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Research Article

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Activation of Adenylate Cyclase by Cholera Toxin in Rat Liver Homogenates

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ABSTRACT The effect of cholera toxin on adenylate cyclase from rat liver has been studied in a broken cell preparation. The activation of the enzyme in this in vitro preparation requires the addition of nicotinamide adenine dinucleotide (NAD) to the incubation medium and the presence of cell components other than the membrane-bound adenylate cyclase. Once the activation of the cyclase is produced, the effect persists despite repeated washing or solubilization of the enzyme. The effect can be obtained with concentrations of cholera toxin as low as 0.4 nM after 15 min of incubation at 22°C, and stimulation can be detected after only 5 min of incubation at 37°C. The activation of the enzyme is still apparent after at least 2 h at 0°C. Preincubation with choleragenoid in vitro does not interfere with this effect of the toxin. Animals pretreated by an intravenous injection of cholera toxin do not respond to the in vitro addition of cholera toxin and NAD to the same extent as untreated animals; i.e., the effects overlap to suggest that the in vitro effect is the same as that in vivo. Responses to isoproterenol, glucagon, and NaF were also similar in the in vitro broken cell-activated system, as previously reported for the enzyme activated in vivo.

INTRODUCTION

The role of cyclic AMP in the cellular effects of cholera toxin has been extensively studied in recent years. It is known that in every animal tissue so far studied in which cyclic AMP plays a regulatory role, cholera toxin mimics the effects of the cyclic nucleotide. Measurements of cyclic AMP as well as determinations of adenylate cyclase activity have shown that after the intact cells of the tissue are exposed to cholera toxin, a large stimulation of the activity of the enzyme occurs after a latent period (1–3). Once the effect is achieved, it is persistent and cannot be eliminated by washing of the membranes (4) or even solubilization of the enzyme (5), both treatments known to eliminate the activation of the enzyme produced by hormones. However, the addition of cholera toxin to broken cell preparations or crude membranes has usually failed to elicit the effect observed in vivo.

Recently, Gill has reported that NAD is required for the stimulation of adenylate cyclase by cholera toxin in a broken cell preparation of pigeon erythrocytes (6). In this respect the NAD requirement is similar to that for the action of diphtheria toxin, which acts by the enzymatic incorporation of adenosine-5′-diphosphate-5′-ribose onto the ribosomal protein elongation factor 2, the enzyme that catalyzes the translocation of polypeptidyl tRNA on eucaryotic ribosomes (7, 8). This does not indicate that the mechanism is similar, since many enzymes require NAD.

This paper reports studies we have performed to follow up this observation and to describe the characteristics of stimulation of adenylate cyclase in broken cell preparations of rat liver.

METHODS

Animals to be used were anesthetized with ether, and their livers were exposed and perfused through the portal vein with a cold solution of 75 mM Tris-HCl, pH 7.5, containing 12.5 mM $MgCl_2$. Immediately, a piece was cut off and homogenized in 10 vol of the same buffer by 10 strokes of a Dounce homogenizer. Aliquots of the homogenate were incubated in the presence or absence of NAD and/or cholera toxin in a total volume of 240 μl. At the end of the incubation period (5–60 min), an excess (5 ml) of cold buffer was added, and the samples were mixed on a Vortex stirrer (Scientific Industries, Inc., Queens Village, N. Y.) for 5 s and immediately centrifuged at 1,200 g for 5 min. The pellets were resuspended in 75 mM Tris-HCl buffer, pH 7.5, 25 mM $MgCl_2$ with three strokes of the same homogenizers and the product was immediately assayed for cyclase activity. When adenylate cyclase was solubilized, 2.5 ml of homogenate was used for the control or test incubations, followed by washing in 50 ml of cold buffer.

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After being centrifuged at 1,200 g for 5 min, the pellet was resuspended with 20 strokes of a Dounce homogenizer in a cold solution containing 75 mM Tris-HCl, 225 mM sucrose, 1 mM EDTA, 0.25% Lubrol PX (obtained from ICI America, Inc., Stamford, Conn.) pH 7.5. The product was centrifuged at 200,000 g for 30 min and adenylate cyclase activity was assayed in the supernate.

Adenylate cyclase activity was assayed by the method of Krishna et al. (9). 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 phosphoenol-pyruvate, 0.07 IU pyruvate kinase, 0.7 IU myokinase, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5. The amount of protein in the incubation mixture varied between 15 and 25 μg. The incubation was carried out for 5 min at 37°C. These were determined as the 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 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 0.25 M $ZnSO_4$ and 0.25 M $Ba(OH)_2$, 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]ATP in the incubation mixture and amount of [^{3}H]cyclic recovered minus reaction blank. Reaction blanks were incubations performed in the absence of homogenate and were always less than 10% of the basal activity. Results are expressed in picomoles of cyclic AMP per milligram of protein per 5 min.

