

# Mechanisms of Lysosomal Enzyme Release from Human Leukocytes

## II. EFFECTS OF cAMP AND cGMP, AUTONOMIC AGONISTS, AND AGENTS WHICH AFFECT MICROTUBULE FUNCTION

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**ABSTRACT** Selective release of inflammatory materials from leukocyte lysosomes is reduced by compounds which increase cyclic 3',5'-adenosine monophosphate (cAMP) levels in suspensions of human leukocytes and is augmented by agents which increase cyclic 3',5'-guanosine monophosphate (cGMP) levels in these cell suspensions. Lysosomal enzymes are released in the absence of phagocytosis when cytochalasin B (5  $\mu$ g/ml) converts polymorphonuclear leukocytes (PMN) to secretory cells: lysosomes merge directly with the plasma membrane upon encounter of PMN with zymosan, and cells selectively extrude substantial proportions of lysosomal, but not cytoplasmic enzymes.  $\beta$ -Adrenergic stimulation of human leukocytes produced a dose-related reduction in  $\beta$ -glucuronidase release (blocked by  $10^{-6}$  M propranolol) whereas  $\alpha$ -adrenergic stimulation (phenylephrine plus propranolol) was ineffective. In contrast, the cholinergic agonist carbamylcholine chloride enhanced enzyme secretion, an effect blocked by  $10^{-6}$  M atropine. Incubation of cells with exogenous cAMP or with agents that increase endogenous cAMP levels (prostaglandin  $E_1$ , histamine, isoproterenol, and cholera enterotoxin) reduced extrusion of lysosomal enzymes; in contrast, exogenous cGMP and carbamylcholine chloride (which increases endogenous cGMP levels), increased  $\beta$ -glucuronidase release. Whereas colchicine ( $5 \times 10^{-4}$  M), a drug which impairs microtubule integrity, reduced selective enzyme release, deuterium oxide,

which favors microtubule assembly, enhanced selective release of lysosomal enzymes. The data suggest that granule movement and acid hydrolase release from leukocyte lysosomes requires intact microtubules and may be modulated by adrenergic and cholinergic agents which appear to provoke changes in concentrations of cyclic nucleotides.

### INTRODUCTION

Phagocytic cells become the center of inflammatory lesions (1) due, in part, to release of substances previously sequestered within lysosomes. During uptake of particles such as zymosan and immune complexes, polymorphonuclear leukocytes of human blood (PMN)<sup>1</sup> remain viable and selectively extrude lysosomal, but not cytoplasmic, enzymes (2-5). Selective lysosomal enzyme release proceeds independently of particle ingestion when cytochalasin B (CB) converts PMN to "secretory" cells in which an intracellular event (fusion of lysosomes with phagosomes) becomes converted to an extracellular event (fusion of lysosomes with the plasma membrane). Under these conditions leukocytes release substantial proportions of lysosomal enzymes. Zymosan particles are not ingested but remain adherent to the cell surface (6-8).

Intracellular movement of lysosomes, and of secretory granules in a variety of cell types, appears to be

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Received for publication 13 March 1973 and in revised form 7 August 1973.

<sup>1</sup> Abbreviations used in this paper: carbachol, carbamylcholine chloride; CB, cytochalasin B; DMSO, dimethyl sulfoxide;  $D_2O$ , deuterium oxide; LDH, lactate dehydrogenase;  $PGE_1$ , prostaglandin  $E_1$ ; PMN, polymorphonuclear leukocytes, SRS-A, slow-reacting substance of anaphylaxis.

regulated by an as yet unclear interaction between cyclic nucleotides, microtubules, and microfilaments (9). Although microtubules are in a dynamic state of assembly and disassembly, it is probably in their aggregated state that microtubules exert their influence on cell mechanics (10). Exogenous cyclic 3',5'-adenosine monophosphate (cAMP) and colchicine both favor disassembly of microtubules (11) and both reduce selective enzyme release (2, 3, 8, 12, 13). Exogenous cAMP, or compounds which increase its cellular concentration, also reduces antigen-induced histamine release from sensitized leukocytes (14, 15) and lung tissue (16, 17). In contrast, deuterium oxide (D<sub>2</sub>O) favors formation of microtubules (18, 19) and augments histamine release from leukocytes (20, 21). Moreover, it has been reported that  $\alpha$ -adrenergic agents (which decrease cAMP levels) and cholinergic agonists, enhance the immunologic release of histamine and slow-reacting substance of anaphylaxis (SRS-A) from human lung fragments (22). Cholinergic stimulation of heart and brain preparations produces increases in levels of cyclic guanosine 3',5'-monophosphate (cGMP) (23) and the introduction of 8-bromo-cGMP to sensitized lung tissue (22) was associated with enhancement of antigen-induced release of histamine and SRS-A. It therefore seemed possible that movement of lysosomes to phagosomes or the plasma membrane might also be controlled by autonomic agonists, cyclic nucleotides, and agents which influence assembly of microtubules.

