone stimulation decreases with time, from 17-fold at 8 min, to fivefold at 35 min (Fig. 2).

Properties of the Accelerating Phase of Gpp(NH)p Stimulation. It was of interest to examine the unusual time course of Gpp(NH)p activation in more detail. The delay, most likely, is not explained by slow transport of Gpp(NH)p into ghosts, since similar kinetics are obtained after vigorous sonication of membranes in the presence of Gpp(NH)p and hormone (not shown). Also, the delay does not represent an interaction with one of the components in the preincubation medium since an accelerating phase is still observed when membranes are incubated with Gpp(NH)p in a simple Tris buffer (not shown).

The rate of acceleration of activation is proportional to the concentration of Gpp(NH)p (Fig. 4) within certain limits, and is further increased

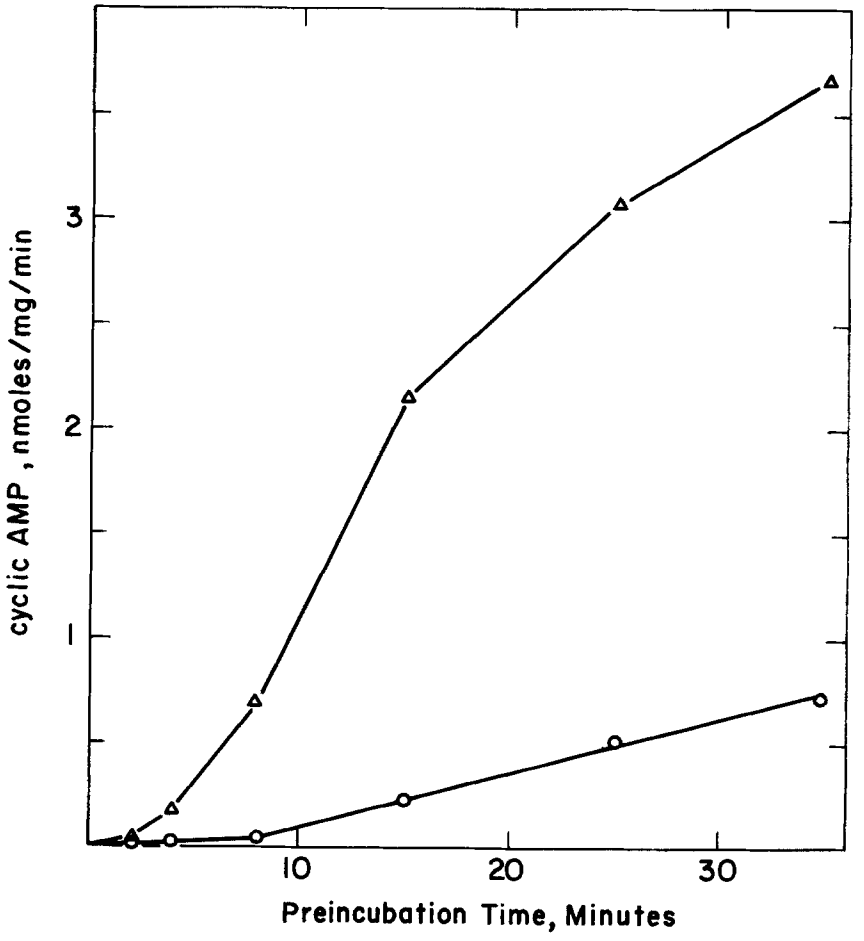

Fig. 2. Time course of activation of adenylate cyclase during preincubation of toad erythrocyte plasma membranes with Gpp(NH)p (50 μM) (○) or Gpp(NH)p (50 μM) and (−)-isoproterenol (50 μM) (△). Erythrocytes (3 ml packed cells) were washed and lysed (*see* Materials and Methods) and the plasma membranes suspended (0 °C) in 8 ml buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (62 mM), ATP (0.62 mM), pyruvate kinase (70 μg/ml), about 1 mg/ml of membrane protein and Gpp(NH)p (50 μM). The suspension was divided into two equal portions, and (−)-isoproterenol (final concentration, 50 μM) added to one of these. After 10 min at 0 °C, the membranes were warmed to 30 °C (zero time) and aliquots (0.5 ml) were removed at various times and added to centrifuge tubes (0 °C) containing 10 ml of Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), KCl (50 mM), $MgCl_2$ (1 mM), and propranolol (17 μM). The samples were centrifuged at 35,000 × g for 30 min (0 °C), and the resulting membrane pellets were resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (18 min at 30 °C). The data are corrected for the activity at zero time (0.1 nmoles/min/mg for both (○) and (△)), and are expressed as the mean of triplicate determinations. In parallel experiments, membranes incubated under identical conditions at 30 °C in the absence of Gpp(NH)p demonstrate no appreciable change in adenylate cyclase activity. Similarly, membranes incubated at 0 °C with Gpp(NH)p or Gpp(NH)p and (−)-isoproterenol demonstrate essentially no stimulation relative to control samples