When cholera toxin was administered in vivo, a dose of 1 μg/g rat wt dissolved in saline was injected through a PE 10 polyethylene catheter in the right jugular vein after light ether anesthesia. The incision was sutured and 4 h later the rats were treated as described for preparation of the liver homogenate.

Quantitation of the extent of cell breakage due to the homogenization procedure was performed. The tissue was homogenized in the same buffer (75 mM Tris HCl, pH 7.5, 12.5 mM $MgCl_2$) by 10 strokes of a Dounce homogenizer. Aliquots of the homogenate were placed on a Clay Adams hemocytometer (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.), and the free nuclei and unbroken cells were counted during a period not longer than 10 min under Nomarski optics.

Purified cholera toxin was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Texas.

Tests for significance were by Student's t test on paired data.

RESULTS

Breakage of cells. To estimate the effectiveness of the homogenization, free nuclei and unbroken cells were counted after preparation of the homogenates under conditions similar to those of the incubation with NAD and cholera toxin. Free nuclei equaled 123,000±6,500/mg liver ($n = 6$), unbroken cells from the same preparations were 1,920±570/mg liver, the ratio of unbroken cells to nuclei being 0.016.

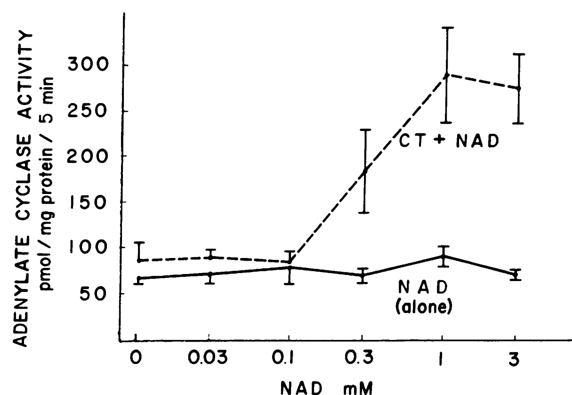


FIGURE 1 Stimulation of adenylate cyclase activity by cholera toxin (4 μg/ml) in the presence of different concentrations of NAD. Homogenates were incubated with the two agents for 15 min at 22°C before washing and assay ($n = 4$). Stimulation by cholera toxin was significant in the presence of 0.3 mM ($P < 0.02$), 1 mM ($P < 0.01$), and 3 mM ($P < 0.01$) NAD.

Dose-response characteristics for NAD. The results of experiments carried out in the presence and absence of 4 μg/ml of cholera toxin and different concentrations of NAD are shown in Fig. 1. Preincubation for 15 min at 22°C was performed before washing and assay for adenylate cyclase. In the absence of cholera toxin, NAD at concentrations of 0.03–3 mM had no effect upon the activity of adenylate cyclase. Cholera toxin alone had no effect upon adenylate cyclase activity nor was any response detected in the presence of 0.03 or 0.1 mM NAD. However, in combination with concentrations of NAD of 0.3 mM and higher, significant stimulation of adenylate cyclase activity was seen. The effect appeared to be maximal at 1 mM, as no further increase in activity occurred with 3 mM NAD.

Dose-response characteristics for cholera toxin. Experiments were performed by incubating the liver preparation for 15 min at 22°C with different concentrations of cholera toxin with or without 1 mM NAD before assay. From the results shown in Fig. 2 it can be seen that cholera toxin in the presence of NAD stimulated adenylate cyclase activity significantly ($P < 0.01$) at a concentration of 0.03 μg/ml (equivalent to 0.4 nM if the mol wt of the toxin is 84,000). Increasing the concentration of cholera toxin from 0.03 to 30 μg/ml resulted in continually increasing activation of adenylate cyclase. The lack of effect of cholera toxin alone is shown on Table I.

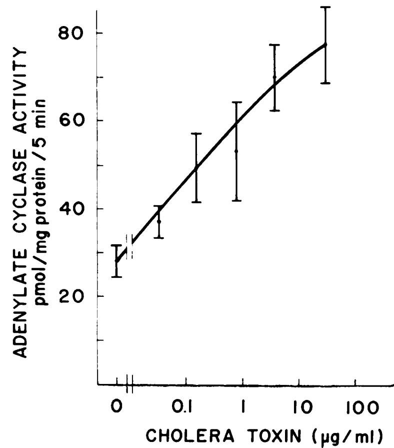
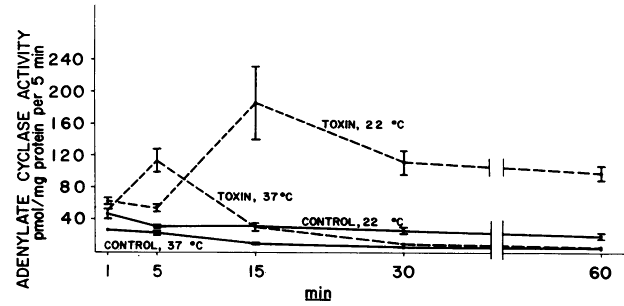