Consequently, following the experimental protocol suggested by Kaliner, Orange, and Austen (22), CB-treated human leukocytes were incubated with a series of autonomic agonists before zymosan challenge. The results obtained in these studies indicate that, in CB-treated human leukocytes, release of acid hydrolases may be modulated by adrenergic and cholinergic agents as well as by cyclic nucleotides. It is further demonstrated that agents which increase cAMP levels in suspensions of CB-treated human PMN (isoproterenol, prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), histamine, cholera enterotoxin) reduce enzyme release from these cells whereas carbamylcholine chloride (carbachol), which raises cGMP levels, increases lysosomal enzyme release.

The role of microtubules in lysosomal enzyme release was also evaluated by means of D<sub>2</sub>O and colchicine, following the protocol suggested by Gillespie and Lichtenstein (21). It was found that D<sub>2</sub>O enhanced enzyme release whereas colchicine, a compound known to impair microtubule integrity (10) and reduce selective enzyme extrusion (2, 8, 12, 13) modified the D<sub>2</sub>O effect.

## METHODS

*Medium.* Cells were incubated in a medium consisting of (millimolars): NaCl, 138; KCl, 2.7; Na<sub>2</sub>HPO<sub>4</sub>, 8.1; KH<sub>2</sub>PO<sub>4</sub>, 1.5; CaCl<sub>2</sub>, 0.6; MgCl<sub>2</sub>, 1.0; pH 7.4. The osmo-

larity of the buffer was within 5% of 300 mosmol by freezing point depression technique. D<sub>2</sub>O replaced H<sub>2</sub>O in the medium as required.

*Human peripheral blood leukocytes* were separated and suspended ( $4 \times 10^6$  cells/ml) as previously described (2, 3). Leukocyte suspensions used in the majority of studies contained  $83 \pm 4\%$  polymorphs; the remaining cells were identified as monocytes and lymphocytes. Hypo-osmolar lysis of erythrocytes, and subsequent differential centrifugation, removed the bulk of contaminating platelets and mononuclear cells. In other experiments purified preparations of human PMN were obtained by means of Hypaque/Ficoll gradients (24), allowing studies of cell suspensions which contained  $98 \pm 1\%$  polymorphs. Portions of cell suspensions (1.0 ml) were dispensed into  $10 \times 75$ -mm test tubes and incubated with CB ( $5 \mu\text{g/ml} \times 5 \text{ min}$ ). Cells were then incubated with the test compounds. A dose of theophylline ( $5 \times 10^{-4} \text{ M}$ ), insufficient to affect enzyme release by itself, was usually added when cells were incubated with cAMP,  $\beta$ -adrenergic agonists, and histamine. Metabisulfite, to a final concentration of 0.01%, was added in experiments with adrenergic agonists. Autologous serum (10% vol/vol) was then added and cells were exposed to zymosan. As detailed in previous studies (3), appropriate control experiments (a) indicated there was no preferential degradation of enzyme activity in resting or treated cells, (b) excluded the possibilities that particles and test compounds interfered with enzyme assays, or (c) that there was selective adsorption of enzymes to cells or particles after their release into the medium. At the end of experiments, tubes were centrifuged at 755 *g* at 4°C. The cell-free supernates were removed for enzyme determination.

*Cytochemical localization of myeloperoxidase* was carried out by a modification of the method of Graham and Karnovsky (25) in which the cells were fixed for 5 h at room temperature in 4% formaldehyde and 5% glutaraldehyde and washed overnight in 0.1 M cacodylate buffer (pH 7.2). They were then incubated at room temperature in a medium that contained 10% 3,3'-diaminobenzidine and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.5 M tris buffer (pH 7.6). The cells were rinsed briefly in distilled water and postfixed in 2% O<sub>3</sub>O<sub>4</sub> in distilled water for 1 h. The fixed cells were washed twice in distilled water, en bloc, stained in 2% aqueous uranyl acetate for 15 min, and washed again in water. They were then dehydrated in ethanol and embedded in Spurr's low molecular weight epoxy. Thin sections were taken with a diamond knife on a Porter-Blum MT 2-B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) and viewed with a Zeiss EM 9s (Carl Zeiss, Inc., New York).