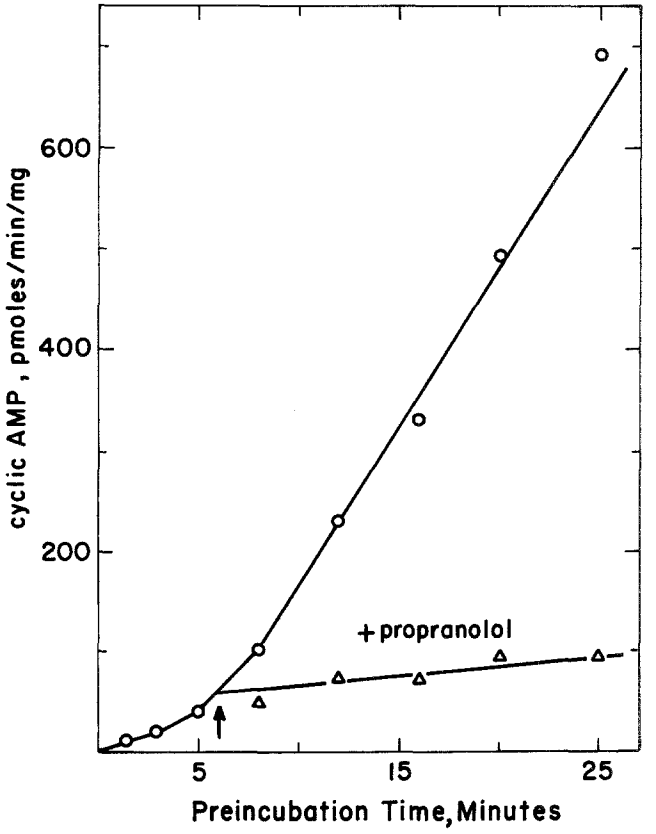

Fig. 3. Interruption of activation of adenylate cyclase following addition of propranolol during preincubation of toad erythrocyte plasma membranes in the presence of Gpp(NH)p and (−)-isoproterenol. Erythrocytes (2.5 ml packed cells) were washed and lysed (*see* Materials and Methods), and the plasma membranes suspended in 8 ml of ice-cold buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (6.2 mM), pyruvate kinase (62 μg/ml), Gpp(NH)p (30 μM) and (−)-isoproterenol (6 μM) at a concentration of about 1 mg/ml of membrane protein. The suspension was divided into two portions, and these were placed in a 30 °C bath (zero time). Aliquots (0.5 ml) were removed at various times and added to centrifuge tubes (0 °C) containing 10 ml of Tris-HCl (50 mM, pH 8), KCl (50 mM), $MgCl_2$ (1 mM) and propranolol (17 mM). After 6 min, propranolol was added to one portion of membranes at a final concentration of 50 μM, and the incubation continued at 30 °C. The samples were finally centrifuged at 35,000 × *g* for 30 min (0 °C) and the membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (18 min at 30 °C). The data are corrected for the activity at zero time (25 pmoles/min/mg) and are expressed as the mean of triplicate determinations

three- to fourfold by addition of (−)-isoproterenol in the presence of maximal levels of the nucleotide. The lag phase is still evident, however, with 50 μM Gpp(NH)p and (−)-isoproterenol (Fig. 2), even though this level of the nucleotide is saturating at 20 min, and about 10-fold higher

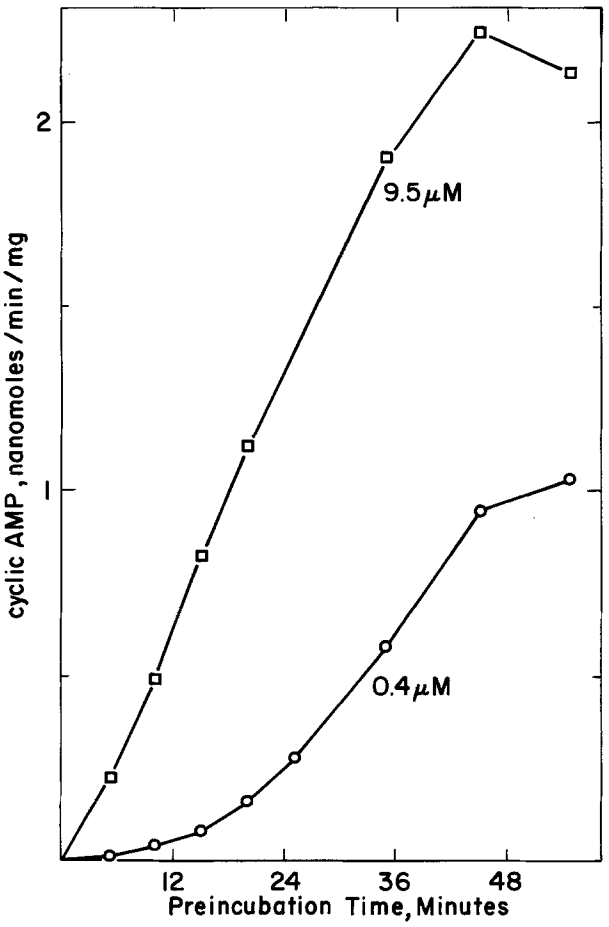

Fig. 4. Influence of the concentration of Gpp(NH)p on the rate of acceleration of activation of adenylate cyclase during preincubation of toad erythrocyte plasma membranes. Erythrocytes (3 ml packed cells) were washed and lysed (*see* Materials and Methods) and the plasma membranes suspended (0 °C) in 9 ml of buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (4.4 mM), $MgCl_2$ (5.5 mM), ATP (0.5 mM), pyruvate kinase (55 μg/ml) and (−)-isoproterenol (10 μM) at a concentration of about 1 mg/ml membrane protein. The suspension was divided into three portions, and Gpp(NH)p was added to two of these at final concentrations of 9.5 μM (□) and 0.4 μM (○). The control sample was diluted as described below without further additions. At zero time the tubes were warmed to 30 °C, aliquots (0.5 ml) removed at various times and immediately diluted with 10 ml of an ice-cold buffer containing Tris-HCl (50 mM, pH 8), KCl (50 mM), $MgCl_2$ (1 mM), dithiothreitol (1 mM), and propranolol (20 μM). The samples were then centrifuged (35,000 × g, 30 min at 0 °C) and the resulting membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (18 min, 30 °C). The data are corrected for the activity of the control sample (0.01 nmoles/min/mg), and are expressed as the mean of triplicate determinations

than the approximate K_a at 1.5 min (Fig. 7). Furthermore, it is not possible to obtain instantaneous zero-order kinetics even at concentrations of Gpp(NH)p and (−)-isoproterenol as high as 10^{-4} M (not shown).

