

Effects of Cholera Enterotoxin on Adenosine 3',5'-Monophosphate and Neutrophil Function

COMPARISON WITH OTHER COMPOUNDS WHICH STIMULATE LEUKOCYTE ADENYL CYCLASE

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ABSTRACT Cholera enterotoxin caused a delayed accumulation of adenosine 3',5'-monophosphate (cyclic AMP) in human leukocytes, associated with an increase in leukocyte adenylyl cyclase activity. The action of cholera enterotoxin contrasted with that of other agents which stimulate adenylyl cyclase: (a) the effects of the toxin were delayed in onset, while prostaglandin- E_1 (PGE $_1$) and isoproterenol acted rapidly; (b) removal of the soluble toxin from the extracellular medium did not abolish its effects on cyclic AMP and inhibition of antigenic histamine release, while removal of PGE $_1$ did prevent its effects; (c) PGE $_1$, but not cholera enterotoxin, stimulated adenylyl cyclase activity when added directly to broken cell preparations. Binding of the toxin to leukocytes was rapid and irreversible, and was followed by a gradual increase in cyclic AMP which was not prevented by cycloheximide.

Cholera enterotoxin caused accumulation of cyclic AMP in purified human neutrophils as well as mononuclear cells, but did not prevent the extrusion of lysosomal hydrolases from phagocytic cells. The toxin only slightly inhibited the ability of human neutrophils to kill *Candida albicans*. Thus these results with the toxin

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cast doubt on previous proposals that cyclic AMP regulates these two functions of neutrophils. The unique action of cholera enterotoxin on cyclic AMP production provides a potentially useful pharmacologic tool, in addition to methylxanthines and dibutyryl cyclic AMP, for testing hypotheses relating cyclic AMP to altered function of leukocytes and, perhaps, of other mammalian cells.

INTRODUCTION

Numerous recent reports have suggested that intracellular adenosine 3',5'-monophosphate (cyclic AMP) can regulate a variety of leukocyte functions in vitro (1-7). Since the purified enterotoxin of *Vibrio cholerae* stimulates accumulation of cyclic AMP in intestinal mucosa (8) and in every other mammalian tissue tested (9), we have used the toxin as a pharmacologic tool for testing hypotheses that relate leukocyte content of cyclic AMP to function of the cells in vitro. As reported in a companion paper (10), the enterotoxin's inhibition of antigenic histamine release from human leukocytes and of immunologically specific cytolytic activity of mouse splenic lymphocytes correlated well with changes in cellular cyclic AMP content, confirming earlier reports (1, 3-6) in which other drugs were used to stimulate production of the nucleotide.

Experiments reported in the present paper use cholera enterotoxin to test two previous suggestions that cyclic AMP inhibits function of human neutrophils: the extrusion of lysosomal hydrolases following phagocytosis

(7) and the neutrophil's ability to kill *Candida albicans* (2). Although the toxin augmented cyclic AMP content of human neutrophils, it failed to inhibit hydrolase release and only minimally inhibited candidacidal activity. These results suggest that cyclic AMP is not a major determinant of the postphagocytic events examined in the human neutrophil.

In the course of these experiments we have compared the action of cholera enterotoxin with that of other agents which stimulate adenyl cyclase. We found that the toxin acts by a mechanism quite distinct from that of other agents, such as prostaglandin- E_1 (PGE $_1$)¹ and isoproterenol.

The potential importance of both sets of observations stems from the growing attention being focused on cyclic AMP in the leukocyte, an easily biopsied tissue. Several laboratories are actively investigating the nucleotide's potential role in leukocyte models of hormone action (11), immunologic reactivity (12, 13), antibody formation (14, 15), cell-mediated immunity (5, 6), host defense mechanisms against microorganisms (2), release of hydrolytic enzymes and inflammatory mediators (3, 4, 7), phagocytosis (16-18), and diseases such as asthma (19). Our results demonstrate the toxin's usefulness as a pharmacologic tool for investigating effects of cyclic AMP in leukocyte subpopulations: experiments with the toxin strongly confirm the inhibitory role of cyclic AMP in models of immediate hypersensitivity (basophils) and cell-mediated immunity (lymphocytes) (10), but cast considerable doubt on the postulated importance of cyclic AMP in regulating neutrophil function.

METHODS

Leukocytes were obtained from venous blood of normal or allergic volunteer subjects as previously described (4). All cell incubations were performed at 37°C in a Tris-buffered salt solution containing albumin (4), unless otherwise noted. Before assay of adenyl cyclase or phosphodiesterase, the leukocyte preparations were subjected to hypotonic lysis of erythrocytes, centrifuged, resuspended in 0.32 M sucrose at 0°C, and sonicated as described previously (2).

Adenyl cyclase. The assay was that of Krishna, Weiss, and Brodie (20), modified for leukocytes as described elsewhere (2). Each value reported is the mean of duplicate determinations, differing by not more than 10%.

Phosphodiesterase. The method was that of Beavo, Hardman, and Sutherland (21), involving conversion of [³H]cyclic AMP (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) to radioactive 5'-AMP, followed by hydrolysis to [³H]adenosine by *Crotalus atrox* venom (Ross Allen Reptile Institute, Silver Springs, Md.). Adenosine was separated from other adenine nucleotides by ion-exchange chromatography. Enzyme activity was linear with time (0-30 min) and increasing amounts of leukocyte protein (0.1-2.0 mg). In parallel incubations the rate and extent of appear-

¹ Abbreviations used in this paper: PBS, phosphate-buffered saline; PGE $_1$, prostaglandin- E_1 .

ance of adenosine was the same as the disappearance of [³H]cyclic AMP, measured as described previously (2). In the experiments reported here, enzyme activity was assayed at 1.0×10^{-6} M substrate concentration, and at two dilutions of sonicated leukocytes (between 0.5 and 2.0 mg protein per assay tube) for a period of 20 min at 30°C.

Cyclic AMP. At appropriate times, 0.2 ml 20% trichloroacetic acid (TCA) was added to 0.6-ml leukocyte suspensions in buffer ($1.0-2.0 \times 10^7$ cells per tube). After two cycles of freezing and thawing in ethanol-dry ice, particulate material was removed by centrifugation and the supernatant fluid was applied to an 0.5×2.0 cm column of Dowex 50-H⁺ (X-8, 100-200 mesh) previously equilibrated with water. Cyclic AMP was eluted with water and collected in the 2nd and 3rd ml of eluate. This fraction was frozen and lyophilized overnight. The cyclic AMP was then redissolved in 0.25 ml 50 mM sodium acetate buffer, pH 4.0, and assayed for cyclic AMP in duplicate or at two dilutions by the protein-binding competition method of Gilman (22).