*Viability of cells.* The integrity of leukocytes in the pellets at the end of experiments was assayed by several techniques. (a) Exclusion of eosin Y: a cell pellet was diluted in 0.5% eosin Y in saline, mixed, and cells were counted in a hemocytometer. Nonviable cells lost their ability to exclude eosin Y and stained pink. In no instance did the proportion of nonviable cells exceed 3%. (b) Phagocytosis: the percent of untreated cells capable of phagocytosis (85%) when zymosan was added after a 5 h incubation period was no less than when cells were incubated immediately with zymosan, and CB-treated cells regained their capacity to the same extent in CB-free media. (c) Release of cytoplasmic lactate dehydrogenase (LDH) was used as an indicator of cell death, since rupture of cells by detergent (Triton X-100), sonication, or hypo-osmolar lysis (60 mosmol buffer) leads to release of total assayable LDH.

**Enzyme assays.**  $\beta$ -Glucuronidase was determined at 18 h of incubation with phenolphthalein glucuronic acid as substrate, as previously described (26). LDH was determined by the method of Wacker, Ulmer, and Vallee (27).

The platelet:leukocyte ratio of the cell suspensions was 5:1. Platelets, present even in 20-fold excess, contained negligible proportions of total  $\beta$ -glucuronidase activity (3). The contribution to total enzyme activity of leukocyte subpopulations was determined after separation of cells by means of Hypaque/Ficoll gradients (24).

**cAMP determinations.** At appropriate times, 0.1 ml 60% trichloroacetic acid (TCA) was added to purified preparations of PMN suspensions ( $10^7$  cells). After sonication (Sonifier Cell Disruptor; Heat System-Ultrasonics, Inc., Plainview, N. Y.) particulate material was removed by centrifugation and TCA was extracted from the supernatant fluid by four washes with 5 vol of water-saturated ether. Duplicate 50- $\mu$ l samples were assayed for cAMP by the protein-binding competition method of Gilman (28). The following modifications were employed: (a) Protein kinase was purified from frozen bovine heart muscle through the ammonium sulfate fractionation step and subsequent dialysis (29). (b) Each tube containing standard cAMP (Calbiochem, San Diego, Calif.), solutions also contained a volume of ether-extracted TCA solution equal to the volume of the unknown sample to minimize any possible effects of these substances on the assay. (c) Dextran-coated charcoal was used to separate free cAMP from protein kinase-bound cAMP according to the method of Mashiter, Mashiter, Hauger, and Field (30). Liquid scintillation counting of the supernate was performed in 10-ml samples of a 1:2 mixture of Triton X-100: Permafluor-toluene solution (Packard Instrument Co., Inc., Downers Grove, Ill).

Although addition to assay tubes of lysosomal enzyme-rich supernates from zymosan stimulated PMN suspensions completely inhibited binding of cAMP, a finding in agreement with observations of a cAMP-binding inhibitor in human PMN lysosomes (31), there was no inhibition of cAMP in PMN preparations from which protein had been removed by TCA precipitation and centrifugation. Recoveries were  $100 \pm 5\%$ . Individual values reported for cAMP are the means of duplicate determinations from separate incubation tubes. Duplicate determinations varied by no more than 10%.

**cGMP determinations.** Hypaque/Ficoll-purified PMN suspensions were prepared (TCA addition, sonication, ether extraction) as outlined for cAMP determinations. Extracts were frozen and lyophilized overnight. The powder was redissolved in 0.05 M sodium phosphate buffer pH 6.2 and cGMP determined by radioimmunoassay as detailed by Steiner, Parker, and Kipnis (32), with minor modifications. Sodium phosphate buffer was substituted for sodium acetate since it has greater buffering capacity at pH 6.2. Separation of free and bound forms of cyclic nucleotide was achieved by addition of dextran-coated charcoal instead of by second antibody precipitation. Contents of the assay tubes were mixed with 1 ml of dextran-coated charcoal adsorption mixture (2.5% charcoal, 0.3% dextran, 0.25% heated human plasma protein fraction, 20 mM  $\text{KH}_2\text{PO}_4$ , pH 6.0). The mixture was allowed to stand at  $4^\circ\text{C}$  for 10 min before centrifugation (760 g) for 15 min. Radioactivity of the supernates was determined.