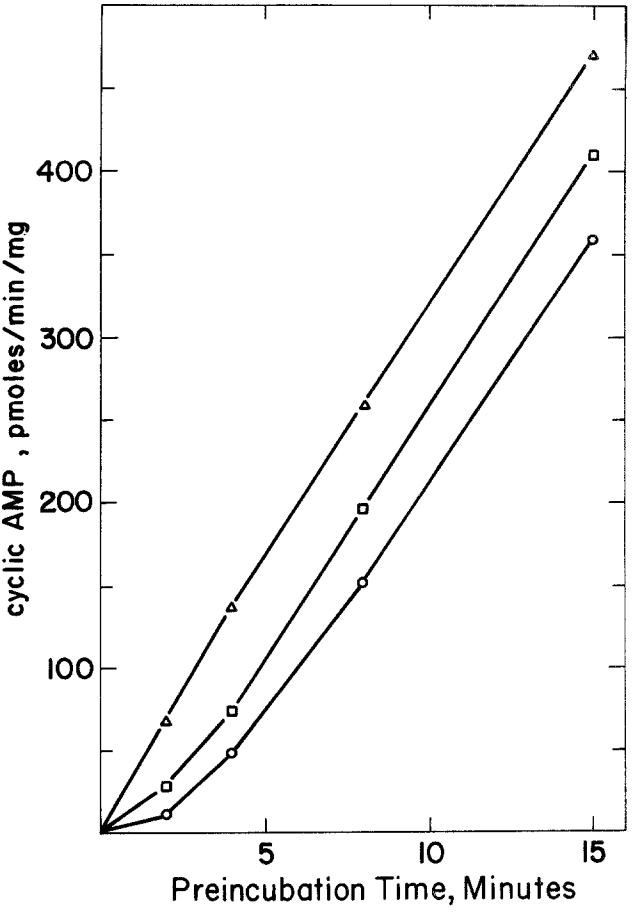

Fig. 5. Influence of pretreating membranes at 0 (○), 20 (□), or 30 °C (△) in the absence of Gpp(NH)p or (−)-isoproterenol on the subsequent time course of activation of adenylate cyclase during preincubation with Gpp(NH)p and (−)-isoproterenol at 30 °C. Toad erythrocytes (3 ml packed cells) were washed and lysed (*see* Materials and Methods), and the plasma membranes suspended (0 °C) in a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (6.2 mM), ATP (0.6 mM) and pyruvate kinase (60 μg/ml) at a concentration of about 1 mg/ml of membrane protein. The suspensions were divided into three equal portions and incubated for 8 min at either 0 (○), 20 (□) or 30 °C (△). At zero time, Gpp(NH)p and (−)-isoproterenol were added to each sample at final concentrations of 3.7 μM and 7.5 μM, respectively, and the tubes were placed in a 30 °C bath. Aliquots (0.5 ml) were removed at various times and immediately added to tubes (0 °C) containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), KCl (50 mM), $MgCl_2$ (1 mM), and propranolol (17 M). The samples were centrifuged (35,000 × *g*, 30 min at 0 °C) and the membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (18 min, 30 °C). The data are corrected for the activity of samples at zero time [17 pmoles/min/mg (○); 20 pmoles/min/mg (□), (△)], and are expressed as the mean of triplicate determinations