Column chromatography was used in order to avoid the repeated washes with ether described by Gilman (22) and previously used in our laboratory (4, 6). This extraction method also allows assay of cyclic AMP in the presence of large concentrations of ATP, ADP, and salts, since these substances (like TCA) are eluted by water before cyclic AMP appears. A tracer amount of [³H]cyclic AMP (0.3-0.5 pmol, with a specific activity of 16.3 Ci/mmol, obtained from Schwarz/Mann Div.) was carried through the entire extraction procedure with each tube. Overall recoveries after lyophilization averaged $60 \pm 10\%$, and were used to correct individual determinations of leukocyte cyclic AMP content.

Individual values reported for cyclic AMP are the means of duplicate or triplicate determinations from separate incubation tubes, except where otherwise specified. Duplicate determinations varied by 10%.

Histamine release. The release of histamine from leukocytes of allergic human subjects, stimulated by purified antigen, was measured as described in the accompanying paper (10) and elsewhere (4). All values are the means of at least two determinations, differing by about 5%.

Candidacidal activity. Death of *C. albicans* ingested by neutrophils was measured in mixed leukocyte populations as previously described (2). Leukocytes were preincubated with cholera enterotoxin (10 ng/ml) or buffer for 90 min at 37°C before the candida cells were added. Toxin-treated leukocytes did not differ from control cells in their uptake of *C. albicans* under these experimental conditions.

Hydrolase extrusion. Leukocytes were suspended at a concentration of 3×10^8 cells per ml in phosphate-buffered saline (PBS, Grand Island Biological Co., Grand Island, N. Y.) to which Ca⁺⁺ (6×10^{-4} M), Mg⁺⁺ (1×10^{-3} M), and autologous serum (10%, vol/vol) were added. The cells were incubated for 90 min with test compounds at 37°C, and were then exposed to particles for 1 h at 37°C. Incubations were terminated by centrifugation at 755 g at 4°C, and the cell supernates were removed for enzyme determination.

Zymosan particles (ICN Nutritional Biochemicals Div., Cleveland, Ohio), 3-5 μ m in diameter, were suspended in saline, boiled, washed twice, and resuspended (5 mg/ml) in PBS. Cell suspensions (1.0 ml) were incubated with zymosan (0.1 ml) containing 1×10^8 particles. Aggregated human gamma globulin was prepared from Cohn fraction II (courtesy Dr. Edward C. Franklin) by heating at 63°C for 20 min. The antigen, present in excess (1.0 ml of a 1% solution in 0.15 M NaCl) was added to 5 ml of a high titer (1:5120) rheumatoid serum (antibody) and reacted at 4°C for 24 h.

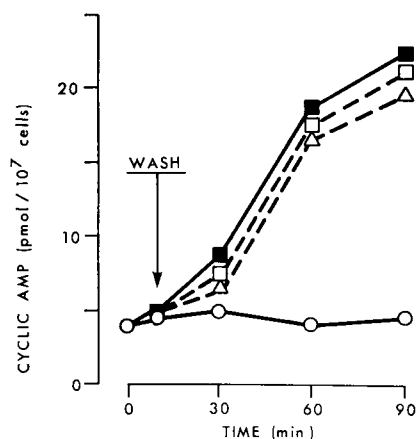


FIGURE 1 Leukocyte cyclic AMP after exposure to cholera enterotoxin, 10 ng/ml. Symbols, (■), toxin present during entire 90 min incubation; (□), soluble toxin removed by washing cells after first 10 min; (△), cells incubated with toxin for first 10 min at 0°C, after which cells were washed and resuspended in toxin-free buffer and incubated at 37°C; (○), no toxin present at any time.

The precipitate was washed three times in 0.15 M NaCl and resuspended in PBS containing Ca^{++} and Mg^{++} . The preparation is referred to as rheumatoid factor complex (RF-*algG*). Leukocyte suspensions (1.0 ml) were exposed to 0.5 ml of immune precipitates containing 400 μg protein.

β -Glucuronidase was determined as previously described (23), and lactate dehydrogenase was measured by the method of Wacker, Ulmer, and Vallee (24).

Cell separation procedures. Blood was obtained from healthy young adults, heparinized, and centrifuged at 100 *g* for 10 min. The supernatant plasma was removed, centrifuged at 1500 *g* for 10 min to deposit the platelets, and then added back to the blood cells. Mixed leukocytes were prepared from 75 to 120 ml of this reconstituted blood by previously described methods (2). The remaining platelet-poor blood was diluted with an equal volume (180–220 ml) of PBS and separated by minor modifications of the method of

TABLE I
Inhibition of Histamine Release after Removal of Unbound Cholera Enterotoxin*

Cells exposed to toxin† for 10 min and washed Then:	Inhibition of histamine release		
	Exp. 1	Exp. 2	Exp. 3
	%		
Antigen added immediately in toxin-free buffer	6	6	0
60 min incubation in buffer containing toxin before addition of antigen	91	77	63
60 min incubation in toxin-free buffer before addition of antigen	100	62	63

* The extent of histamine release was determined as described previously (4) after cells had been incubated with antigen for 1 h. Percent inhibition of histamine release, calculated as described elsewhere (4), was based on the histamine release from cells preincubated without cholera enterotoxin.

† Concentration, 16 ng/ml.

Böyum into granulocyte-enriched and mononuclear-cell fractions (25). In some experiments, additional purification of the mononuclear cell fraction was accomplished by passing these cells through a 1 cm wide plastic column that contained a 7 cm length of scrubbed nylon fibers (3 denier, Fenwal Laboratories, Morton Grove, Ill.). This procedure removed virtually all of the monocytes from the mononuclear cell preparations, leaving highly purified lymphocytes.

All leukocyte preparations were washed three times in PBS at 100 *g* and suspended in Tris-buffered salt solution (4) at a concentration of 2×10^7 or 4×10^7 cells per ml, before exposure to test drugs.

Differential counts (2000–4000 cells) were performed on smears that had been rapidly air-dried (to preserve basophil structure) and stained with Wright's stain.

Materials. PGE_1 was a gift of Dr. John Pike of The Upjohn Co., Kalamazoo, Mich. Cholera enterotoxin and cholera toxoid, prepared as described elsewhere (26), were obtained from Dr. R. A. Finkelstein, Dallas, Tex. The cholera antitoxin was raised in a dog immunized with the cholera toxin. This antiserum had 1354 antitoxin U by comparison to a standard cholera antitoxin made by The Swiss Serum and Vaccine Institute and distributed by Dr. John Seale (National Institutes of Health Standards).² Other drugs were obtained commercially.