Radioimmunoassay of cGMP as developed by Steiner et al. (32) did not require purification of the nucleotide from TCA extracts of tissues or cells. TCA extracts of human

PMN, however, contained a factor(s) which interfered with the binding to antibody of cGMP and recovery of known amounts of cGMP added to TCA extracts of PMN suspensions averaged  $70 \pm 10\%$ . We are currently investigating a simplified purification procedure to eliminate the cGMP-binding inhibitor from extracts of PMN suspensions. Individual values reported for cGMP are the means of duplicate determinations and are not corrected for the decreased recovery. Duplicate determinations varied by no more than 5%.

## MATERIALS

Prostaglandins were kindly furnished by Dr. John Pike, Upjohn Co., Kalamazoo, Mich.; theophylline, Mann Research Lab Inc., New York; cyclic nucleotides, adrenergic and cholinergic agents, histamine, and atropine, Sigma Chemical Co., St. Louis, Mo.; propranolol (Inderal) provided by Ayerst Laboratories, New York; zymosan: ICK Nutritional Biochemicals Div., Cleveland, Ohio; CB: ICI Research Laboratories, Alderley Park, Cheshire, England. CB was dissolved in 0.1% dimethyl sulfoxide (DMSO). This concentration of DMSO did not alter cell morphology or enzyme release (6). Cholera enterotoxin was provided by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Tex., who prepared it according to published procedures (33, 34). There is evidence that cholera enterotoxin loses activity rapidly when dilute solutions are prepared in protein-free phosphate-buffered saline.<sup>2</sup> Portions of enterotoxin were therefore added to PMN suspensions (containing serum) within 30 min of their preparation. In previous studies, where cholera enterotoxin did not reduce lysosomal enzyme release from phagocytic cells (35), care was not taken to use the enterotoxin soon after dilution with phosphate-buffered saline. Indeed, dilute solutions were often stored frozen and used after thawing.

## RESULTS

**Time of preincubation with pharmacological agents.** The time of preincubation with  $\beta$ -adrenergic agents was critical for reduction of enzyme release to be demonstrated. Preliminary experiments indicated that a lag period of 5 min after addition of agents was required for inhibition. Peak effects were observed when cells were incubated 5–20 min. Consequently, cells were preincubated 15 min with  $\beta$ -adrenergic agents in all experiments. Zymosan-induced release of  $\beta$ -glucuronidase from CB-treated cells was assayed at timed intervals after stimulation with carbachol. Enhancement of enzyme release was evident at 2 min, reached a peak effect within 5 min, and was minimal by 10 min. In experiments with carbachol and cGMP, 3 min proved to be the optimum time for preincubation (Fig. 1).

**Distribution of enzyme activity.** Suspensions of mixed leukocytes contained  $83 \pm 4\%$  polymorphonuclear cells. To exclude the possibility that mononuclear cells contributed significantly to  $\beta$ -glucuronidase release, cells were separated by means of Hypaque/Ficoll gradients

<sup>2</sup> Craig, J. 1973. Personal communication.

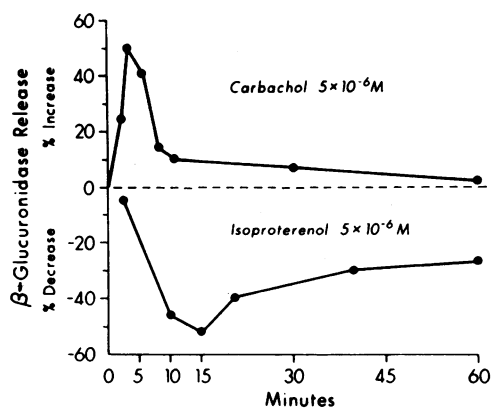


FIGURE 1 Lysosomal enzyme release from CB-treated human leukocytes: effect of incubation time with autonomic agonists before exposure of cells to zymosan. Leukocytes ( $4 \times 10^6$ /ml) incubated at  $37^\circ\text{C}$  with  $5 \times 10^{-6}$  M carbachol or isoproterenol for times indicated, then exposed to zymosan (particle:cell ratio = 30:1) for 30 min. The percent change in  $\beta$ -glucuronidase release is compared with enzyme release from untreated control cells.