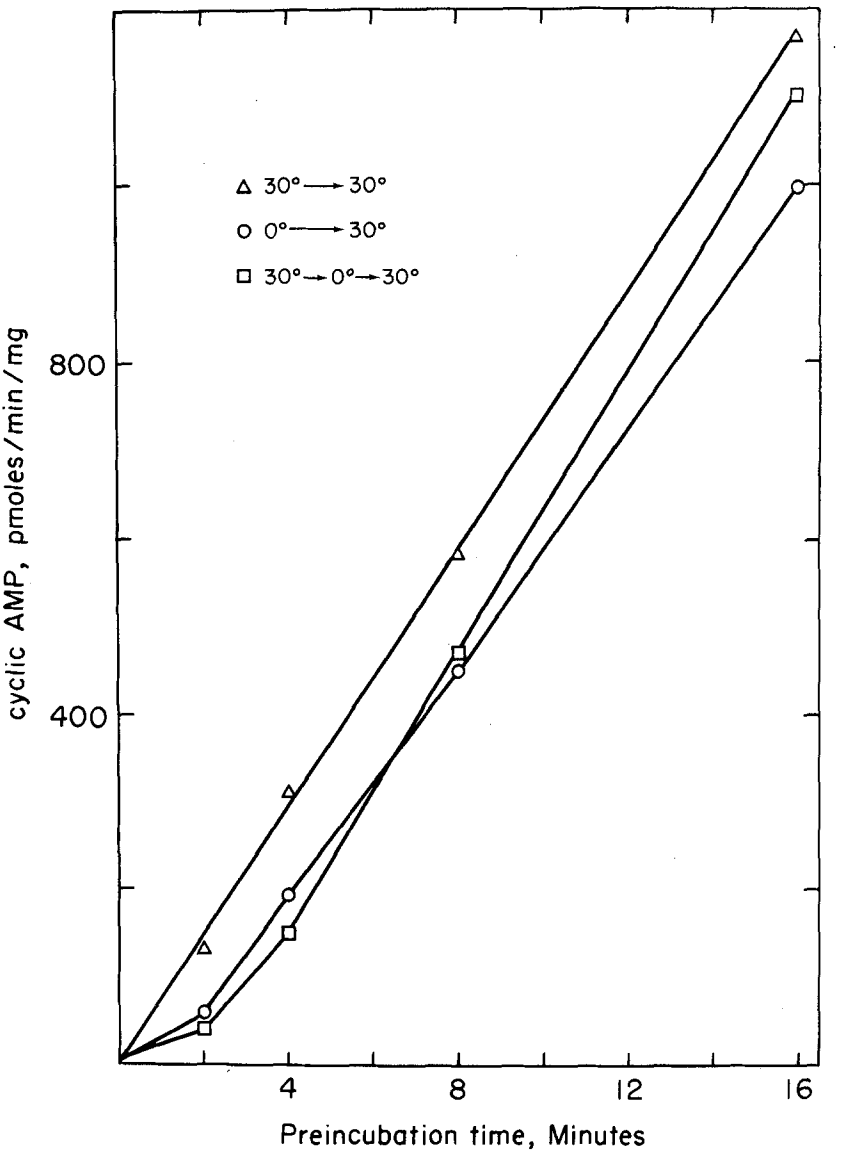

Fig. 6. Return of the accelerating phase in the time course of Gpp(NH)p-activation of adenylate cyclase following chilling of prewarmed membranes. Toad erythrocyte plasma membranes were suspended in the absence of Gpp(NH)p or (−)-isoproterenol as described in Fig. 5, and divided into three equal portions. These were preincubated either for 20 min at 0 °C (○), 10 min at 0 °C followed by 10 min at 30 °C (△) or 10 min at 30 °C followed by 10 min at 0 °C (□). At zero time, Gpp(NH)p and (−)-isoproterenol were added to each sample at final concentrations of 1.9 μM and 20 μM, respectively, and the tubes were placed in a 30 °C bath. Aliquots (0.5 ml) were removed at various times and added to tubes containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), KCl (50 mM), $MgCl_2$ (1 mM), and propranolol (10 μM). These samples were centrifuged (30,000 × g, 30 min at 0 °C), the membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (15 min, 30 °C). The data are corrected for the activity of samples at zero time [6 pmoles/min/mg (○); 12 pmoles/min/mg (□); 6 pmoles/min/mg (△)], and are expressed as the mean of triplicate determinations

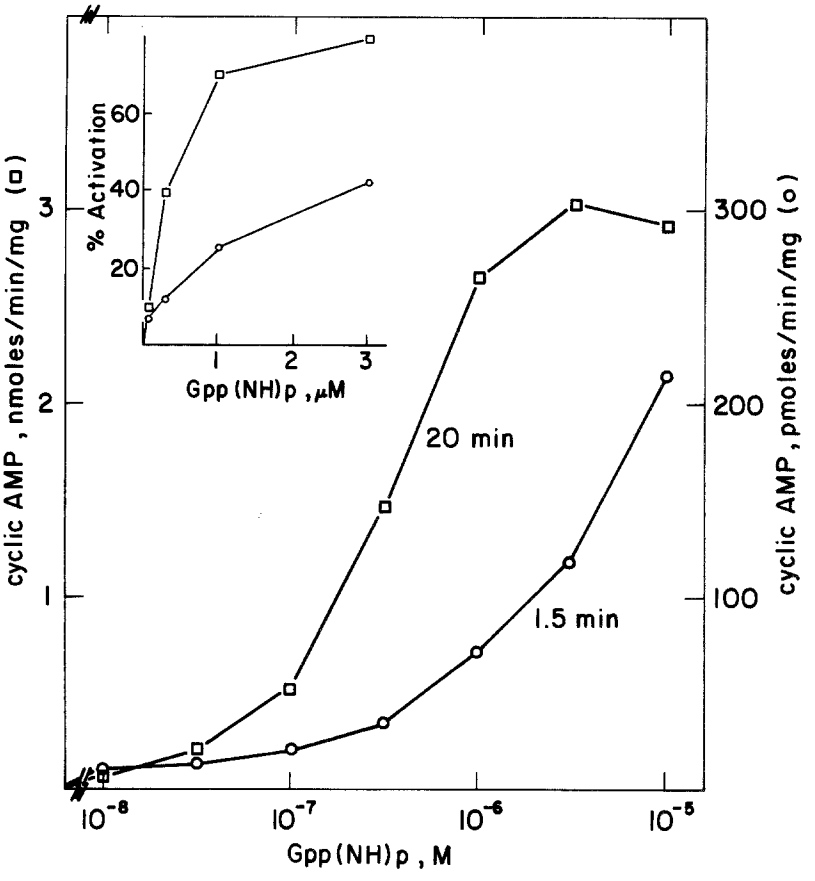

Fig. 7. Influence of the time of incubation on the concentration dependence of Gpp(NH)p activation of adenylate cyclase during pretreatment of toad erythrocyte plasma membranes. Erythrocytes (3 ml packed cells) were washed and lysed (*see* Materials and Methods), and the plasma membranes suspended (0 °C) in 8 ml of a buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (6.2 mM), ATP (0.6 mM), pyruvate kinase (60 μg/ml) and (−)-isoproterenol (12 μM) at a concentration of about 1 mg/ml of membrane protein. The suspension was divided into 0.5 ml portions and to these were added various concentrations of Gpp(NH)p. At zero time, the tubes were warmed to 30 °C; after 1.5 and 20 min, a set of samples was chilled to 0 °C and each was rapidly diluted with 10 ml of an ice-cold buffer containing Tris-HCl (50 mM, pH 8), dithiothreitol (1 mM), KCl (50 mM), $MgCl_2$ (1 mM), and propranolol (17 μM). The samples were centrifuged (30,000 × *g*, 30 min at 0 °C), and the membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (15 min at 30 °C). The data are expressed as Gpp(NH)p-stimulated activity and are corrected for the activity of control samples incubated under identical conditions in the absence of Gpp(NH)p [0.01 nmoles/min/mg (○); 0.02 nmoles/min/mg (□)]. The percent activation (insert) was calculated based on a maximal activity estimated from double-reciprocal plots of activity versus Gpp(NH)p concentrations. The values were determined in triplicate