RESULTS

Cholera enterotoxin: comparison with other agents

As reported in a companion paper (10), cholera enterotoxin stimulates accumulation of cyclic AMP in human leukocytes and mouse splenic lymphocytes. These effects were delayed in onset compared with those of prostaglandins, biogenic amines, and methylxanthines, previously described (2–4). Accordingly, we undertook a more detailed comparison of cholera enterotoxin with PGE_1 and isoproterenol. Measurement of inhibition of histamine release was used to complement measurements of leukocyte cyclic-AMP content.

Time course. Cholera enterotoxin, PGE_1 , and isoproterenol presented three distinctly different time courses, whether cyclic AMP content or inhibition of histamine release was measured. The rise in leukocyte cyclic-AMP content after exposure to cholera enterotoxin (10 ng/ml) was perceptible at 30 min and increased progressively for the following 60 min (Fig. 1). The enterotoxin had little or no detectable effect on histamine release before 30 min (Table I) but was increasingly inhibitory thereafter (10).

The effect of PGE_1 on both cyclic AMP and histamine release was immediate, reaching a maximum at about 10 min, and persisted almost undiminished thereafter (Fig. 2). Isoproterenol also produced an immediate rise in cyclic AMP and inhibition of histamine release (Fig. 3). In contrast to PGE_1 , however, the inhibitory effect of low concentrations of isoproterenol on histamine release was transient (Fig. 3).

² Pierce, N. F. Personal communication.

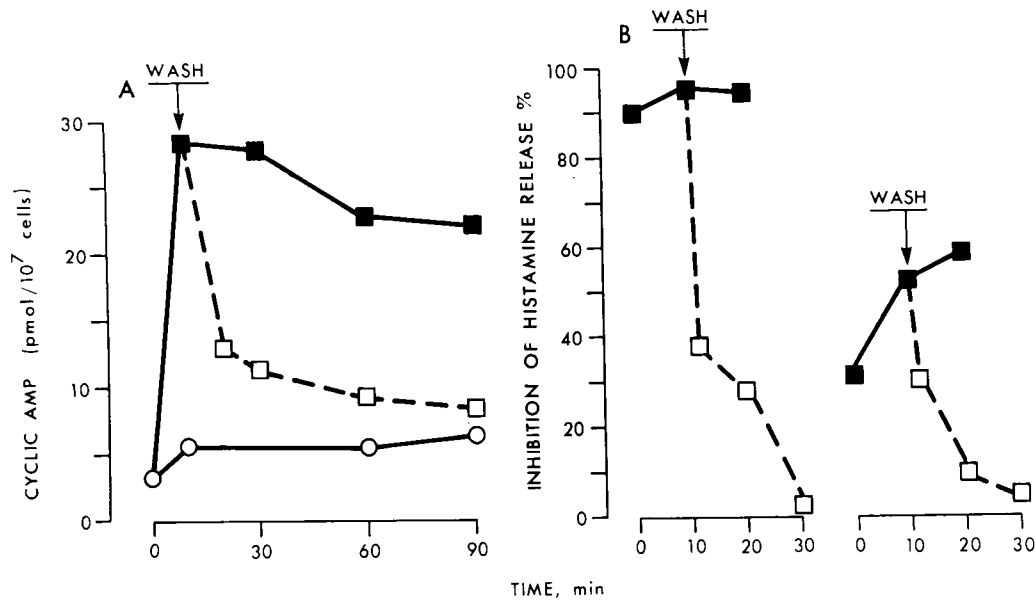


FIGURE 2 Disappearance of PGE₁ effect on leukocytes after removal of PGE₁ from extracellular medium. Cells were incubated with PGE₁ for 10 min, then washed and resuspended in drug-free medium (□) or medium containing the same concentration of PGE₁ (■). (A) Time-course of changes in leukocyte cyclic AMP after exposure to PGE₁, 1 × 10⁻⁵ M. Open symbols (○) indicate control incubations (no drug present). (B) Time course of inhibition of antigenic histamine release from leukocytes of two subjects after exposure to PGE₁ (left = 1 × 10⁻⁵ M; right = 1 × 10⁻⁶ M). Time points refer to time of addition of antigen after addition of PGE₁ (at zero time), although the extent of histamine release was measured 1 h later (see Methods and reference 4).

Specific antagonists. The rise in leukocyte cyclic AMP caused by cholera enterotoxin is antagonized by a canine antibody to the toxin molecule and by a naturally occurring, physiologically inactive toxoid mole-

cule (10, 26, footnote 2). Both antagonists were found to be specific for cholera enterotoxin, in that they did not prevent the rise in cyclic AMP caused by PGE₁, isoproterenol, or histamine. Conversely, the beta ad-

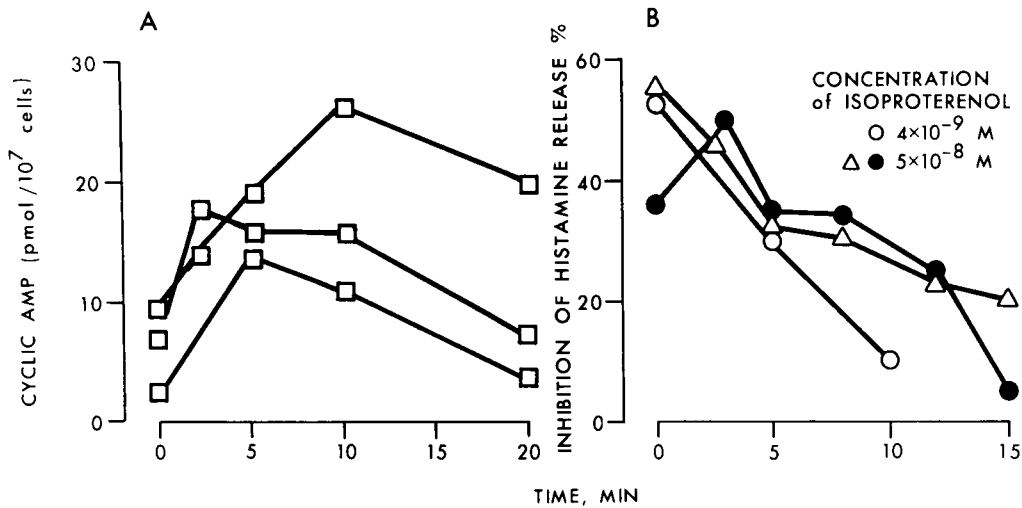


FIGURE 3 Time course of effects of isoproterenol on leukocytes. (A) Cyclic AMP in leukocytes of three subjects exposed to isoproterenol, 1 × 10⁻⁴ M. (B) Inhibition of histamine release in leukocytes of three subjects exposed to antigen at varying times after addition of isoproterenol. These leukocytes were treated with two different concentrations of isoproterenol: (○), 4 × 10⁻⁹ M; (●), and (△), 5 × 10⁻⁸ M.

