Effects of Cholera Toxin on Adenylate Cyclase

STUDIES WITH GUANYLYLIMIDODIPHOSPHATE

JORGE FLORES and GEOFFREY W. G. SHARP

From the Departments of Medicine and Physiology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT Similarities exist between the properties of adenylate cyclase after stimulation by cholera toxin and after stimulation by guanylylimidodiphosphate (Gpp(NH)p). Thus a strong stimulation is achieved by both agents, the stimulation is essentially irreversible, the action of certain hormones is enhanced and the enzyme can be solubilized with Lubrol PX in the activated state. Because of these similarities the interaction of cholera toxin and Gpp(NH)p on adenylate cyclase was examined. It was found that prior activation of rat liver adenylate cyclase by cholera toxin in vivo, or by cholera toxin and NAD in homogenates, blocked the stimulatory effect of Gpp(NH)p. Furthermore under conditions in which the effect of Gpp(NH)p was less than that of cholera toxin, inhibition of stimulation by cholera toxin was seen. Stimulation of adenylate cyclase by maximal concentrations of Gpp(NH)p, but not by submaximal concentrations, blocked the stimulatory effect of cholera toxin. The mutual interference of the actions of these two agents suggests a common target in the regulatory mechanism of the adenylate cyclase complex.

INTRODUCTION

While the effect of cholera toxin on adenylate cyclase has been widely studied in a variety of animal tissues (1-8), the mechanism by which the toxin stimulates the enzyme is not understood. The activation characteristically occurs after a lag period which is variable according to the tissue studied. Once the effect is produced it is essentially irreversible and can resist dilution, washing, or solubilization of the preparation (8). Simultaneously with the increase in basal activity, cholera toxin produces an enhancement of the effects of some hormones on adenylate cyclase. Thus catecholamine responses are enhanced (5, 7, 8) and the apparent affinity of glucagon for adenylate cyclase is reported to be increased in tissues treated with cholera toxin relative to control tissues (6). The ability to respond to fluoride has been shown to be decreased by the toxin treatment (5).

Some of the characteristics of the cholera toxin effect resemble those of guanyl nucleotides on adenylate cyclase. Since the early reports of the effect of GTP on the enzyme by Rodbell et al. (9) a number of laboratories have found that GTP enhances the effect of hormones on adenylate cyclase, and can stimulate basal activity of the enzyme (10-12). Such effects have been reported to be present in many different eucaryotic cells (13). Nonhydrolyzable analogues of GTP such as guanylylimidodiphosphate Gpp(NH)p[1] and guanylylmethylenediphosphate [Gpp(CH_2)p] have a similar but much greater effect than GTP on both basal and hormone-stimulated adenylate cyclase activity (12-16) presumably because of the stability of the terminal phosphate group. The stimulatory effects are irreversible, resisting dilution, washing, or solubilization. Interference with the stimulatory effect of sodium fluoride by GTP or Gpp(CH_2)p has also been reported (9).

Thus the effects of cholera toxin and the analogues of GTP are similar in that both stimulate adenylate cyclase, both enhance the effects of certain hormones, and both produce an essentially irreversible stimulation of the enzyme which can then be solubilized in the activated state. These similarities prompted us to examine the interrelationship between cholera toxin and Gpp(NH)p. A recently developed procedure to stimulate adenylate cyclase in broken cell preparations (17, 18) allowed the possibility of sequential incubations with the two agents to study this interaction.

METHODS

Stimulation of adenylate cyclase in vivo was achieved by intravenous injection into rats of 1 μg/g of cholera toxin after light ether anesthesia. 5 h later the animals were

Received for publication 20 May 1975 and in revised form 26 August 1975.

[1] *Abbreviations used in this paper:* Gpp(CH_2)p, guanylylmethylenediphosphate; Gpp(NH)p, guanylylimidodiphosphate.