FIGURE 2 Stimulation of adenylate cyclase activity by different concentrations of cholera toxin in the presence of 1 mM NAD. Homogenates were incubated with the two agents for 15 min at 22°C before washing and assay ($n = 6$). Statistics in the text.



		1	5	15	30	60
22 °C	Δ±SE	17±3	22±2	153±36	88±17	78±13
	P	<0.05	<0.01	<0.05	<0.05	<0.05
	n	3	3	3	3	3
37 °C	Δ±SE	–	90±13	21±4	–	–
	P	–	<0.05	<0.05	–	–
	n	1	3	3	1	1

FIGURE 3 Time-course and temperature dependence of the stimulation of adenylate cyclase by cholera toxin (4 μg/ml) and NAD (1 mM). Homogenates were incubated with the two agents for different periods of time at either 22°C or 37°C before washing and assay. Statistical analysis was by paired t test. The probability (P) of significance refers to the increase in adenylate cyclase activity at each time and temperature relative to control homogenates incubated similarly but without cholera toxin and NAD.

Since the cholera toxin preparation available to us contains sodium azide, the effect of different concentrations of azide in the absence or presence of 1 mM NAD was studied. Azide was not found to have any effect on adenylate cyclase under these conditions, even at doses much higher than those in the preparation of cholera toxin used for these experiments (results not shown).

Time-course of the effect and temperature requirements. Fig. 3 shows the results of experiments in which the liver homogenate was incubated in the presence and absence of 4 μg/ml cholera toxin and 1 mM NAD for different periods of time and at two different temperatures, 22° and 37°C, before assay for adenylate cyclase (5 min at 37°C). Under control conditions, basal adenylate cyclase activity decreased with time in accord with the known lability of the enzyme. At 37°C in the presence of cholera toxin and NAD, adenylate cyclase activity was stimulated after only 5 min preincubation. The stimulated enzyme activity was less after 15 min incubation than after 5 min, and was similar to control values after 30 and 60 min incubation. At 22°C, 5 min preincubation was insufficient to stimulate the enzyme, whereas after 15 min, a profound stimulation of adenylate cyclase resulted, the mean activity being more than fivefold that of basal activity. 30 and 60 min incubation also caused marked increases in adenylate cyclase activity, though this was less than that seen with the 15-min incubations.

TABLE I

Lack of Effect of Cholera Toxin on Adenylate Cyclase Activity of Rat Liver Homogenates in the Absence of NAD

	Mean±SEM				
	Control	Toxin	Δ±SEM	P	n
100 μg/ml toxin	46±7	42±6	−4.4±3.4	>0.2	15
20 μg/ml toxin	35±5	36±6	1.6±2.8	>0.9	20
4 μg/ml toxin	32±2	33±2	0.9±1.4	>0.5	8
0.8 μg/ml toxin	33±2	29±2	−3±3	>0.2	17

Homogenates were incubated with different concentrations of cholera toxin for 15 min at 22°C before washing and assay.

Persistence of the activation of adenylate cyclase. In these experiments, the preparation of liver was incubated with 4 μg/ml toxin and 1 mM NAD for 15 min at 22°C. Aliquots were assayed immediately after the membranes were washed at the end of the incubation period and the remaining washed particulate preparation was placed on ice. Aliquots were then assayed for adenylate cyclase activity at 30, 60, and 120 min. Similar studies were performed on the liver particulate preparation alone, and on preparations treated only with NAD. NAD alone had no effect upon adenylate cyclase activity, and the results were similar to control values. The combination of cholera toxin and NAD resulted in a 1.7-fold increase in enzyme activity. The preparations showed a loss of activity with time approximating 40% over the first 60 min. As a consequence, the increased activity observed initially was also observed at 30 and 60 min. Thus the ratio of stimulated to control activity was 2.7 at zero time, 2.9 and 2.6 at 30 and 60 min, respectively, and declined to 2.1 at 120 min. The results are shown in Table II.