The membranes, however, exhibit the unusual property that prewarming for 8–10 min at 30 °C in the absence of Gpp(NH)p or (−)-isoproterenol results in the immediate onset of linear activation with a rate which nonheated membranes achieve only after incubation for 8–10 min (Fig. 5). Pretreatment at 20 °C also increases the rate of acceleration but not the same extent (Fig. 5). This behavior does not reflect a delay in thermal equilibrium, since direct measurement of the temperature of the membrane suspensions indicates that they reach 30 °C within 1 min. The events occurring during prewarming of the membranes at 30 °C are readily reversible, since treating membranes at 30 °C for 10 min followed by chilling to 0 °C for 10 min results in a return to the identical time course of membranes maintained at 0 °C until addition of Gpp(NH)p (Fig. 6).

Concentration-Response Relationships for Gpp(NH)p activation

The nonlinear time course of cyclase activation and the slow rate of this process complicates interpretation of concentration-response curves for Gpp(NH)p, particularly when these are performed by adding the nucleotide during the assay. The apparent affinity for activation of cyclase by Gpp(NH)p during preincubation with membranes is markedly dependent on the time of exposure to the nucleotide (Fig. 7). Half-maximal activation is achieved with about 0.4 μM Gpp(NH)p after 20 min, while approximately 5 μM Gpp(NH)p is required for half-maximal stimulation during a 1.5-min incubation (Fig. 7). Furthermore, the rate of activation obtained with 0.4 μM Gpp(NH)p increases with time until after 35 min of incubation, when the rate approaches the maximal velocity observed in the presence of 9.5 μM Gpp(NH)p (Fig. 4), a concentration of the nucleotide which is saturating at 20 min (Fig. 7).

The erythrocyte membranes also exhibit the novel property of an increased sensitivity to Gpp(NH)p when they are prewarmed at 30 °C in the absence of Gpp(NH)p or (−)-isoproterenol (Table 2). In these experiments, membranes were pretreated at 30 °C with no additions, and then preincubated with (−)-isoproterenol and Gpp(NH)p, for 5 min at 30 °C. There is a 60–70% increase in the response of prewarmed membranes to concentrations of Gpp(NH)p up to 1 μM, while higher levels of the nucleotide stimulate both sets of membranes equally (Table 2). An analogous increase in sensitivity of prewarmed membranes to (−)-isoproterenol at a fixed concentration of Gpp(NH)p also occurs (Table 3). In the case of the hormone, the enhancement is also most apparent at low concentrations (Table 3).

Table 2. Increased sensitivity of adenylate cyclase to activation by Gpp(NH)p following pretreating membranes at 30 °C in the absence of Gpp(NH)p or (−)-isoproterenol

Additions		Gpp(NH)p Stimulated-adenylate cyclase activity[a] (pmoles/min/mg)	
		Membrane pretreatment[b]	
		0→30 °C	30→30 °C
(−)-isoproterenol[c] + Gpp(NH)p	3×10^{-8} M	1 ± 0.9	9 ± 2
	10^{-7} M	13 ± 3	21 ± 2
	3×10^{-7} M	40 ± 3	68 ± 6
	10^{-6} M	90 ± 9	149 ± 6
	3×10^{-6} M	191 ± 17	211 ± 5
	10^{-5} M	364 ± 24	383 ± 20

[a] Expressed as the difference (stimulated-basal) ± one standard deviation; mean of triplicate determinations.

[b] Erythrocyte plasma membranes (Materials and Methods) were suspended (0 °C) in 2 ml of buffer containing Tris (50 mM, pH 8), dithiothreitol (1 mM), phosphoenolpyruvate (5 mM), $MgCl_2$ (5 mM), ATP (0.5 mM) and pyruvate kinase (50 μg/ml), at a concentration of about 4 mg/ml of membrane protein. The suspension was divided into two equal portions; one of these was warmed to 30 °C and the other maintained on ice. After 7 min, aliquots (0.1 ml) were removed at staggered times and added to tubes (30 °C) containing the same buffer (0.4 ml) and (−)-isoproterenol and various concentrations of Gpp(NH)p. After 5 min at 30 °C, the samples were diluted with 10 ml of ice-cold buffer containing Tris-HCl (50 mM, pH 8), KCl (50 mM), $MgCl_2$ (1 mM), dithiothreitol (1 mM) and propranolol (10^{-5} M). The suspensions were centrifuged (35,000 × g, 30 min at 0 °C) and the membrane pellets resuspended in Tris-HCl (50 mM, pH 8) and assayed for adenylate cyclase activity (15 min, 30 °C). The activity of the sample incubated in the absence of Gpp(NH)p was 12 pmoles/min/mg.

[c] 10 μM.

Discussion

The present studies examine some aspects of the irreversible stimulation of adenylate cyclase of toad erythrocyte plasma membranes by Gpp(NH)p, a phosphoramidate analog of GTP. Toad erythrocytes were chosen because membranes from these cells respond extremely well to Gpp(NH)p (up to 100-fold stimulation) with activities as high as 10,000 pmoles of cyclic AMP formed per min per mg of protein,[3] and because the cyclase of these cells withstands well the multiple washes and prolonged incubations employed in these experiments. Frog erythrocytes have also been utilized for similar reasons (Lefkowitz, 1974; Lefkowitz & Caron, 1975; Schramm & Rodbell, 1975). The activation of cyclase of toad erythrocytes by Gpp(NH)p exhibits properties very similar to those described in other

3 The activity is such that the membrane preparations could conceivably be used for the enzymatic synthesis of [^{32}P] cyclic AMP.