TABLE II
Leukocyte Cyclic AMP after Removal of Unbound Cholera
Enterotoxin or PGE₁*

Drug	Cells washed at 10 min	Cyclic AMP, 90 min after exposure to drug			
		Exp. 1	Exp. 2	Exp. 3	
				A†	B‡
<i>pmol/10⁷ cells</i>					
None	No	6.0	2.0	11	9.0
	Yes	5.0	3.0	9.6	9.5
Cholera enterotoxin, 10 ng/ml	No	52	70	38	36
	Yes	54	72	35	40
PGE ₁ , 1 × 10 ⁻⁸ M	No	103	94	—	—
	Yes	10	6.0	—	—

* Each of the three experiments was performed on cells of a different normal donor. The cyclic AMP assay is described in Methods, and the washing procedure under Results.

† A, entire incubation at 37°C; B, first 10 min at 0°C.

renergic antagonist propranolol did not prevent the effect of cholera enterotoxin, although it specifically blocked the effect of isoproterenol, as reported previously (4, 11). (For the sake of brevity, these results are not shown).

Initial interaction of cholera enterotoxin with leukocytes

The slow rise in cyclic AMP after exposure of leukocytes to cholera enterotoxin, as compared with the rapid effect of PGE₁, could be due to slow penetration of the cell by the toxin, or to slow metabolism of toxin to an active form. To investigate these possibilities, leukocytes were exposed to cholera enterotoxin or PGE₁ for 10 min, then washed twice and resuspended in drug-free buffer. Removal of soluble toxin from the extracellular medium did not prevent the subsequent rise in cyclic AMP (Fig. 1, Table II) or inhibition of histamine release (Table I). In contrast, the effects of PGE₁ on both cyclic AMP and histamine release diminished rapidly after the cells were washed (Fig. 2, Table II).

Titration experiments with extracellular fluid separated from cells incubated with cholera enterotoxin (initially 10 ng/ml) showed that at least 90% of the pharmacologic activity was still present, as measured by ability to augment cyclic AMP content of cells not previously exposed to the toxin (results not shown). This suggested that the initial interaction of enterotoxin with leukocytes required only a small fraction of the toxin available at this concentration.

If the toxin's initial interaction with cells is rapid and irreversible, specific pharmacologic antagonists might prevent the rise in cyclic AMP only if present in the early stages of exposure to cells to the toxin.

This proved to be the case (Fig. 4): neither canine antitoxin nor cholera toxoid prevented the effect of cholera enterotoxin when added to incubation mixtures 10 min after the toxin, even though no change in cyclic AMP could be measured at that time. Both antagonists completely prevented the toxin's effect when added shortly before the toxin, but inhibition was only partial if the antagonists were added 2 min after the toxin.

In several experiments leukocytes were preincubated for 10 min in tubes immersed in ice water, then exposed to cholera enterotoxin for 10 min at the same temperature. The cells were then centrifuged and washed twice with cold buffer before being suspended in buffer at 37°C and incubated for a further 80 min. The rise in cyclic AMP was identical with that in cells which had initially interacted with cholera enterotoxin at 37°C (Fig. 1, Table II).

Mechanism of action of cholera enterotoxin

The elevated cyclic AMP of gut mucosa exposed to cholera enterotoxin was related to increased activity of adenylyl cyclase (8). The following experiments were designed to confirm a similar mechanism in leukocytes, to rule out an effect of the toxin on cyclic AMP degradation, and to test the possibility that the leukocyte response to cholera enterotoxin required continuing protein synthesis.

Adenylyl cyclase. When leukocytes were incubated with cholera enterotoxin for 90 min, then washed and subjected to sonication, adenylyl cyclase activity (in the absence of added drugs) was more than threefold

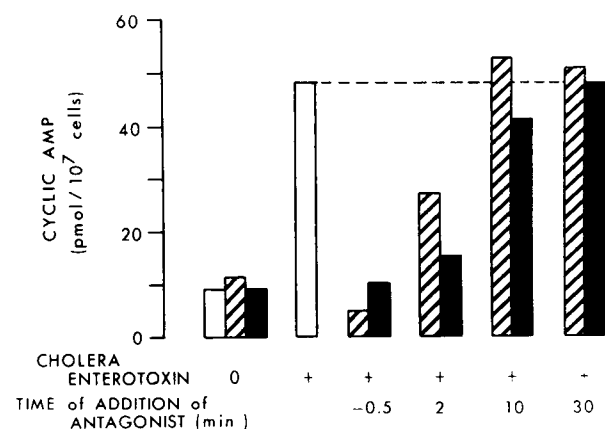


FIGURE 4 Inhibition of cholera enterotoxin-induced accumulation of leukocyte cyclic AMP by specific antagonists added before or at various times after the toxin. Cyclic AMP was measured after 90-min incubations. Drug concentrations: cholera enterotoxin, 10 ng/ml; canine antitoxin, 1:1000 dilution of antiserum (cross-hatched bars); cholera toxoid, 1.0 µg/ml (black bars). The antagonists were added either 0.5 min before or at various times after cholera enterotoxin, as indicated below the figure.

TABLE III
Effects of Cholera Enterotoxin and PGE₁ on Leukocyte Adenyl Cyclase Activity

Drugs preincubation*	Direct addition‡	Adenyl cyclase		
		Exp. 1	Exp. 2	Exp. 3
None (control)	None (basal)	1.0	1.2	3.2
	NaF, 1 × 10 ⁻² M	8.2	9.8	48
	PGE ₁ , 1 × 10 ⁻⁵ M	—	—	15.2
	Toxin, 30 ng/ml	—	1.1	3.3
Toxin, 30 ng/ml	None (basal)	4.7	4.1	11
	NaF, 1 × 10 ⁻² M	7.9	9.5	53
PGE ₁ , 1 × 10 ⁻⁵ M	None (basal)	—	—	3.3
	NaF, 1 × 10 ⁻² M	—	—	50

* Intact leukocytes incubated with drug for 90 min, then washed, sonicated, and assayed for adenyl cyclase (see Methods).

‡ Drug present in enzyme assay tube.

greater than in leukocytes preincubated in toxin-free buffer (Table III). Preincubation with the enterotoxin did not affect adenyl cyclase activity measured in the presence of NaF (1 × 10⁻² M). As with broken cell preparations from gut mucosa (8), direct incubation of toxin with sonicated leukocytes did not stimulate the enzyme (Table III, experiments 2 and 3). Higher toxin concentrations (up to 3 μg/ml) also failed to stimulate the cyclase in the sonicated preparations (not shown).

PGE₁ has been shown to stimulate adenyl cyclase activity directly when added to broken preparations of human leukocytes (2, 11), as in experiment 3 of Table III. However, preincubation of intact cells with PGE₁, in contrast to cholera enterotoxin, produced no measurable effect on adenyl cyclase in washed and sonicated cells (Table III).