TABLE II

Persistence of the Stimulation of Adenylate Cyclase by Cholera Toxin and NAD

	Control	Toxin	Δ±SEM	*P*	Toxin/ Control	*n*
0 min	82±14	222±37	140±33	<0.02	2.7	5
30 min	52±8	150±29	98±23	<0.02	2.9	5
60 min	52±5	133±20	82±18	<0.02	2.6	5
120 min	43±5	89±12	46±6	<0.02	2.1	5

Homogenates were incubated with 4 μg/ml cholera toxin and 1 mM NAD for 15 min at 22°C and then placed on ice. Adenylate cyclase was then measured, immediately, and subsequently at30, 60, and 120 min.

Solubilization of adenylate cyclase. When homogenates were incubated for 15 min at 22°C with 4 μg/ml cholera toxin and 1 mM NAD and subsequently solubilized with Lubrol PX, the adenylate cyclase in the 200,000 *g* supernate was significantly greater than the control solubilized preparation. The results are shown in Table III.

Effect of sequential additions of cholera toxin and NAD. Liver homogenates were incubated for two 15-min periods at 22°C. The preparations were washed with excess cold buffer at the end of each period. Addition of cholera toxin alone (4 μg/ml) or NAD alone (1 mM) during the first period and the complementary agent (NAD or cholera toxin, respectively) during the second period did not result in stimulation of adenylate cyclase. In contrast, cholera toxin and NAD added simultaneously in the first period caused the expected stimulation of adenylate cyclase. The results of these experiments are shown in Fig. 4.

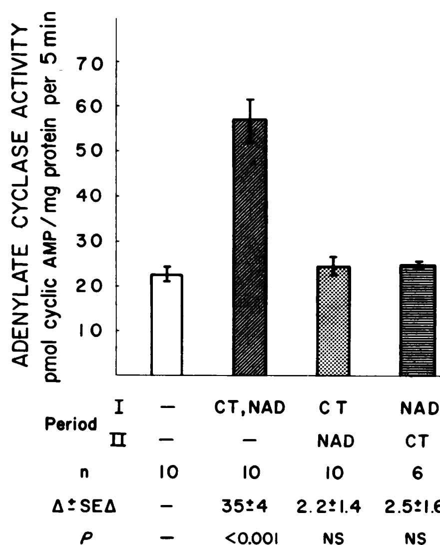
Supernate requirement. The effect of cholera toxin (4 μg/ml) and NAD (1 mM) was tested on the crude homogenate and a washed particulate preparation. Thus

TABLE III

Effect of Solubilization of Adenylate Cyclase on the Persistence of the Activation by Cholera Toxin and NAD

	Mean±SEM	Δ±SEM	*P*	*n*
	pmol cAMP/mg protein/5 min			
Control	51±6			
		28±8	<0.01	10
Toxin	79±13			

2.5-ml aliquots of homogenate were incubated with 4 μg/ml cholera toxin and 1 mM NAD for 15 min at 22°C. At the end of the incubation, an excess of cold buffer was added and the product centrifuged at 1,200 *g*. The pellet was taken up in 75 mM Tris HCl, 225 mM sucrose, 1 mM EDTA, 0.25% Lubrol PX, pH 7.5, by 20 strokes of a Dounce homogenizer followed by centrifugation at 200,000 *g* for 30 min. Adenylate cyclase was assayed in the supernate.



Period	I	—	CT, NAD	CT	NAD
	II	—	—	NAD	CT
n		10	10	10	6
Δ±SEΔ		—	35±4	2.2±1.4	2.5±1.6
P		—	<0.001	NS	NS

FIGURE 4 Lack of effect of separate incubations with cholera toxin (CT) (4 μg/ml) and NAD (1 mM). The left-hand bar shows results for the homogenate (control), incubated for 15 min at 22°C, washed, and incubated again similarly before further washing and assay of adenylate cyclase activity. In the three remaining bars we see results for the homogenates incubated for 15 min at 22°C in the presence of cholera toxin and NAD, cholera toxin alone, and NAD alone. This was followed by a wash and the pellet was then incubated either without addition, with NAD, or with cholera toxin, respectively. Adenylate cyclase activity was measured as for the control preparation after a further wash.

the adenylate cyclase-containing membranes in the homogenate were treated before and after purification by washing. The results of these experiments are shown in Fig. 5. Each wash procedure consisted of the addition of 5 ml cold buffer and centrifugation at 1,200 *g* for 5 min. The pellets were then resuspended in the assay buffer. The two left-hand bars show the effect of toxin plus NAD, a 1.7-fold increase of adenylate cyclase activity in the homogenate. The control and test preparations were washed once after the incubation and before the assay. The two right-hand bars shows results for the control and test particulate fractions of the homogenate, washed three times before incubation with toxin and NAD, and once after incubation. No activation of adenylate cyclase occurred. The two center bars show that the washing procedures per se, as distinct from the time of washing, did not affect the result; in these experiments the control and test homogenates were incubated without and with toxin and NAD and then washed four times before assay. Activation in this preparation was 2.8-fold, as in the preparation washed only once after incubation. There was, however, a slight decrease in the absolute value of enzyme activity in both the control and test preparations. Thus the par-