(24). The fraction enriched in mononuclear cells (lymphocytes and monocytes) proved to contain only 1.5% of the total  $\beta$ -glucuronidase activity and 9.2% of the total LDH activity of the lymphocyte/monocyte-contaminated leukocyte preparation. For example, the total  $\beta$ -glucuronidase (released by 0.2% Triton X-100) in the lymphocytes and monocytes normally present in a suspension of  $4 \times 10^6$  leukocytes (83% PMN) was 0.34  $\mu\text{g}$  phenolphthalein/h, whereas the total  $\beta$ -glucuronidase content of the leukocyte suspension was 22.2  $\mu\text{g}$  phenolphthalein/h. (numbers are means;  $n = 3$ ).

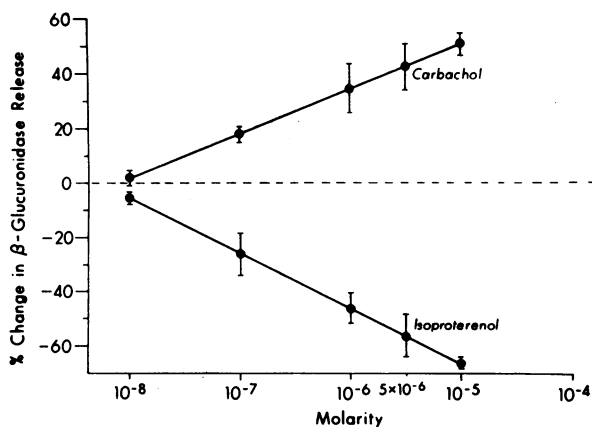


FIGURE 2 Pharmacological regulation of lysosomal enzyme release from CB-treated human leukocytes. Leukocytes ( $4 \times 10^6$ /ml) incubated 5 min with  $5 \mu\text{g}/\text{ml}$  CB, then 15 min with isoproterenol (plus  $5 \times 10^{-4}$  M theophylline), or 3 min with carbachol, then exposed to zymosan (particle:cell ratio = 30:1) for 30 min. Control cells released  $25.6 \pm 2.8\%$  of total  $\beta$ -glucuronidase ( $5.2 \pm 0.7 \mu\text{g}/\text{phenolphthalein}/h/4 \times 10^6$  cells).

*Effect on enzyme release of autonomic agonists.* Cell suspensions from several ( $n = 7$ ) subjects were exposed to the  $\beta$ -adrenergic agonist isoproterenol. Inhibition of enzyme release varied directly with the concentration of isoproterenol. In direct contrast, exposure to carbachol resulted in a dose-related enhancement of  $\beta$ -glucuronidase release (Fig. 2). In the absence of zymosan, carbachol did not enhance enzyme release. Epinephrine produced inhibition equivalent to that of isoproterenol (measured at 30 min).

In leukocytes from a minority of subjects ( $n = 3$ ), enzyme release measured at 30 min appeared to be unaffected by autonomic agonists. These were studied in detail and kinetic analysis demonstrated that the rate of enzyme release was invariably modified: decrease in the presence of isoproterenol, enhancement with carbachol. Moreover, when the concentrations of agonists were raised by one log order, the expected results were again obtained. An experiment with leukocytes from such a subject is shown in Fig. 3. The rate of enzyme release was decreased by isoproterenol (Fig. 3A) and increased by carbachol (Fig. 3B), compared with control cells, during the first 15 min of zymosan exposure, despite the fact that cumulative enzyme release after 30 min of zymosan contact was similar in control and treated cells.