Phosphodiesterase. Leukocyte phosphodiesterase activity, measured in cells from six subjects, ranged from 30 to 50 pmol/mg leukocyte protein per min. Cholera enterotoxin, at concentrations up to 30 ng/ml, produced no change in activity of this enzyme, whether the toxin was incubated with the cells before sonication or was added directly to the sonicate (results not shown).

Cycloheximide. The delayed rise in leukocyte cyclic AMP after exposure to cholera enterotoxin raised the possibility that the increase in adenyl cyclase activity (Table III) was due to *de novo* synthesis of the enzyme. At concentrations (up to 1 × 10⁻³ M) which almost completely prevented protein synthesis in human leukocytes (27), however, cycloheximide failed to prevent the rise in cyclic AMP or the inhibition of histamine release caused by cholera enterotoxin (results not shown).

Neutrophil function

The inhibitory effects of PGE₁ and theophylline on neutrophil candidacidal activity and extrusion of hydrolases following phagocytosis have been adduced as evidence that these processes in the neutrophil are modulated by intracellular cyclic AMP. Accordingly, we exposed leukocytes for 90 min to cholera enterotoxin, 10 ng/ml, which produced a maximal rise in cyclic AMP (10), before adding phagocytic particles or *C. albicans*.

Hydrolase extrusion. As reported previously (7), prostaglandins E₁ and A₂, as well as theophylline, prevented the appearance of lysosomal hydrolases, such as β-glucuronidase, in the extracellular medium after phagocytosis of zymosan particles or RF-*algG* complexes (Table IV). Cholera enterotoxin, however, did not prevent release of β-glucuronidase, even when the cells were preincubated with the toxin at concentrations between 10 and 1800 times greater than the maximally effective concentration for increasing cyclic AMP (Table IV).

Candidacidal activity. The effects of cholera enterotoxin (10 ng/ml) on the ability of normal neutrophils to kill ingested *C. albicans* was studied in a series of paired experiments on blood from nine normal sub-

TABLE IV
Enzyme Release from Phagocytic Leukocytes*

Compound concentration	Percent of control β-glucuronidase release‡	
	Exp. A	Exp. B
None	100.0 ± 2.8	100.0 ± 3.1
(Control)		
Cholera		
18 μg/ml	93.9 ± 1.7	—
3.6 μg/ml	103.0 ± 1.9	—
1.0 μg/ml	89.1 ± 3.4	94.0 ± 4.7
10 ⁻¹ μg/ml	96.0 ± 2.1	89.7 ± 5.9
10 ⁻² μg/ml	96.5 ± 0.6	97.4 ± 5.0
10 ⁻³ μg/ml	101.4 ± 2.7	—
10 ⁻⁴ μg/ml	100.4 ± 3.0	—
10 ⁻⁵ μg/ml	101.2 ± 1.9	—
PGE ₁		
2.8 × 10 ⁻⁴ M	73.7 ± 4.2§	68.9 ± 3.8§
Theophylline		
10 ⁻³ M	68.4 ± 6.1§	64.2 ± 4.8§
2-Chloroadenosine		
10 ⁻³ M	72.8 ± 5.4§	66.1 ± 4.9§
PGE ₁ +		
Theophylline		
10 ⁻³ M	48.7 ± 4.3§	38.2 ± 3.7§
PGE ₁ +		
2-Chloroadenosine		
10 ⁻³ M	44.1 ± 3.9§	40.1 ± 2.8§
Cholera +		
1.0 μg/ml	64.9 ± 5.9§	61.9 ± 4.0§
Theophylline		
10 ⁻³ M		
Cholera +		
10 ⁻¹ μg/ml	68.4 ± 4.9§	61.4 ± 4.4§
Theophylline		
10 ⁻³ M		

* 90 min incubation with drug alone (37°C) followed by 1 h with 10⁸ zymosan particles (Exp. A) or 0.5 ml aggregated IgG-rheumatoid factor complex (Exp. B). See methods for details.

‡ Each value represents mean ± SE of three to five samples. Control activity of β-glucuronidase, expressed as μg phenolphthalein, 3 × 10⁶ leukocytes/h: Exp. A, 3.04 ± 0.29; Exp. B, 2.71 ± 0.34.

§ P < 0.01 vs. controls (paired samples).

TABLE V
Theophylline Effects on Leukocyte Cyclic AMP

Drugs		Cyclic AMP*		
PGE ₁	Theophylline	Exp. 1	Exp. 2	Exp. 3
M	M	pmol/10 ⁷ cells		
0	0	10	1.6	6.3
0	1 × 10 ⁻³	8.6	1.7	5.1
0	3 × 10 ⁻³	—	3.4	8.1
0	1 × 10 ⁻²	18	5.9	11
1 × 10 ⁻³	0	66	—	29
1 × 10 ⁻³	1 × 10 ⁻³	63	—	31
1 × 10 ⁻³	3 × 10 ⁻³	—	—	46
1 × 10 ⁻³	1 × 10 ⁻²	220	—	75

* Assayed after 10 min exposure to drugs.

jects. The toxin exerted a small but consistent inhibitory effect on this function in all experiments. Overall, the toxin-treated leukocytes killed ingested organisms 67.0±4.8% as effectively as their paired controls (mean ±SEM, $P < 0.005$). The absolute values of the percentage of nonviable *C. albicans* after the 60 min incubation with leukocytes were 17.8±2.3% (control cells) and 11.7±1.5% (toxin treated).

Theophylline. The substantial inhibition of hydrolyase extrusion (Table IV, reference 7) and candidacidal activity (2) by theophylline, 1 × 10⁻³ M, has been used to implicate cyclic AMP in regulation of these processes, since theophylline inhibits cyclic nucleotide phosphodiesterase in many tissues. Accordingly, we tested the ability of theophylline to raise cyclic AMP content of leukocytes or to potentiate the effect of PGE₁. No detectable change in cyclic AMP occurred at 1.0 × 10⁻³ M theophylline, although higher concentrations produced a modest increase in nucleotide content and potentiation of the action of PGE₁ (Table V).

Cyclic AMP in leukocyte subpopulations

One possible reason for the failure of cholera enterotoxin to inhibit extrusion of lysosomal hydrolases could be that the toxin stimulates accumulation of cyclic AMP in cells other than the neutrophil. Therefore we measured the accumulation of cyclic AMP in subpopulations of leukocytes separated by a modification of the method of Böyum (25) (see Methods). Mixed leukocytes and two fractions, one enriched with neutrophils, the other predominantly mononuclear cells, were exposed to cholera enterotoxin, 10 ng/ml, and to maximally effective concentrations of PGE₁, histamine, and isoproterenol.