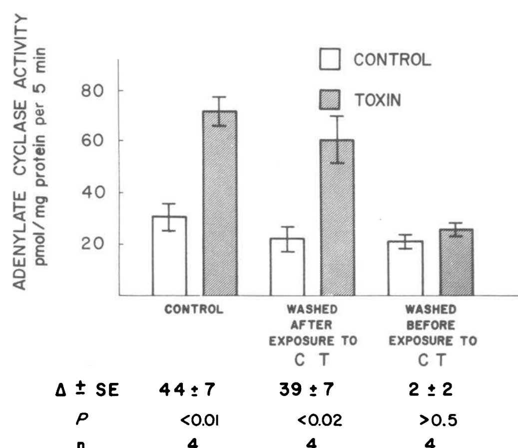


FIGURE 5 The effects of cholera toxin (CT) (4 μg/ml) and NAD (1 mM) on adenylate cyclase activity in the presence and absence of the 1,200 *g* supernate. The bars represent adenylate cyclase activity in 1,200 *g* pellets from liver homogenates. In the left-hand bars the shaded bar represents the activity when homogenates were incubated with toxin and NAD for 15 min at 22°C and subsequently washed once before assay. The middle bars represent the adenylate cyclase activity when toxin and NAD were incubated with the homogenate at 22°C for 15 min and subsequently washed four times before assay. The right-hand bars represent the adenylate cyclase activity after the homogenates were washed three times, then incubated with toxin and NAD for 15 min at 22°C, washed once more, and assayed.

ticulate fraction of homogenates, in the absence of the 1,200 *g* supernate, does not respond to toxin and NAD. Furthermore, the activation caused by toxin and NAD in homogenates is not eliminated by subsequent washing.

Because the washed particulate preparation does not responded to cholera toxin and NAD, separate experiments were performed in which the 1,200 *g* supernates from control homogenates were saved and added subsequently to washed 1,200 *g* pellets under various conditions. The results are presented in Table IV. In experiments A and B, the homogenate was incubated at 22°C for 15 min, then washed, and subsequently treated with supernate (A) or supernate + cholera toxin + NAD (B) for a further 15-min incubation. After a further wash, adenylate cyclase was measured. It can be seen that in the presence of supernate, cholera toxin and NAD stimulated adenylate cyclase. In experiments C, D, and E, similar sequential incubations were performed. In C, the homogenate was treated during the first period with cholera toxin and NAD. After washing no addition was made during the second period. Adenylate cyclase, as expected, was stimulated by the treatment. In D, cholera toxin was added alone during the first period and NAD and 1,200 *g* supernate during the second period. Again, adenylate cyclase was stimulated. In experiment E, NAD was added alone during the first period, and after washing cholera toxin and supernate during the second period. No stimulation of adenylate cyclase was detected.

Further studies have shown that the supernate factor is not precipitated by centrifugation at 200,000 *g*. Thus, when pellets from homogenates (200 μl) treated with cholera toxin alone (4 μg/ml) and washed were exposed to 200 μl of the 200,000 *g* supernate and 1 mM NAD, the adenylate cyclase rose from 15±1 pmol/mg protein/5 min in control preparations (cholera toxin→ wash→NAD) to 37±5 in toxin-treated preparations exposed to the 200,000 *g* supernate and NAD. (Δ± SEM = 22±4, $P < 0.02$, $n = 4$).

These experiments, and also experiment D in Table IV, rule out the possibility that NAD has a secondary role, in the sense that it might simply prevent the breakdown of cholera toxin and thus permit stimulation of adenylate cyclase. The reason for this is that cholera toxin was present for at least 15 min in the absence of NAD during the first period of incubation To strengthen this conclusion and to compare directly the effect of cholera toxin followed by NAD with the effect of the two agents added simultaneously, a separate series of experiments was performed. Thus, as shown in Table V, rat liver homogenates were incubated for two successive 15-min periods. The conditions tested were no additions (control), 1 mM NAD during the second 15 min, 4 μg cholera toxin during the first 15 min, toxin during the first 15 min followed by NAD in the second period, and toxin and NAD added simultaneously in the first period. At the end of the incubations, the preparations were washed and assayed for adenylate cyclase activity. It can be seen that, as expected, neither NAD or cholera toxin alone had any effect, whereas both combinations of toxin and NAD stimulated the enzyme. Furthermore, there was no significant difference between the stimulations achieved by toxin and NAD additions simultaneously or sequentially. Thus NAD does not exert a protective effect on the toxin and is intimately involved in the stimulatory process.