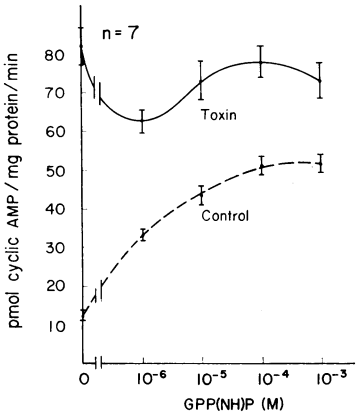

FIGURE 1 Effect of different concentrations of Gpp(NH)p on adenylate cyclase from control rat livers (- - - -) and from livers of rats injected with 1 μg/g cholera toxin and sacrificed after 5 h (——). The liver homogenates were washed once by mixing them with an excess of cold 75 mM Tris-HCl buffer, pH 7.5 and centrifuging at 1,200 *g* for 5 min. The pellets were resuspended and assayed for adenylate cyclase activity in the presence or absence of Gpp(NH)p. Inhibition of the toxin-treated adenylate cyclase by 10^{-6} M Gpp(NH)p was statistically significant ($P < 0.01$).

again anesthetized and their livers perfused through the portal vein with a cold solution of 75 mM Tris-HCl buffer pH 7.5, containing 12.5 mM $MgCl_2$. Livers from toxin-treated rats and from control rats perfused in the same way were cut out and homogenized by 10 strokes of a Dounce homogenizer in 10 vol of the same buffer. Aliquots were washed with 25 vol of buffer and centrifuged at 1,200 *g* for 5 min.

Stimulation of adenylate cyclase in the broken cell preparations was achieved by incubating aliquots of control homogenates (200 μl) with or without 4 μg/ml cholera toxin and 1 mM NAD for 15 min at room temperature; the incubations were terminated by adding an excess (5 ml) of cold buffer and were immediately centrifuged at 1,200 *g* for 5 min.

Pellets from both in vivo and in vitro treated preparations were resuspended in 75 mM Tris-HCl, pH 7.5, containing 25 mM $MgCl_2$, by three strokes of the same homogenizer. The volume of the buffer added was adjusted to obtain a final protein concentration between 1 and 1.5 mg/ml.

Adenylate cyclase activity was assayed using the method of Krishna et al. (19). The final incubation medium (50 μl) consisted of 0.1 mM ATP, including 1 μCi of [^{32}P]ATP, 0.1 mM cyclic AMP, 5 mM phosphoenolpyruvate, 0.07 IU pyruvate kinase, 0.7 IU myokinase, 10 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5. The amount of protein in the incubation varied between 20 and 30 μg. The incubation was carried out for 5 min at 37°C. These were optimal conditions for the assay. The reaction was stopped by adding 1 ml of a cold solution containing 100 μg cyclic AMP and 200 μg ATP, [^{3}H]cyclic AMP was also added for calculation of the recovery. Tube contents were pipetted onto 0.6 × 4-cm chromatographic columns which had been prepared by pipetting 2 ml of 50% (vol/vol) aqueous suspension of Bio-Rad resin AG-50W-X2, 200–400 mesh, H^+ form (Bio-Rad Laboratories, Richmond, Calif.) into tubes containing glass wool supports. The cyclic AMP was eluted by water in the 4th and 5th ml. Impurities in this fraction were removed by two precipitations with $ZnSO_4$ and $Ba(OH)_2$, care being taken that the final pH was close to 7.5. After centrifugation for 5 min at 1,200 *g* the precipitation was repeated. Then the supernates were transferred to scintillation vials containing 15 ml of naphthalene-dioxane solvent system. ^{3}H and ^{32}P were estimated simultaneously in a liquid scintillation spectrometer. Recovery of cyclic AMP as estimated with [^{3}H]cyclic AMP was 50–70%. The amount of cyclic AMP formed was calculated from the specific activity of ^{32}P in the incubation mixture and amount of [^{3}H]cyclic AMP recovered minus the reaction blank. Reaction blanks were always less than 10% of the lowest basal activity. Results are expressed as picomoles of cyclic AMP per milligrams of protein per minute.

Purified cholera toxin was prepared under contract for the National Institute of Allergy and Infectious Diseases by R. A. Finkelstein, Ph.D., The University of Texas Southwestern Medical School, Dallas, Tex. (20). Adenosine 5′-α-[^{32}P]triphosphate (13–20 Ci/mmol) and 8-[^{3}H]cyclic AMP (12–18 Ci/mmol) were obtained from New England Nuclear, Boston, Mass.). Nicotinamide adenine dinucleotide was obtained from Sigma Chemical Co., St. Louis, Mo. Gpp(NH)p was obtained from International Chemical and Nuclear Corporation, Irvine, Calif. All statistical analyses were performed by Student's *t* test.