*The effect of autonomic antagonists on enzyme release.* The effects on enzyme release of isoproterenol and epinephrine were prevented when cells were incubated with  $\beta$ -adrenergic-blocking agent propranolol for 2 min before  $\beta$ -adrenergic stimulation, whereas introduction of the antimuscarinic agent, atropine sulfate, (2 min before addition of carbachol to CB-treated cells) prevented the cholinergic effect (Table I). Propranolol ( $10^{-6}$  M) and atropine ( $10^{-6}$  M) had no effect on enzyme release. Neither norepinephrine nor the relatively specific  $\alpha$ -adrenergic agent phenylephrine ( $10^{-12}$ – $10^{-8}$  M) had sig-

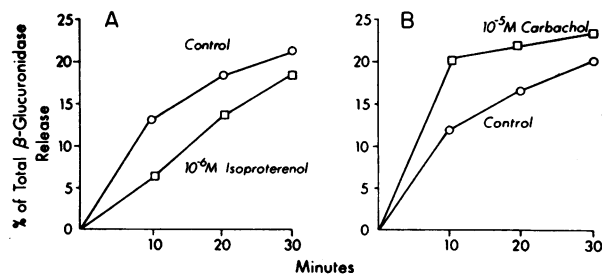


FIGURE 3 Kinetics of enzyme release from CB-treated human leukocytes: regulation by autonomic agonists. Leukocytes ( $4 \times 10^6$ ) incubated 5 min with  $5 \mu\text{g}/\text{ml}$  CB then 15 min with isoproterenol (Fig. 3A), or 3 min with carbachol (Fig. 3B). Curves document reduction by isoproterenol and acceleration by carbachol of rate of  $\beta$ -glucuronidase release despite the similarity in cumulative release at 30 min in control and treated cells.

TABLE I  
Pharmacological Regulation by Autonomic Agonists of Lysosomal Enzyme Release  
from Human Leukocytes\*

Compound (agonist $\pm$ antagonist)	Enzyme release		
	$\beta$ -Glucuronidase		LDH percent total $\ddagger$
	Percent total $\ddagger$	Percent ( $\pm$ ) change	
Resting	1.1 $\pm$ 0.6	—	0.7 $\pm$ 0.4
Stimulation (zymosan)	24.5 $\pm$ 1.7	—	1.1 $\pm$ 0.7
<i>(<math>\beta</math>-Adrenergic)</i>			
Isoproterenol	10.8 $\pm$ 1.0	-56.5	1.4 $\pm$ 0.7
Isoproterenol + propranolol ( $10^{-6}$ M)	24.0 $\pm$ 1.9	0	1.7 $\pm$ 1.0
Epinephrine ( $5 \times 10^{-6}$ M)	14.7 $\pm$ 0.9	-40.0	1.7 $\pm$ 0.9
Epinephrine + propranolol ( $10^{-6}$ M)	22.3 $\pm$ 1.1	0	1.8 $\pm$ 0.7
<i>(<math>\alpha</math>-Adrenergic)</i>			
Phenylephrine	20.8 $\pm$ 1.4	-15.1	1.9 $\pm$ 1.1
Phenylephrine + propranolol ( $10^{-6}$ M)	23.9 $\pm$ 1.3	0	2.4 $\pm$ 0.9
<i>(Cholinergic)</i>			
Carbachol	34.9 $\pm$ 2.6	+42.6	2.1 $\pm$ 0.7
Carbachol + atropine ( $10^{-6}$ M)	23.9 $\pm$ 2.1	0	2.0 $\pm$ 1.1
cGMP	35.6 $\pm$ 3.0	+45.2	1.7 $\pm$ 1.0

\* All incubations at 37°C. Leukocytes ( $4 \times 10^6$ ) pretreated with CB ( $5 \mu\text{g/ml} \times 10 \text{ min}$ ). Agonists present at  $5 \times 10^{-6}$  M. Cells exposed to zymosan (particle:cell ratio:30:1)  $\times 30 \text{ min}$ . Values = mean  $\pm$  SEM.  $n = 4$ .

$\ddagger$  Expressed as percent of total (100%) activity released by 0.2% Triton X-100; 100%  $\beta$ -glucuronidase =  $26.4 \pm 1.6 \mu\text{g phenolphthalein}/4 \times 10^6 \text{ leukocytes/h}$ ; 100% LDH =  $1718 \pm 101 \text{ AU}/4 \times 10^6 \text{ leukocytes}$ .

nificant effects on lysosomal enzyme release. At the higher concentrations, phenylephrine displayed only its  $\beta$ -adrenergic effect. Addition of propranolol with the  $\alpha$ -adrenergic agents did not significantly alter enzyme release.

*Effect of exogenous cyclic nucleotides on enzyme release.* Incubation of cells with cGMP, the presumed mediator of cholinergic stimulation (22), before challenge by zymosan, resulted in augmented  $\beta$ -glucuronidase release (Table I). In contrast, incubation of leukocytes with exogenous cAMP, with its dibutyryl analogue, or with compounds which augment endogenous cAMP concentrations in mixed leukocyte preparations, consistently reduced lysosomal enzyme release from CB-treated cells exposed to zymosan (Table II).