Effect of choleragenoid on the adenylate cyclase response to cholera toxin and NAD. The effect of choleragenoid (40 μg/ml) on the adenylate cyclase response to 0.4 and 4 μg/ml cholera toxin in the presence of 1 mM NAD was tested. In these experiments the liver homogenates were incubated for 10 min at 22°C with choleragenoid. Then without washing, cholera toxin and NAD were added for another 10 min. After this, the homogenates were washed and the pellets resuspended and assayed for adenylate cyclase activity. It is shown in Table VI that preincubation with choleragenoid did not affect the response of adenylate cy-

TABLE IV

Effect of Readdition of Supernate to Washed 1,200 g Pellets on the Ability of Adenylate Cyclase to Respond to Cholera Toxin and NAD

	1st Period	2nd Period	Mean±SEM	Δ±SEM	P
		pmol cAMP/mg protein/5 min			
A		Supernate, NAD	26±2	—	—
B		Supernate, cholera toxin, NAD	38±4	13±3	<0.01
C	Cholera toxin NAD	—	67±6	42±5	<0.001
D	Cholera toxin	NAD, supernate	56±5	30±4	<0.001
E	NAD	Cholera toxin, supernate	28±2	2±2	NS

Two incubations at 22°C for 15 min each were performed and the preparations washed after each one. A, washed pellets were incubated alone during the first period and washed, and 1 mM NAD and 200 μl of supernate were added for the second period. B, Same procedure as A plus the addition of 4 μg/ml cholera toxin during the second period. In C, D, and E the homogenates were incubated similarly for 15 min with various combinations of cholera toxin and NAD, washed, and incubated again with no addition to C, with 200 μl supernate and 1 mM NAD added to D, and 200 μl supernate and 4 μg/ml cholera toxin added to E. After the second 15-min incubation, all the preparations were washed once more and resuspended for adenylate cyclase assay. $n = 6$.

clase to the subsequent addition of cholera toxin and NAD, even when a low concentration of toxin was used.

Effect of cholera toxin and NAD on liver homogenates already activated by cholera toxin administered to the rats in vivo. Five rats were injected with 1 μg/g cholera toxin in the right jugular vein. Six control rats were injected with saline solution similarly. 4 h later all animals were killed and liver homogenates prepared. The effect of cholera toxin (4 μg/ml) and NAD (1 mM) on adenylate cyclase activity was then measured on liver homogenates from the control and toxin-treated animals. Basal adenylate cyclase activity in the control animals was 45 pmol/mg protein per 5 min and in the toxin treated animals 162 pmol/mg protein per 5 min. Treatment of these preparations with cholera toxin and NAD in vitro resulted in the control preparations in a significant increase in activity to 210 pmol/mg protein 5 min. Enzyme activity in the homogenates of liver from rats pretreated with cholera toxin and then subjected to toxin and NAD in vitro showed a nonsignificant increase in activity from 162 to 216 pmol/mg protein per 5 min. Thus the effects of cholera toxin in vivo and in vitro were overlapping and not additive. The results are shown in Fig. 6.

Effect of NaF on adenylate cyclase activity in control and in vitro-stimulated liver preparations. Control preparations and preparations stimulated by treatment

TABLE V

Effects on Adenylate Cyclase Activity of 4 μg/ml Cholera Toxin and 1 mM NAD Added Either Alone, Simultaneously, or Sequentially to Homogenates of Rat Liver

0 min 15 min	— —	— NAD	Toxin	Toxin NAD	Toxin + NAD —	Δ±SEM	P	n
				pmol/mg protein/5 min				
	47.4±3.1	52.8±5.9				5.4±4.0	0.2	6
	47.4±3.1		48.9±3.1			1.5±3.4	0.7	6
	47.4±3.1			93.7±10.3		46.3±10.0	<0.001	6
	47.4±3.1				84.6±11.5	37.2±11.5	<0.05	6
				93.7±10.3	84.6±11.5	−9.1±11.5	0.5	6

The homogenates were subjected to two 15-min incubation periods. One control (no additions) and four experimental situations were studied. The latter were NAD added at 15 min; cholera toxin at 0 min; toxin at 0 min, and NAD at 15 min; and toxin and NAD both added at 0 min. After the two incubations periods, the preparations were washed with cold buffer, centrifuged, and the pellets were resuspended for assay. Adenylate cyclase activity is expressed as pmol/mg protein/5 min, $n = 6$.