Table 3. Increased sensitivity of adenylate cyclase to (−)-isoproterenol during activation by Gpp(NH)p at 30 °C following pretreating membranes at 30 °C in the absence of Gpp(NH)p or (−)-isoproterenol

Membrane treatment[c]		Adenylate cyclase activity[a]	Isoproterenol-stimulated activity[b]
1st Incubation	2nd Incubation		
30°, no addition	30°, no addition	10	
30°, no addition	30°, +Gpp(NH)p[d]	124	
30°, no addition	30°, +Gpp(NH)p+ (−)-isoproterenol, 1 μM	405	281±12
30°, no addition	30°, +Gpp(NH)p+ (−)-isoproterenol, 3 μM	529	405±51
30°, no addition	30°, +Gpp(NH)p+ (−)-isoproterenol, 10 μm	740	616±54
30°, no addition	30°+Gpp(NH)p+ (−)-isoproterenol, 50 μM	780	656±70
0°, no addition	30°, no addition	7	
0°, no addition	30°+Gpp(NH)p	110	
0°, no addition	30°+Gpp(NH)p+ (−)-isoproterenol, 1 μM	237	127±11
0°, no addition	30°+Gpp(NH)p+ (−)-isoproterenol, 3 μM	445	335±18
0°, no addition	30°+Gpp(NH)p+ (−)-isoproterenol, 10 μM	566	456±26
0°, no addition	30°+Gpp(NH)p+ (−)-isoproterenol, 50 μM	813	703±20

[a] Picomoles/min/mg protein; mean of triplicate determinations.
[b] Expressed as the difference (stimulated-basal)±one standard deviation.
[c] Erythrocyte plasma membranes (*see* Materials and Methods) were suspended (0 °C) as described in Table 2 and divided into two equal portions; one of these was warmed to 30 °C and the other kept at 0 °C. After 8 min, aliquots (0.1 ml) were removed at staggered times and added to tubes (30 °C) containing buffer (Table 2), Gpp(NH)p and various concentrations of (−)-isoproterenol. After 5 min at 30 °C the reaction was stopped and membranes prepared (Table 2) and assayed for adenylate cyclase activity (15 min, 30 °C).
[d] 5 μM.

tissues. The stimulation is not reversed by removal of the nucleotide, by prolonged incubation with the competitive inhibitor, GTP, or even by solubilization of the membranes in Lubrol PX, as reported previously for rat fat cells (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975), rat liver (Salomon *et al.*, 1975) and frog (Lefkowitz & Caron, 1975; Schramm & Rodbell, 1975) and turkey (Cuatrecasas *et al.*, 1975*a*) erythrocytes. The

activation by Gpp(NH)p is considerably amplified by (−)-isoproterenol (by as much as 20-fold) (Table 1, Figs. 2 and 3), as described for frog erythrocytes (Lefkowitz & Caron, 1975; Schramm & Rodbell, 1975) and turkey erythrocytes (Spiegel & Aurbach, 1974). Gpp(NH)p activation of toad erythrocyte adenylate cyclase occurs at a relatively slow rate, and the time course of stimulation exhibits a lag or accelerating phase when the nucleotide is added directly during the assay (Fig. 1), which has also been observed in other tissues (Londos *et al.*, 1974; Lefkowitz & Caron, 1975; Salomon *et al.*, 1975; Schramm & Rodbell, 1975).

The irreversible nature of Gpp(NH)p activation and the gradual rate of the process are unusual features which render kinetics and concentration-response relationships impossible to interpret when activation is examined simultaneously with assay of enzyme activity. Under these conditions of progressively increasing enzyme velocity (Fig. 1), the observed accumulation of product represents a composite of enzyme activities in various stages of activation. A solution to this problem is to study the activation process idependently from the determination of enzyme activity by washing the membranes to remove free nucleotide and hormone prior to assay under standard conditions. This method, which has been adopted recently (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975), offers the advantage that the irreversible activation by Gpp(NH)p may be specifically studied without the complication of reversible complexes of the cyclase system with hormones or Gpp(NH)p. Also, activation may be performed with various buffers, temperatures, etc., while the assay is conducted under conditions optimal for enzymatic activity. Furthermore, the possible interference of Gpp(NH)p or its metabolites with the catalytic step is avoided, as is the converse possibility that some component of the assay medium alters activation by Gpp(NH)p. The technique of sequential additions and separate incubations also permits examination of the temporal nature of the synergism between hormones and Gpp(NH)p.

When Gpp(NH)p stimulation of toad erythrocyte adenylate cyclase is examined by preincubating the membranes and washing them prior to assay, it is evident that the process is indeed quite slow, and continues for up to 45 min at 30 °C (Figs. 2–4). The rate of activation increases as a function of the concentration of Gpp(NH)p, but will not exceed certain limits (Fig. 7) even with very high concentrations of the nucleotide (up to 0.5 mM). It is notable, however, that in the presence of (−)-isoproterenol the rate of activation increases by 10- to 20-fold above that obtainable with maximal concentrations of Gpp(NH)p alone (Figs. 2 and 3). The facts that the rate of irreversible activation is saturable with respect to Gpp(NH)p,

and that hormones increase the rate beyond that possible with maximal concentrations of the nucleotide, have also been reported for rat fat cell membranes (Jacobs *et al.*, 1975) and rat liver membranes (Salomon *et al.*, 1975). This has been interpreted (Jacobs *et al.*, 1975) as evidence for a rate-limiting, hormone-controlled event (step) occurring separately from binding of Gpp(NH)p to its regulatory site.