*Studies with purified PMN.* Experiments with purified (98 $\pm$ 1%) preparations of PMN obtained from Hypaque/Ficoll gradients indicated that these cells were capable of selective enzyme extrusion; this could be reduced by addition to cells of agents known to increase endogenous cAMP (Table II). Whereas reduction of zymosan induced enzyme release by dibutyryl cAMP and PGE<sub>1</sub> was comparable in PMN and mixed leukocyte preparations, the effects of histamine, isoproterenol, and

cholera enterotoxin were less marked in PMN than in mixed leukocyte suspensions.

*Measurements of cAMP and cGMP in PMN.* It has been demonstrated (35, 36) that isoproterenol and PGE<sub>1</sub>

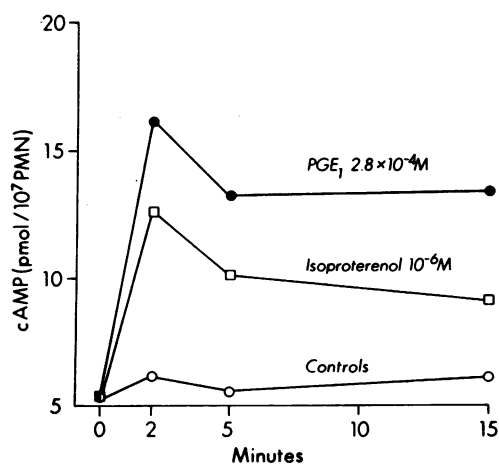


FIGURE 4 Stimulation of cAMP in suspensions of Hypaque/Ficoll-purified human PMN. PMN ( $10^7$ ) incubated at 37°C with  $5 \times 10^{-4}$  M theophylline and with or without (controls) test compounds. Each point represents the mean of three experiments.

TABLE II  
Pharmacological Regulation of Lysosomal Enzyme Release from Human Leukocytes\*

Compound	Mixed leukocyte suspensions			Purified PMN preparations (Hypaque/Ficoll)		
	$\beta$ -Glucuronidase		LDH Percent total‡	$\beta$ -Glucuronidase		LDH, Percent total‡
	Percent total*	Percent change		Percent total‡	Percent change	
Resting	2.9±1.1	—	2.8±0.8	3.6	—	4.0
Stimulation (zymosan)	23.4±2.0	—	3.7±1.0	26.2	—	2.0
Theophylline, $5 \times 10^{-4}$ M	24.1±1.2	0	2.4±1.1	25.4	0	1.6
cAMP, $10^{-3}$ M*	16.6±1.4	-28.7	1.9±0.6	ND	ND	ND
dibutyryl cAMP, $10^{-3}$ M*	14.1±2.1	-39.6	2.3±1.0	16.0	-38.8	5.5
PGE <sub>1</sub> , $2.8 \times 10^{-4}$ M§	13.4±1.1	-43.7	2.6±1.2	14.0	-46.7	3.4
Histamine, $10^{-4}$ M*	18.8±1.6	-39.4	2.8±1.0	18.5	-29.4	2.6
Isoproterenol, $10^{-6}$ M	12.6±1.9	-46.2	1.7±0.9	19.0	-27.4	2.4
Cholera enterotoxin, 10 ng	7.7±2.0	-67.1	2.9±1.3	14.5	-44.6	3.9

\* All incubations at 37°C. Leukocytes ( $4 \times 10^6$ ) pretreated with CB (5  $\mu$ g/ml, 5 min), incubated with compounds 15 min then exposed to zymosan for 30 min. Theophylline ( $5 \times 10^{-4}$  M) in all tubes.

‡ Expressed as percent of total activity (100%) released by 0.2% Triton X-100.

§ 30 min incubation.

|| 60 min incubation.