Table VI
Effect of Preincubation with Choleragenoid on the Adenylate Cyclase Response to Cholera Toxin and NAD

1st Period	2nd Period	Mean ±SEM	Δ±SEM	P
		pmol/mg protein /5 min		
Control	—	52±5		
			−1±4	NS
Choleragenoid	—	51±5		
—	Cholera toxin, 0.4 μg/ml	64±4		
			1±6	NS
Choleragenoid	Cholera toxin, 0.4 μg/ml	65±3		
—	Cholera toxin, 4 μg/ml	88±8		
			−3±4	NS
Choleragenoid	Cholera toxin, 4 μg/ml	85±5		
0.4 μg cholera toxin vs. control			12±4	<0.05
4 μg cholera toxin vs. control			36±6	<0.01

Homogenates were incubated with 40 μg/ml choleragenoid at at 22°C; after the incubation had proceeded for 10 min, cholera toxin (0.4 μg/ml or 4 μg/ml) and NAD (1 mM) were added to the incubation for another 10-min period; the preparations were then washed with cold buffer and the pellets resuspended for assay. $n = 6$.

with NAD and cholera toxin were washed once and adenylate cyclase activity was measured in the presence and absence of 10 mM NaF. From the results in Fig. 7 it can be seen that both preparations were stimulated by F^- but the stimulation in the toxin-treated preparations was much less than in the control. The two F^--treated preparations were not significantly different. Thus, under these conditions the effects of toxin plus NAD and of F^- were overlapping.

Effect of isoproterenol and glucagon on adenylate cyclase activity in control and in vitro-stimulated liver preparations. Control preparations of liver and preparations stimulated by treatment with NAD and cholera toxin were washed once, and adenylate cyclase activity was measured in the presence and absence of isoproterenol (0.1 mM) and of glucagon (0.1 μM). Both preparations were stimulated by the hormones. Under control conditions, isoproterenol caused a small (15%) but significant stimulation of activity. Glucagon caused a large (140%) stimulation of activity. The results are shown in Table VII.

NAD and cholera toxin increased the adenylate cyclase activity to twice the control values, and on this stimulated preparation, the hormones further stimulated activity. The effect of isoproterenol, while still small, was significantly enhanced relative to its effect on the control preparation. Glucagon stimulated activity to the same extent in absolute terms as in the control preparation.

DISCUSSION

The experiments presented here demonstrate that cholera toxin can stimulate adenylate cyclase in a broken cell preparation. The requirements for the effect are membranes containing adenylate cyclase, cholera toxin, NAD, and a component in the supernate of a 200,000 *g* centrifugation. The stimulatory effect can be achieved with concentrations of the toxin as low as 0.4 nM, in the presence of 0.3–1.0 mM NAD. Under the conditions of our experiments with liver homogenate, maximal activation was achieved by incubating with cholera toxin and NAD for 15 min at room temperature, followed by washing of the membranes and assay for 5 min at 37°C. A more prompt stimulation was achieved by incubating at 37°C, when only 5 min was required to stimulate the enzyme. Longer periods of incubation resulted in stimulated but lower levels of adenylate cyclase activity, probably because of the known lability of this enzyme under these conditions.

Of particular interest are the experiments in which washed membrane preparations fail to respond to toxin and NAD by a stimulation of adenylate cyclase. It appears that a component of the cell in the 1,200 *g* supernate is an essential part of the stimulation process along with the toxin and NAD, since readdition of this supernate to previously washed homogenates results in restoration of the adenylate cyclase responsiveness to cholera toxin and NAD. Incubation of cholera toxin alone or NAD alone with the homogenates, fol-

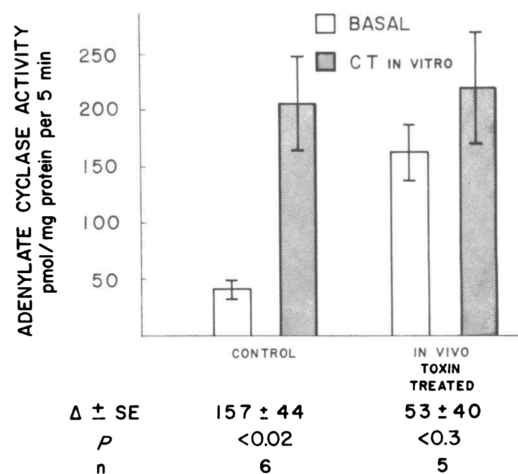


Figure 6 The effects of cholera toxin (CT) (4 μg/ml) and NAD (1 mM) on adenylate cyclase activity of control liver homogenates and of homogenates in which adenylate cyclase had been stimulated by intravenous injection of cholera toxin 4 h before the animal was killed.

lowed by washing and addition of the complementary agent (NAD or cholera toxin, respectively), in a second incubation did not evoke a stimulatory effect, suggesting a necessity for the simultaneous presence of the three agents (cholera toxin, NAD, and supernate).