RESULTS

Effect of Gpp(NH)p on adenylate cyclase activity from control and in vivo toxin-treated preparations. Dose response data for Gpp(NH)p on control and in vivo toxin-treated adenylate cyclase activity were obtained by incubating the washed membranes with different concentrations of the nucleotide. Gpp(NH)p was added during the adenylate cyclase assay which took place during 5 min at 37°C. In control tissue marked stimulation of adenylate cyclase activity was achieved with 10^{-6} M Gpp(NH)p. Maximal stimulation was seen at 10^{-4} M. Treatment with cholera toxin resulted in a sevenfold activation of basal adenylate cyclase activity. In contrast to control tissue, Gpp(NH)p was unable to stimulate cyclase activity from tissues pretreated with cholera toxin in vivo. 10^{-6} M Gpp(NH)p proved to inhibit the activity of cholera toxin-treated adenylate cyclase. The results are shown in Fig. 1.

Effect of Gpp(NH)p on adenylate cyclase from control and in vitro toxin-treated preparations. Incubation of control homogenates with 1 mM NAD and 4 μg/ml cholera toxin for 15 min at room temperature resulted in a sixfold increase in basal cyclase activity. Again, control adenylate cyclase responded to Gpp(NH)p with increased activity whereas toxin-treated adenylate cyclase was inhibited by Gpp(NH)p at all concentrations tested. Results are shown in Fig. 2.

Inhibition of the Gpp(NH)p effect by preincubation with cholera toxin. In a separate series of experiments a 15-min preincubation period with cholera toxin and NAD at room temperature preceded the addition of 10^{-4} M Gpp(NH)p which was then present for a further 15-min period. The homogenates were washed with

an excess of buffer between the two incubations and again after the second incubation. The pellet from the second wash was resuspended and adenylate cyclase assayed immediately afterwards. From the results presented in Fig. 3 it can be seen that cholera toxin and NAD stimulated adenylate cyclase to a similar extent as 10^{-4} M Gpp(NH)p. When Gpp(NH)p was added after the treatment with cholera toxin, no further stimulation of adenylate cyclase occurred. Thus treatment with cholera toxin and NAD completely blocked the effect of Gpp(NH)p added subsequently for 15 min.

Inhibition of the cholera toxin-NAD effect by preincubation with Gpp(NH)p. To determine the maximally effective concentration of Gpp(NH)p when incubations are performed at room temperature, liver homogenates were treated with high concentrations of the nucleotide for 20 min. It is shown in Table I that 10^{-3} M Gpp(NH)p produced a significantly greater stimulation of adenylate cyclase than 10^{-4} M Gpp(NH)p. It is also shown that 10^{-3} M Gpp(NH)p was the maximally effective concentration as no greater effect was caused by 5×10^{-3} M or 10^{-2} M Gpp(NH)p. Submaximal (10^{-4} M) and maximal (10^{-3} M) concentrations of Gpp(NH)p were then incubated with the homogenates for 20 min. Cholera toxin and NAD were added at time 10 min and the adenylate cyclase activity of the preparation was assayed after washing with an excess of cold buffer at the end of the 20 min. It is shown in Fig. 4 that the combination of cholera toxin and NAD can stimulate adenylate cyclase after preincubation with a submaximal concentration of Gpp(NH)p; however, the stimulation in excess of the Gpp(NH)p effect is less than the stimulation achieved on control homogenates. Furthermore, it is shown in Fig. 5 that 10^{-3} M Gpp(NH)p completely prevents the toxin from acting on the enzyme. That is, in the presence of maximally effective concentrations of Gpp(NH)p, cholera toxin does not stimulate adenylate cyclase.

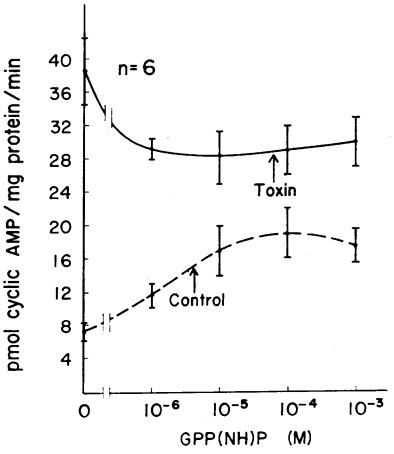

FIGURE 2 Effect of different concentrations of Gpp(NH)p on membranes obtained from control homogenates and homogenates treated in vitro with 4 μg/ml cholera toxin and 1 mM NAD for 15 min at 22°C. The homogenates were washed as described and adenylate cyclase activity assayed in the resuspended pellets in the presence or absence of Gpp(NH)p. Inhibition of the toxin-treated adenylate cyclase by all concentrations of Gpp(NH)p tested was statistically significant ($P < 0.05$).