The results in leukocytes obtained from three normal subjects are shown in Table VI. The mononuclear cell fraction, made up of lymphocytes (75–86%), monocytes (12–24%), and a few basophils, responded to all four agents with a much greater rise in cyclic AMP content than the standard mixed leukocyte population. The polymorphonuclear fraction, composed mostly of neutrophils (79–95%) and some eosinophils (2–6%), had a consistently lower basal cyclic AMP content than the mononuclear fraction or the mixed cells. Both PGE₁ (at 10 min) and cholera enterotoxin (at 90 min) produced 73–278% increases in cyclic AMP in the polymorphonuclear fraction which could not be accounted for by contamination with a small percentage of lymphocytes and monocytes. The polymorphonuclear fraction did not respond to isoproterenol or histamine.

Because the responses of the polymorphonuclear preparations were small, leukocyte populations of a fourth subject were exposed to PGE₁ and cholera enterotoxin. Each cell population was treated with the drugs in triplicate or quadruplicate, and the experiment was repeated in the same subject 1 wk later, in

TABLE VI
Cyclic AMP in Leukocyte Subpopulations: Response to Drugs*

Fraction.....	Time, min	Exp. 1			Exp. 2			Exp. 3		
		Polymorph.	Mono-nuclear	Mixed	Polymorph.	Mono-nuclear	Mixed	Polymorph.	Mono-nuclear	Mixed
Drug										
0	10	3.4	13	7.4	6.4	26	13	3.7	18	9.1
Isoproterenol, 1 × 10 ⁻⁴ M	10	1.9	18	11	7.0	63	31	4.8	35	11
Histamine, 1 × 10 ⁻⁴ M	10	1.8	24	6.2	6.8	45	30	5.2	30	12
PGE ₁ , 1 × 10 ⁻⁵ M	10	11	42	15	14	136	49	14	94	18
0	90	2.4	7.4	5.9	6.9	22	17	3.3	27	4.3
Cholera toxin, 10 ng/ml	90	6.0	45	16	12	86	30	11	75	14

* Cell separation methods and the assay for cyclic AMP are described in Methods. Each value for cyclic AMP (pmol/10⁷ cells) represents the mean of duplicate determinations, which differed by not more than 10%. Each of the three experiments was performed on leukocytes of a different normal subject.

TABLE VII
Cyclic AMP in Leukocyte Subpopulations: Neutrophils vs. Lymphocytes*

Fraction‡	Time, min	Exp. 4			Exp. 5		
		Polymorph.	Lymphocytes	Mixed	Polymorph.	Lymphocytes	Mixed
0	10	2.38±0.13	13.2	2.54 ±0.22	4.27 ±1.08	11.5	3.8 ±0.28
PGE ₁ , 1 × 10 ⁻⁵ M	10	3.9§±0.18	33.5	7.3§ ±0.67	11.2§ ±0.65	39.9	11.7§ ±1.9
0	90	2.72±0.19	9.7	2.06 ±0.14	2.84 ±0.30	7.43	2.34 ±0.46
Cholera toxin, 10 ng/ml	90	4.2§±0.33	22.8	5.82§±0.64	4.57§±0.23	28.8	4.43§±0.43

* Cell separation methods and the assay for cyclic AMP are described in Methods. Both experiments were performed on leukocytes obtained from the same normal subject, with an interval of 1 wk between experiments.

‡ Determinations on mixed cells and the polymorphonuclear fraction were performed in quadruplicate. The mean of these values is reported (pmol/10⁷ cells), with standard deviation. Determinations in lymphocytes were performed in duplicate or values is reported (pmol/10⁷ cells), with standard deviation. Determinations in lymphocytes were performed in duplicate or triplicate, the values differing by not more than 10%.

§ Value significantly different from control, $P < 0.05$ (standard t test).

order to detect and minimize possible variation in the experimental procedure (Table VII, experiments 4 and 5). This subject's mononuclear cells were subjected to a further purification procedure to obtain lymphocytes free of significant contamination by monocytes and basophils (i.e., less than 1% contamination; see Methods).

The mixed leukocytes of this subject had a consistently low basal content of cyclic AMP, and were predominantly neutrophils (84.3 and 83.4% on the two occasions). Responses of this subject's purified lymphocytes to PGE₁ at 10 min (150 and 247% increases) and cholera enterotoxin at 90 min (135 and 288% increases) were considerably greater than those measured in the mixed cells or the "heavy" cell fraction. The latter fraction, 97% neutrophils, showed a small but consistent and statistically significant increase in cyclic AMP in response to PGE₁ at 10 min (60 and 136%) and to cholera enterotoxin at 90 min (55 and 61% increases). Assuming that any mononuclear cells contaminating these neutrophil preparations responded exactly like the purified lymphocytes in the same experiments, they could have accounted for 16 and 7% of the responses to PGE₁ and 11 and 21% of the responses to cholera enterotoxin in the neutrophil fractions of experiments 4 and 5, respectively.

Like the responses to PGE₁ and cholera enterotoxin, the basal concentration of cyclic AMP in the various leukocyte preparations in these five experiments tended to correlate with the percentage of mononuclear cells present (Tables VI and VII). This suggests that one possible source of the previously noted variation in cyclic AMP content of human leukocyte preparations (4) may be the degree of neutrophilia.

The leukocytes in these experiments were contaminated with platelets (less than 0.5 platelets per leukocyte). The possibility that this number of platelets could contribute to the rise in cyclic AMP after exposure to any of the agents was tested. The platelet-rich plasma of subject 1 (Table VI) was exposed to PGE₁ and cholera enterotoxin in parallel with the leukocyte incubations. The cyclic AMP content of these platelets, even after drug stimulation, did not exceed 0.6 pmol/10⁷ platelets, and consequently could have accounted for no more than a very small percentage of the cyclic AMP measured in any of the leukocyte subpopulations.

DISCUSSION

Recent reports from our laboratories suggested that increased intracellular cyclic AMP inhibited two functions of neutrophils in vitro: extrusion of lysosomal hydrolases after phagocytosis (7) and candidacidal activity (2).

Both hypotheses were based on observed inhibitory effects of PGE₁, theophylline, and dibutyryl cyclic AMP, compounds which were thought to increase synthesis of cyclic AMP, block its degradation, and mimic its intracellular action, respectively. The experiments reported here used cholera enterotoxin as an additional pharmacologic tool to test our hypotheses; the results cast doubt on both:

(a) Cholera enterotoxin failed to inhibit postphagocytic release of β -glucuronidase (Table IV), even at concentrations much higher than those required to produce a maximal rise in leukocyte cyclic AMP. The rise in cyclic AMP caused by cholera enterotoxin was com-

parable with that produced by PGE₁, which did inhibit hydrolase release.