The experiments in which cholera toxin alone was incubated with homogenates, and washed, before incubation with supernate and NAD suggest that cholera toxin bound during the first incubation can interact with the supernate and NAD during the second incubation to produce stimulation of the adenylate cyclase. However, the failure of large amounts of choleragenoid to prevent the action of the toxin suggests that binding of cholera toxin to the membrane is not an essential requirement in the activation process.

Several tests were applied to the stimulated adenylate cyclase, after treatment with cholera toxin and NAD, to determine whether the stimulation achieved in the broken cell state had the same characteristics as the stimulation achieved by toxin treatment of intact cells. Thus studies were performed routinely to determine the persistence of the stimulation. As for the intact cell stimulation, the effect of cholera toxin is persistent and cannot be reversed by washing or solubilization. A comparison was also made of the effects of cholera toxin and NAD on homogenates from control livers and on homogenates from livers of rats pretreated with cholera toxin 4 h before obtaining the livers. No evidence for a difference in the nature of the stimulation was obtained, in that there was no additivity and the effect of the toxin and NAD in vitro overlapped with that obtained in vivo. Similarly, and as reported previously (5, 10, 11), the effects of fluoride and toxin were partially overlapping. Final points of similarity were obtained by a comparison of the effects of hormones on control and toxin plus NAD-stimulated liver preparations. As reported previously (5), isoproterenol and glucagon both stimulated adenylate cyclase under control and toxin-treated conditions; isoproterenol has been shown to have enhanced activity in toxin-stimulated conditions (5, 12); in the study reported here isoproterenol had enhanced activity in the preparation activated by cholera toxin and NAD in vitro. Glucagon stimulated adenylate cyclase in both control and in vitro toxin-treated preparations, as it does in the in vivo toxin-stimulated liver (5). Thus in all comparative tests made, the stimulation of adenylate cyclase by cholera toxin and NAD in the broken cell preparation appears similar to that achieved in vivo or in the isolated but intact cells.

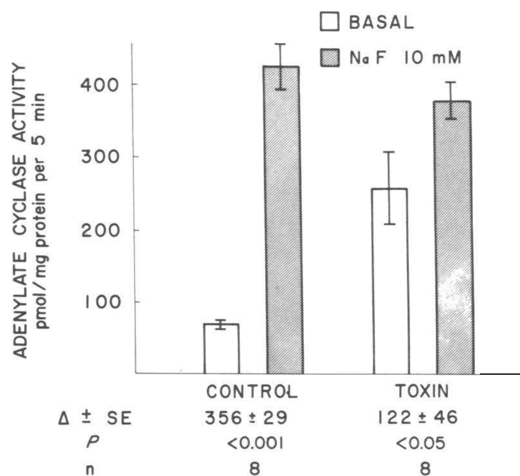


FIGURE 7 The effect of NaF (10 mM) on adenylate cyclase activity of control liver homogenates and of homogenates in which adenylate cyclase had been stimulated by incubation with 4 μg/ml cholera toxin and 1 mM NAD for 15 min at 22°C before washing and assay.

TABLE VII

Effects of Isoproterenol and Glucagon on Adenylate Cyclase Activity in Control Homogenates and in Homogenates Activated by Incubation with 4 μg/ml Cholera Toxin and 1 mM NAD for 15 min at 22°C before Washing and Assay

	Mean ±SEM	Δ±SEM	P	n
Control				
Basal	67±7	—	—	6
Isoproterenol, 0.1 mM	77±7	10±2	<0.01	6
Glucagon, 0.1 μM	150±12	83±13	<0.01	6
Toxin				
Basal	139±12	—	—	6
Isoproterenol, 0.1 mM	156±12	17±3	<0.01	6
Glucagon, 0.1 μM	239±23	100±12	<0.001	6
Control vs. toxin				
Basal		72±10	<0.001	6
Isoproterenol-basal		7±2	<0.02	6
Glucagon-basal		17±17	>0.3	6

The findings that cholera toxin can stimulate adenylate cyclase in broken cell preparations and that NAD is an essential requirement are important for two reasons: (*a*) the in vitro broken cell preparations should facilitate studies on the mechanism of action of cholera toxin and (*b*) it suggests the possibility that cholera toxin acts as an enzyme to produce its effect. In this regard it may be useful to look for parallels with the action of diphtheria toxin, which acts via the incorporation of ADP-ribose from NAD onto the polypeptide chain elongation factor 2 (7, 8). The possibility exists that cholera toxin may act similarly by incorporating NAD or a fraction of the molecule onto a component of

the adenylate cyclase complex, which regulates the activity of this enzyme.

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