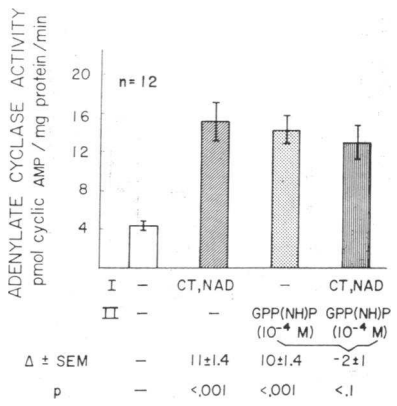

FIGURE 3 Effect of preincubating liver homogenates with cholera toxin (4 μg/ml) and NAD (1 mM) on the response to Gpp(NH)p. The liver homogenates were incubated with or without cholera toxin and NAD for 15 min at 22°C. An excess of cold buffer was added and the product spun at 1,200 *g* for 5 min. The pellets were resuspended in a volume of buffer equivalent to the original volume of the homogenate. A second incubation was then performed with or without 10^{-4} M Gpp(NH)p for 15 min at 22°C. The product was again washed and assayed for adenylate cyclase.

DISCUSSION

The results presented in this work reveal an interesting relationship between the effects of cholera toxin and a

TABLE I

Effect of High Concentrations of Gpp(NH)p Incubated with the Homogenates for 20 min at 22°C on Adenylate Cyclase Activity

Experiment I					
0	10^{-4}	10^{-3}	Δ±SEM	*P*	*n*
pmol/mg protein/1 min					
7±1	28±4		21±2	<0.01	6
	28±4	39±5	11±1	<0.01	6

Experiment II						
0	10^{-3}	5×10^{-2}	10^{-2}	Δ±SEM	*P*	*n*
pmol/mg protein/1 min						
4±1	36±5			32±5	<0.01	6
	36±5	36±4		0±4	NS	6
		36±4	34±8	−1±6	NS	6

The homogenates were washed before the assay. Two sets of experiments were performed to test different ranges of Gpp(NH)p concentrations.

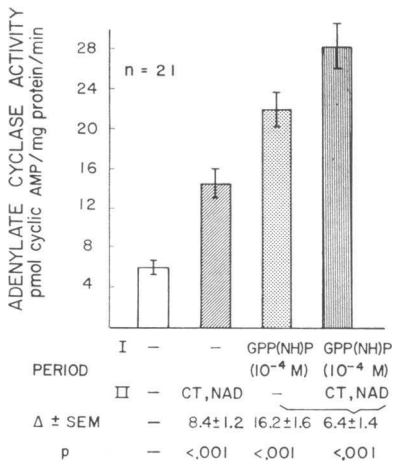

FIGURE 4 Effect of preincubating the liver homogenates with or without 10^{-4} M Gpp(NH)p on the response to cholera toxin and NAD. 4 μg/ml cholera toxin and 1 mM NAD were added after 10 min incubation with Gpp(NH)p at 22°C. After a second 10 min period, the preparation was washed and the pellets resuspended and assayed for adenylate cyclase activity.

nonhydrolizable analogue of GTP, Gpp(NH)p, on adenylate cyclase activity. Cholera toxin is shown to block the ability of Gpp(NH)p to stimulate adenylate cyclase, and Gpp(NH)p is shown to block the effect of cholera toxin. When Gpp(NH)p is added to the enzyme preparation after treatment with cholera toxin and when the Gpp(NH)p effect alone is less than that of the toxin, inhibition of the stimulation due to toxin is

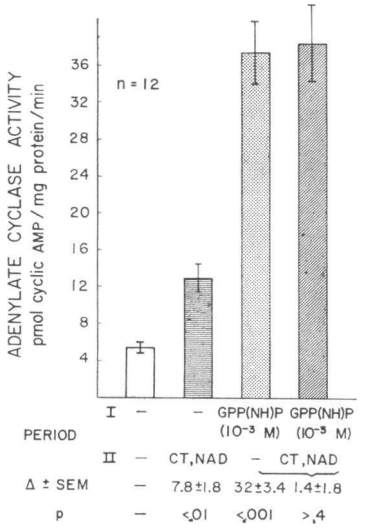

FIGURE 5 Effect of 10^{-3} M Gpp(NH)p on the subsequent response to cholera toxin and NAD. The experimental conditions were similar to those in Fig. 4 except that the dose of Gpp(NH)p employed was 10^{-3} M instead of 10^{-4} M.