(b) Cholera enterotoxin did produce a small but consistent inhibition of neutrophil candidacidal activity, comparable with that produced by PGE₁ (2). If the effect of cholera enterotoxin is truly confined to a rise in production cyclic AMP, the nucleotide may indeed block candidacidal activity. The toxin's inhibitory effect was small, however, and the much greater inhibition previously demonstrated with theophylline and dibutyryl cyclic AMP (2) was misleading. In fact, theophylline at a concentration, 1×10^{-3} M, which produced 60–70% inhibition of candidacidal activity (2) or hydrolase release (Table IV, reference 7), had no measurable effect on leukocyte cyclic AMP concentration (Table V). Much higher concentrations were required to produce a small increase in concentration of the nucleotide. It seems likely that the effects of theophylline on the complex neutrophil functions measured in these experiments were not mediated by intracellular cyclic AMP. In other tissues, significant effects of theophylline and other methylxanthines on intracellular distribution of calcium (independent of cyclic AMP) and enzymes other than phosphodiesterase have been described (28–30).

Similarly, the relative ineffectiveness of cholera enterotoxin in inhibiting neutrophil functions suggests that exogenous cyclic AMP and its dibutyryl derivative did not faithfully reproduce the action of cyclic AMP production within the neutrophil. In view of the complexity of both hydrolase release and candidacidal activity, it is not surprising that high concentrations of methylxanthines and cyclic AMP analogs might produce misleading effects. Precedents for such an inconsistency do exist: e.g., under certain conditions, exogenous cyclic AMP actually inhibited lipolysis in adipose tissue (31).

An additional potential pitfall in interpreting the role of cyclic AMP lies in the fact that peripheral blood leukocytes are not a homogeneous tissue. For example, the increase in leukocyte cyclic AMP after exposure of the cells of phagocytic particles (17) has been shown by Manganiello and others to occur in the mononuclear fraction, rather than in neutrophils (18). Our experiments with purified neutrophils (Tables VI and VII) demonstrated a similar lessened reactivity of these cells: As compared with mononuclear cells, neutrophils contained less cyclic AMP in the basal state, and responded less vigorously to PGE₁ and cholera enterotoxin; the rise in cyclic AMP caused by isoproterenol and histamine was confined entirely to the mononuclear fraction.

This heterogeneity, in fact, dictates several important qualifications to our conclusions: (a) the preparation of purified cell populations may have preferentially damaged the neutrophil adenyl cyclase system; (b) our

methods for extraction and assay of cyclic AMP may not have been accurate for neutrophils, since with their increased content of hydrolytic enzymes and cationic proteins they might contain inhibiting substances; (c) it is possible that most acid hydrolases released from mixed leukocyte populations are derived from monocytes, rather than neutrophils. These possibilities are under active investigation in our laboratories.

We have used cholera enterotoxin simply as a pharmacologic tool, in applying to leukocytes an important criterion for defining the role of endogenous cyclic AMP as a second messenger controlling cell function. The criterion, proposed by Sutherland, Robison, and Butcher (32), is that tissue content of cyclic AMP should correlate quantitatively and temporally with the functional effect thought to be mediated by the nucleotide. In a companion paper, cholera enterotoxin was used to confirm the premise that endogenous cyclic AMP inhibits antigenic release of histamine by human basophils and immunologically specific cytolytic activity of mouse splenic lymphocytes (10). In this paper, by the same criterion, the premise that cyclic AMP inhibits neutrophil candidacidal activity and hydrolase extrusion from neutrophils after phagocytosis has been found wanting.

Cholera enterotoxin has been shown to elevate cyclic AMP content of every tissue so far tested, including thyroid, adipocytes, platelets, and liver, as well as gut mucosa (8, 9). The purified toxin is an extremely potent stimulator of adenyl cyclase. The maximally effective concentration of the toxin for human leukocytes (10 ng/ml) is approximately 1.1×10^{-10} (molecular weight = 90,000 [9]), which is comparable with effective concentrations in other tissues (9). In addition, the toxin exhibits a distinctive delayed time course, and specific pharmacologic antagonists (the antitoxin and cholera toxoid) are available. These characteristics suggest that the toxin may prove to be an ideal agent, perhaps superior to dibutyryl cyclic AMP and the methylxanthines, for evaluating the role of cyclic AMP in other mammalian cells, as well as leukocytes.

If the toxin is to prove truly useful as a pharmacologic tool, its unusual mechanism of action must be more completely understood. We cannot be sure, for example, that the toxin specifically and uniquely stimulates production of cyclic AMP in leukocytes. Some other effect, perhaps on the neutrophil's plasma membrane, could counteract a true inhibitory action of cyclic AMP on complex cell functions like hydrolase release or candidacidal activity. Such an effect, if demonstrated, would nullify our negative conclusions regarding neutrophil cyclic AMP.

Whereas we have not ruled out additional effects of the toxin, our experiments shed some light on the way

in which this potent compound activates adenylyl cyclase. As in the gut and other tissues (8, 9), the effect of cholera enterotoxin on cyclic AMP in leukocytes was delayed, in contrast with the almost instantaneous effect of other agents (Figs. 1-3). The initial stage of the toxin's interaction with leukocytes, however, was rapid and could not be reversed by washing the cells or by later addition of specific antagonists (Figs. 1 and 4, Tables, I and II). It appeared likely that this early step involved binding of the toxin to the cell, rather than cellular metabolism, since it was equally effective at 0° or 37°C (Fig. 1, Table II).

Later steps in the action of cholera enterotoxin have not been elucidated. They do not appear to require continued protein synthesis, at least in leukocytes, since cycloheximide did not prevent the toxin's effects. In vivo administration of pharmacologic inhibitors of protein synthesis prevented the stimulation of small intestinal secretion of water and electrolytes by cholera enterotoxin (9), but cyclic AMP was not measured.

Whatever the underlying mechanism, the toxin's effect on leukocyte cyclic AMP is clearly different from the action of agents like PGE₁. The latter drug presumably binds reversibly to a cell receptor, to an extent dependent on the concentration of PGE₁ in the extracellular medium, as shown by the prompt reversal of its effects after washing the cells (Fig. 2, Table II). The toxin's effect on leukocytes is also strikingly similar to its action in the gut: A rapid irreversible binding of the toxin to small intestine is suggested, without data, in one review (9); this is followed by a slow increase in adenylyl cyclase activity (8). It seems likely that the toxin interacts in a similar fashion with most mammalian cells. If so, the relative simplicity of handling leukocytes in vitro may eventually provide more detailed understanding of the enzyme, adenylyl cyclase, or even of the biochemical pathogenesis of cholera.