The following mechanisms have been considered (Jacobs *et al.*, 1975):

1) $\text{Gpp(NH)p} + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Gpp(NH)p} - E^{**} \xrightarrow[\text{hormone}]{k_2} \rightarrow \text{p(NH)p} \cdot E$ (active)

2) $E_A \underset{\text{hormone}}{\overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}}} E_B + \text{Gpp(NH)p}^{**} \xrightarrow{k_2} \rightarrow \text{p(NH)p} \cdot E$ (active)

where E refers to the cyclase system. The irreversible step in each case is the formation of a covalent p(NH)p-cyclase complex which is thought to be relatively resistant to enzymatic hydrolysis (Cuatrecasas *et al.*, 1975*a*, *b*; Jacobs *et al.*, 1975). According to this hypothesis, GTP also forms pyrophosphate-cyclase intermediates, although these are rapidly degraded. Such a pyrophosphate-transfer reaction may occur slower with the analogs, which could explain the diminished rate of activation by Gpp(NH)p. The concentration-response relationships for such a slow, irreversible process would not be expected to conform to behavior based on assumptions of thermodynamic equilibrium. The present studies demonstrate that the apparent K_a for activation by Gpp(NH)p decreases with more prolonged incubations (Fig. 7), and that a nearly maximal rate of activation may be obtained after 35-min incubation with a concentration of Gpp(NH)p which gives only half-maximal stimulation at 20 min (Figs. 4 and 7). Increasing concentrations of Gpp(NH)p thus decrease the time required to obtain a maximal rate of activation. It is of further interest that the apparent sensitivity to Gpp(NH)p may be increased by simply prewarming membranes in the absence of Gpp(NH)p or hormones (Table 2). Any correlation between the affinity of binding of [^{3}H] Gpp(NH)p to its membrane sites (which is most likely not rate-determining) to the K_a for activation thus may be only fortuitous.

The synergism between Gpp(NH)p and (−)-isoproterenol requires the simultaneous presence of both compounds (Table 1) and, moreover, the process of activation may be interrupted by addition of the β-adrenergic blocker, propranolol (Fig. 3). Similar findings have been reported for frog erythrocytes (Schramm & Rodbell, 1975). These experiments exclude the possibility that Gpp(NH)p converts adenylate cyclase to a form which

may be permanently activated by (−)-isoproterenol, and indicate that hormones act at, or prior to, the irreversible step(s) in the stimulation process.

When membranes are treated with Gpp(NH)p (with or without hormone) for a fixed time and subsequently washed, the time course of cyclic AMP production during the assay exhibits linear kinetics (Fig. 1). Similar findings have been reported in other tissues (Salomon *et al.*, 1975; Schramm & Rodbell, 1975). However, if membranes are preincubated for varying times and washed prior to assay, an accelerating phase is clearly evident (Figs. 2–4) which is of similar duration as that observed following addition of Gpp(NH)p in the assay (Fig. 1). It is important to distinguish between such a period of increasing velocity and an absolute delay in onset of activation. Activation by Gpp(NH)p and (−)-isoproterenol may be detected after incubation for as little as 30 sec. Furthermore, semilog plots of activation *vs.* time of preincubation are approximately linear at early time points; moreover, these lines do not extrapolate through the abscissa as would be expected if an absolute lag period were followed by an accelerating phase (not shown). The apparent exponential nature of activation should not be taken too seriously at this time, since a function proportional to $(\text{time})^3$ would, within a certain range, give similar values.

The lag phase of activation occurs in the presence and absence of (−)-isoproterenol although the hormone markedly increases the rate of acceleration (Fig. 2). The primary effect of hormone may be on the *rate* rather than *extent* of activation. However, it was not possible to continue the incubation long enough to establish this point due to the lability of the enzyme.

Although the length of the accelerating period varies markedly with the concentration of Gpp(NH)p (Fig. 4), it is never abolished, even with saturating concentrations of the nucleotide or in the presence of maximal levels of (−)-isoproterenol (Figs. 2 and 7). However, simply prewarming membranes at 30 °C in the absence of Gpp(NH)p or hormone results in the early onset of a linear rate of activation (Fig. 5). The lag is not permanently lost, since chilling membranes following warming affects the return of the usual accelerating time course (Fig. 6). It is of interest to consider possible explanations for these unusual kinetics. It is unlikely that slow equilibration of Gpp(NH)p with its binding site(s) alone could explain the data, since the lag persists in the presence of maximal concentrations of Gpp(NH)p, as reported for rat liver (Salomon *et al.*, 1975). Furthermore, (−)-isoproterenol, which has no effect on the binding of [^{3}H]Gpp(NH)p (Pfeuffer & Helmreich, *unpublished data*), increases the rate of acceleration

in the presence of saturating levels of Gpp(NH)p, which indicates that some process other than binding of the nucleotide is rate limiting. It is also unlikely that prewarming membranes for 8 min at 30 °C should alter the binding behavior of Gpp(NH)p although this treatment abolishes the lag phase (Fig. 5) and increases the sensitivity of cyclase to activation by Gpp(NH)p (Table 2) and (−)-isoproterenol (Table 3). Transport or diffusion of Gpp(NH)p into erythrocyte ghosts should also be considered as a possible rate-limiting step since Gpp(NH)p has little effect on the enzyme of intact cells (Lefkowitz & Caron, *unpublished data*). However, the lag period persists when ghosts are vigorously sonicated in the presence of Gpp(NH)p and (−)-isoproterenol. The fact that prewarming membranes in the *absence* of Gpp(NH)p increases the rate of acceleration argues against rate-limiting transport processes, as well as against the possibility that the lag represents the time required to convert Gpp(NH)p to an active form.