observed (Figs. 1 and 2). The mutual interference of the action of these two agents becomes of considerable interest when taken in conjunction with their similarity of effects on adenylate cyclase. As noted in the introduction to this paper, both cholera toxin and Gpp(NH)p produce a marked stimulation of adenylate cyclase, the stimulation is essentially irreversible, hormone action is enhanced, and the enzyme can be solubilized in the activated state. Thus the possibility exists that cholera toxin and Gpp(NH)p share a common target in the adenylate cyclase complex, the so called guanyl nucleotide-binding (regulatory) protein. While an alternative explanation for these data might be that the two agents act on different parts of the adenylate cyclase complex to produce an activated state that is no longer responsive to the other agent, we are impressed by the inhibition of the toxin stimulation caused by subsequent addition of Gpp(NH)p. This mutual interference of activity and the marked similarities of their effects suggest a common target mechanism.

In considering an action of cholera toxin on the guanyl nucleotide-binding protein one could postulate that cholera toxin or its active subunit acts directly (6) or through a reaction in which NAD is required (17, 18), on the site of the adenylate cyclase complex which is sensitive to the action of Gpp(NH)p to produce essentially the same type of stimulation as the latter. Such reaction between the cholera toxin and NAD may consist in the incorporation of a NAD metabolite onto the Gpp(NH)p target on the enzyme complex. This type of reaction has been found to take place in diphtheria, where the diphtheria toxin acts by incorporating the adenosinediphosphoribose moiety from NAD onto elongation factor 2, a ribosomal protein (21).

Several recent studies have shed light on the effect of guanyl nucleotides on adenylate cyclase (12–16). Pfeuffer and Helmreich (15) have detected a guanyl nucleotide-binding protein which they suggested might regulate the activity of adenylate cyclase; thus interaction of guanyl nucleotides with this membrane bound component of the adenylate cyclase complex would essentially remove an inhibitory restraint on the enzyme activity. Similarly, a model has been developed by Rodbell and his associates for the effect of guanyl nucleotides and glucagon on hepatic adenylate cyclase (16, 22–24). In this model Gpp(NH)p induces the formation of an intermediate transition state with no increase in enzyme activity until the transition state is changed by isomerization to a high activity state. Glucagon would act by accelerating the rate of isomerization. In work on Gpp(NH)p effects on frog erythrocytes it was found that high concentrations of isoproterenol inhibited the stimulation by Gpp(NH)p. It was proposed that this

might be due to the setting up of a new equilibrium position between the transitional and the highly activated states of the enzyme. In this regard it is worth mentioning that high concentrations of isoproterenol have been shown to inhibit the action of cholera toxin in rabbit intestine when added after the stimulated state was attained,—pointing out yet another similarity between the activation of adenylate cyclase by cholera toxin and Gpp(NH)p (25).

It is suggested that cholera toxin and Gpp(NH)p act on the same regulatory mechanism to bring about the stimulation of adenylate cyclase. Further studies on the binding of NAD or a derivative of NAD, in the presence of cholera toxin, to the guanyl nucleotide-binding protein are in progress.

ACKNOWLEDGMENTS

This work was supported in part by grants from the John A. Hartford Foundation, Inc., and by the U. S. Public Health Service research grants HE-06664 from the National Heart Institute and AM 04501 from the National Institute of Arthritis and Metabolic Diseases.