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REFERENCES

- Lichtenstein, L. M., and S. Margolis. 1968. Histamine release *in vitro*: inhibition by catecholamines and methylxanthines. *Science (Wash. D. C.)*. **161**: 902.
- Bourne, H. R., R. I. Lehrer, M. J. Cline, and K. L. Melmon. 1971. Cyclic 3',5'-adenosine monophosphate in the human leukocyte: synthesis, degradation, and effects on neutrophil candidacidal activity. *J. Clin. Invest.* **50**: 920.
- Bourne, H. R., K. L. Melmon, and L. M. Lichtenstein. 1971. Histamine augments leukocyte adenosine 3',5'-monophosphate and blocks antigenic histamine release. *Science (Wash. D. C.)*. **173**: 743.
- Bourne, H. R., L. M. Lichtenstein, and K. L. Melmon. 1972. Pharmacologic control of allergic histamine release *in vitro*: evidence for an inhibitory role of 3',5'-adenosine monophosphate in human leukocytes. *J. Immunol.* **108**: 695.
- Henney, C. S., and L. M. Lichtenstein. 1971. The role of cyclic AMP in the cytolytic activity of lymphocytes. *J. Immunol.* **107**: 610.
- Henney, C. S., H. R. Bourne, and L. M. Lichtenstein. 1972. The role of cyclic 3',5'-adenosine monophosphate in the specific cytolytic activity of lymphocytes. *J. Immunol.* **108**: 1526.
- Weissman, G., P. Dukor, and R. B. Zurier. 1971. Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nat. New Biol.* **231**: 131.
- Kimberg, D. V., M. Field, J. Johnson, A. Henderson, and E. Gershon. 1971. Stimulation of intestinal mucosal adenylyl cyclase by cholera enterotoxin and prostaglandins. *J. Clin. Invest.* **50**: 1218.
- Pierce, N. F., W. B. Greenough, III, and C. C. J. Carpenter, Jr. 1971. *Vibrio cholerae* enterotoxin and its mode of action. *Bacteriol. Rev.* **35**: 1.
- Lichtenstein, L. M., C. S. Henney, H. R. Bourne, and W. B. Greenough, III. 1973. Effects of cholera toxin on in vitro models of immediate and delayed hypersensitivity. Further evidence for the role of cyclic adenosine 3',5'-monophosphate. *J. Clin. Invest.* **52**: 691.
- Bourne, H. R., and K. L. Melmon. 1971. Adenylyl cyclase in human leukocytes: evidence for activation by separate β adrenergic and prostaglandin receptors. *J. Pharmacol. Exp. Ther.* **178**: 1.
- Smith, J. W., A. L. Steiner, W. M. Newberry, Jr., and C. W. Parker. 1971. Cyclic adenosine 3',5'-monophosphate in human lymphocytes. Alterations after phytohemagglutinin stimulation. *J. Clin. Invest.* **50**: 432.
- Smith, J. W., A. L. Steiner, and C. W. Parker. 1971. Human lymphocyte metabolism. Effects of cyclic and noncyclic nucleotides on stimulation by phytohemagglutinin. *J. Clin. Invest.* **50**: 442.
- Ishizuka, M., W. Braun, and T. Matsumoto. 1971. Cyclic AMP and immune responses. I. Influence of poly A:U and cAMP on antibody formation *in vitro*. *J. Immunol.* **107**: 1027.
- Braun, W., and M. Ishizuka. 1971. Cyclic AMP and immune responses. II. Phosphodiesterase inhibitors as potentiators of polynucleotide effects on antibody formation. *J. Immunol.* **107**: 1036.
- Stossel, T. P., F. Murad, R. J. Mason, and M. Vaughan. 1970. Regulation of glycogen metabolism in polymorphonuclear leukocytes. *J. Biol. Chem.* **245**: 6228.
- Park, B. H., R. A. Good, N. P. Beck, and B. B. Davis. 1971. Concentration of cyclic adenosine 3',5'-monophosphate in human leukocytes during phagocytosis. *Nat. New Biol.* **229**: 27.
- Manganiello, V., W. H. Evans, T. P. Stossel, R. J. Mason, and M. Vaughan. 1971. The effect of polystyrene beads on cyclic 3',5'-adenosine monophosphate concentration in leukocytes. *J. Clin. Invest.* **50**: 2741.
- Parker, C. W. 1972. Prostaglandins and the immune response. In *Prostaglandins in Cellular Biology and the Inflammatory Process*. P. W. Ramwell and B. B. Pharriss, editors. Proceedings of Carmel Conference, Plenum Publishing Corp., New York. In press.

20. Krishna, G., B. Weiss, and B. B. Brodie. 1968. A simple, sensitive method for the assay of adenylyl cyclase. *J. Pharmacol. Exp. Ther.* **163**: 379.
21. Beavo, J. A., J. G. Hardman, and E. W. Sutherland. 1970. Hydrolysis of cyclic guanosine and adenosine 3',5'-monophosphates by rat and bovine tissues. *J. Biol. Chem.* **245**: 5649.
22. Gilman, A. G. 1970. A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 305.
23. Brittinger, G., R. Hirschhorn, S. D. Douglas, and G. Weissman. 1968. Studies on lysosomes. XI. Characterization of a hydrolase-rich fraction from human lymphocytes. *J. Cell Biol.* **37**: 394.
24. Wacker, W. E. C., D. D. Ulmer, and B. L. Vallee. 1956. Metalloenzymes and myocardial infarction. II. Malic and lactic dehydrogenase activities and zinc concentrations in serum. *N. Engl. J. Med.* **25**: 449.
25. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest. Suppl.* **97**: 77.
26. Finkelstein, R. A., and J. J. LoSpalluto. 1970. Production of highly purified cholera toxin and cholera toxinogen. *J. Infect. Dis.* **121**: 563.
27. Cline, M. J. 1970. Leukocyte metabolism. In Regulation of Hematopoiesis. A. S. Gordon, editor. Appleton-Century-Crofts, New York. 1045.
28. Sandow, A. 1965. Excitation-contraction coupling in skeletal muscle. *Pharmacol. Rev.* **17**: 265.
29. Sutherland, E. W. 1951. The effect of the hyperglycemic factor and epinephrine on enzyme systems in liver and muscle. *Ann. N. Y. Acad. Sci.* **54**: 693.
30. Robison, G. A., R. W. Butcher, and E. W. Sutherland. 1971. Cyclic AMP. Academic Press, Inc., New York. 120: 164, 199.
31. Vaughan, M. 1960. Effect of hormones on phosphorylase activity in adipose tissue. *J. Biol. Chem.* **235**: 3049.
32. Sutherland, E. W., G. A. Robison, and R. W. Butcher. 1968. Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). *Circulation.* **37**: 279.