The accelerating period could conceivably result from an initial inhibitory agent or process which is slowly overcome with time at 30 °C. This seems unlikely, however, since the lag phase, which is lost by warming membranes, may be regained simply by returning them to 0 °C prior to the preincubation period (Fig. 6). This reversibility also reasonably excludes any enzymatic mechanism as the basis of the slowly developing rate of activation.

Similar accelerating time courses of activation in the presence of allosteric modifiers have been observed with other complex enzyme systems (reviewed by Frieden, 1970). Two general mechanisms appear to be responsible: either a slow, ligand-induced change in configuration of the enzyme or an association (or dissociation) of subunits of the enzyme complex. A slow Gpp(NH)p-dependent isomerization of adenylate cyclase cannot be invoked to explain the lag phase since this may be abolished by prewarming the enzyme in the absence of ligand. However, it is possible that an association of adenylate cyclase with a regulatory subunit (such as a hormone receptor) may be occurring during the lag phase. An inhibitory subunit could, in principle, be dissociating, as proposed by Pfeuffer and Helmreich (1975). However, in view of the striking synergism between Gpp(NH)p and (−)-isoproterenol, it is perhaps simpler to postulate a positive or cooperative interaction between the cyclase and hormone receptors. Adenylate cyclase and known hormone receptors fulfill the criteria for "integral" membrane proteins (Singer & Nicolson, 1972), which are, in many instances, capable of lateral mobility within the plane of the plasma membrane (Singer & Nicolson, 1972; Edidin, 1974; Singer,

1974). In the recently proposed mobile receptor theory of adenylate cyclase-hormone receptor interactions (Cuatrecasas, 1974, 1975; Bennett, O'Keefe & Cuatrecasas, 1975), it has been suggested that the cyclase and receptors may exist separately and that these molecules may collide and form complexes as the result of lateral diffusion within the phospholipid bilayer. Thus, according to this hypothesis, associations may occur between membrane proteins which are analogous to the behavior of proteins in three-dimensional aqueous solution. The activation of cyclase by Gpp(NH)p could occur according to the scheme:

$$H + R \rightleftharpoons H \cdot R$$

$$E + R \rightleftharpoons E \cdot R \xrightarrow[\text{Gpp(NH)p}]{} E \cdot R \text{ (active) (slower)}$$

$$E + H \cdot R \rightleftharpoons E \cdot R \cdot H \xrightarrow[\text{Gpp(NH)p}]{} E \cdot R \text{ (active) (faster)}$$

where E refers to adenylate cyclase, R to hormone receptor and H to hormone. This represents just one of many possibilities, and is intended only to illustrate the feature of complex formation between membrane molecules. For instance, it is not clear whether Gpp(NH)p binds to E or to R, whether complexes can form between E and R in the absence of hormone, which step is rate-determining, or what is the nature of the active form of the enzyme. Unfortunately, it is not a simple matter to evaluate this model by varying the concentrations of E or R as may be done with soluble enzyme systems. However, the recent finding that the number of catecholamine receptors may be reduced by exposing intact tissues to the hormone (Mukherjee, Caron & Lefkowitz, 1975) suggests possible future experiments. Another approach involves dissolution of the membrane with nonionic detergents and evaluation of the molecular weight of cyclase solubilized in the presence or absence of hormones or following activation by Gpp(NH)p. It is pertinent in this regard that the fraction of solubilized adenylate cyclase of rat fat cell membranes which is activated by Gpp(NH)p exhibits a larger Stokes radius (and presumably molecular weight) than the fractions with basal or fluoride-stimulated activities (Bennett & Cuatrecasas, *unpublished*).

The possible existence of a rate-determining step of complex formation between membrane proteins during the initial phase of fractionation suggests a physical basis for the reversible loss of the lag phase by prewarming the membranes. A simple gel-fluid phase transition of membrane

lipids is unlikely since this process occurs within milliseconds or seconds (Tsong, 1974). Mixtures of phospholipids such as occur in biological membranes, however, exhibit the property of "lateral phase separation" of lipids into regions with differing composition and fluidity within a temperature range which may be physiologic (Linden, Wright, McConnell & Fox, 1973; Shimshick & McConnell, 1973; Lee, Birdsall, Metcalfe, Toon & Warren, 1974; Jacobson & Papahadjopoulos, 1975; Wu & McConnell, 1975). Membrane proteins which are incorporated into these lipid bilayers may partition preferentially into more "fluid" areas (Grant & McConnell, 1974). Adenylate cyclase and hormone receptors may have different lipid environments due to their localization on the inner or outer surface of the membrane, and thus could conceivably partition into different areas in the plane of the membrane during a phase separation. Warming the membranes past the transition temperature would result in a rapid intermixing of phospholipids (diffusion constant for lateral diffusion of about 10^{-8} cm^2/sec (Sackmann, Trauble, Galla & Overath, 1973; Scandella, Devaux & McConnell, 1973; Brulet & McConnell, 1975), while the proteins would diffuse at a much slower rate ($10^{-9} - 10^{-10}$ cm^2/sec; Edidin & Fambrough, 1973; Sackmann *et al.*, 1973; Poo & Cone, 1974). These speculations could, in principle, be evaluated by labeling adenylate cyclase and hormone receptors with specific morphological markers and examining their distribution by freeze-etch electronmicroscopy.

A lag or accelerating phase in onset of adenylate cyclase activity has been reported previously in the absence of Gpp(NH)p (Wolff & Cook, 1973; Rodbell *et al.*, 1974; Bennett & Cuatrecasas, 1975*a*), and this may have a similar basis as the phenomenon examined here. The unique permanence of the stimulation by Gpp(NH)p permits dissection of the sequence of events involved in the activation process and thus provides a powerful tool in understanding this complex enzyme system.

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