REFERENCES

1. Sharp, G. W. G., and S. Hynie. 1971. Stimulation of intestinal adenyl cyclase by cholera toxin. *Nature (Lond.)*. **229**: 226–269.
2. Kimberg, D. V., M. Field, J. Johnson, A. Henderson, and I. Gershaw. 1971. Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. *J. Clin. Invest.* **50**: 1218–1230.
3. Chen, L. C., J. E. Rohde, and G. W. G. Sharp. 1971. Intestinal adenyl cyclase activity in human cholera. *Lancet.* **1**: 939–941.
4. Guerrant, R. L., L. C. Chen, and G. W. G. Sharp. 1972. Intestinal adenyl cyclase in canine cholera: correlation with fluid accumulation. *J. Infect. Dis.* **125**: 377–381.
5. Field, M. 1974. Mode of action of cholera toxin stabilization of catecholamine-sensitive adenylate cyclase in turkey erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 3299–3303.
6. Bennett, V., E. O'Keefe, and P. Cuatrecasas. 1975. Mechanism of action of cholera toxin and the mobile receptor theory of hormone receptor-adenylate cyclase interactions. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 33–37.
7. Hewlett, E. L., L. J. Guerrant, D. J. Evans, Jr., and W. B. Greenough, III. 1974. Toxins of *Vibrio cholerae* and *Escherichia coli* stimulate adenyl cyclase in rat fat cells. *Nature (Lond.)*. **249**: 371–373.
8. Beckman, B., J. Flores, P. A. Witkum, and G. W. G. Sharp. 1974. Studies on the mode of action of cholera toxin. Effects on solubilized adenyl cyclase. *J. Clin. Invest.* **53**: 1202–1205.
9. Rodbell, M., L. Birnbaumer, S. L. Pohl, and H. M. J. Kraus. 1971. The glucagon sensitive adenyl cyclase system in plasma membranes of rat liver. An obligatory role of guanyl nucleotides in glucagon action. *J. Biol. Chem.* **246**: 1877–1882.
10. Leray, F., A. Chambaut, and J. Hanoune. 1972. Role of GTP in epinephrine and glucagon activation of adenyl cyclase of liver plasma membrane. *Biochem. Biophys. Res. Commun.* **48**: 1385–1391.
11. Wolff, J. and G. H. Cook. 1973. Activation of thyroid membrane adenylate cyclase by purine nucleotides. *J. Biol. Chem.* **248**: 350–355.
12. Spiegel, A. M., and G. D. Aurbach. 1974. Binding of 5′-guanylyl-imidodiphosphate to turkey erythrocyte membranes and effects of β-adrenergic-activated adenylate cyclase. *J. Biol. Chem.* **249**: 7630–7636.
13. Lefkowitz, R. J. 1975. Guanosine triphosphate binding sites in solubilized myocardium. Relation to adenylate cyclase activity. *J. Biol. Chem.* **250**: 1006–1011.
14. Londos, C., Y. Salomon, M. C. Lin, J. P. Harwood, M. Schramm, J. Wolff, and M. Rodbell. 1974. 5-Guanylylimidodiphosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 3087–3090.
15. Pfeuffer, T., and E. J. M. Helmreich. 1975. Activation of pigeon erythrocyte membrane adenylate cyclase by guanylnucleotide analogues and separation of a nucleotide binding protein. *J. Biol. Chem.* **250**: 867–876.
16. Schramm, M., and J. Rodbell. 1975. A persistent active state of the adenylate cyclase system produced by the combined actions of isoproterenol and guanylylimidodiphosphate in frog erythrocyte membranes. *J. Biol. Chem.* **250**: 2232–2237.
17. Flores, J., P. Witkum, and G. W. G. Sharp. 1975. Stimulation of adenylate cyclase by cholera toxin in rat liver homogenates. *Clin. Res.* **23**: 393A.
18. Gill, D. M. 1975. The involvement of nicotinamide adenine dinucleotide in the action of cholera toxin *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 2064–2068.
19. Krishna, G. B., B. B. Weiss, and B. B. Brodie. 1968. A simple sensitive method for the assay of adenyl cyclase. *J. Pharmacol. Exp. Ther.* **163**: 379–385.
20. Finkelstein, R. A., and J. J. Lospalluto. 1970. Production of highly purified choleragen and choleragenoid. *J. Infect. Dis.* **121S**: 63–91.
21. Gill, D. M., A. M. Pappenheimer Jr., and T. Uchida. 1973. Diphtheria toxin, protein synthesis and the cell. *Fed. Proc.* **32**: 1508–1515.
22. Salmon, Y., M. C. Lin, C. Londos, M. Rendell, and M. Rodbell. 1975. The hepatic adenylate cyclase system. I. Evidence for transition states and structural requirements for guanine nucleotide activation. *J. Biol. Chem.* **250**: 4239–4245.
23. Lin, M. C., Y. Salomon, M. Rendell, and M. Rodbell. 1975. The hepatic adenylate cyclase system. II. Substrate binding and utilization and the effects of magnesium ion and pH. *J. Biol. Chem.* **250**: 4246–4252.
24. Rendell, M., Y. Salomon, M. C. Lin, M. Rodbell, and M. Berman. 1975. The hepatic adenylate cyclase system. III. A mathematical model for the steady state kinetics of catalysis and nucleotide regulation. *J. Biol. Chem.* **250**: 4253–4260.
25. Sharp, G. W. G., S. Hynie, H. Ebel, D. K. Parkinson, and P. Witkum. 1973. Properties of adenylate cyclase in mucosal cells of the rabbit ileum and the effect of cholera toxin. *Biochim. Biophys. Acta.* **309**: 339–348.