ND = Not done; values = Mean  $\pm$  SEM,  $n = 4$ ; purified PMN preparations: mean of two experiments.

stimulate greater accumulations of cAMP in lymphocytes and mixed leukocytes than in PMN. We have confirmed these findings and have observed that preparations of purified PMN accumulated cAMP in response to isoproterenol and PGE<sub>1</sub> maximally within the first 5 min (Fig. 4). In addition, the rapid accumulation and/or release of cAMP which followed exposure of CB-treated

PMN to zymosan was enhanced and sustained when these cells were preincubated with PGE<sub>1</sub>, isoproterenol, histamine, or cholera enterotoxin (Table III). These compounds simultaneously reduced zymosan-induced release of  $\beta$ -glucuronidase.

In contrast, PGF<sub>2a</sub>, which did not increase cAMP levels significantly, did not reduce zymosan-induced

TABLE III  
Pharmacological Regulation of Lysosomal Enzyme Release from Human PMN\*

	n	Preincubation min	cAMP		$\beta$ -Glucuronidase release, percent inhibition
			Before zymosan	20 min	
				after zymosan	
			pmol/10 <sup>7</sup> cells		
Control (no drug)	5	—	5.4	6.8	—
PGE <sub>1</sub> , $2.8 \times 10^{-4}$ M	5	30	18.0	37.0	36.8
PGE <sub>1</sub> , $2.8 \times 10^{-6}$ M	2	30	11.1	16.3	19.5
PGF <sub>2a</sub> , $2.8 \times 10^{-6}$ M	3	30	8.4	10.2	0
Cholera enterotoxin, 1 ng	2	60	11.7	19.9	26.8
Cholera enterotoxin, 10 ng	3	60	16.0	85.0	49.9
Cholera enterotoxin, 100 ng	1	60	32.8	ND	ND
Histamine, $10^{-4}$ M	3	15	12.9	26.1	30.0
Isoproterenol, $10^{-6}$ M	4	15	14.4	23.8	27.9
Theophylline, $5 \times 10^{-4}$ M	5	30	6.1	8.9	0

\* All incubations at 37°C. Hypaque/Ficoll-purified PMN ( $4 \times 10^6$ ) pretreated with CB (5  $\mu$ g/ml  $\times$  5 min), incubated with compounds, then exposed to zymosan for 20 min. Theophylline ( $5 \times 10^{-4}$  M) present in all experiments.

Values are means for number of experiments noted.

ND = Not done.

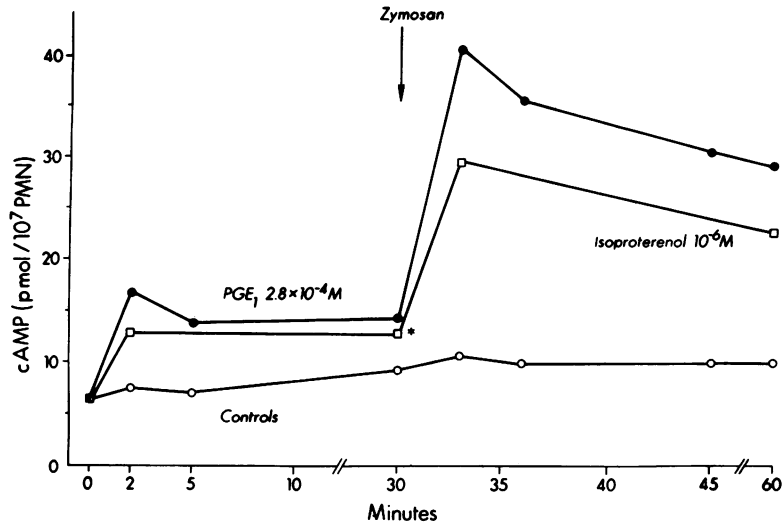


FIGURE 5 Stimulation of cAMP in suspensions of Hypaque/Ficoll-purified human PMN. PMN ( $10^7$ ) incubated at  $37^\circ\text{C}$  with  $5\ \mu\text{g/ml}$  CB plus  $5 \times 10^{-4}\ \text{M}$  theophylline and with or without (controls)  $\text{PGE}_1$  or isoproterenol and then exposed to zymosan. Each point represents the mean of three experiments. (\*) Cells were incubated 30 min with  $\text{PGE}_1$  and 15 min with isoproterenol before their exposure to zymosan.

$\beta$ -glucuronidase release. (Concentrations of  $\text{PGF}_{2\alpha}$  greater than  $2.8 \times 10^{-6}\ \text{M}$ , cause enzyme release by virtue of cell damage [3]). The cAMP response of CB-treated human PMN to  $2.8 \times 10^{-4}\ \text{M}$   $\text{PGE}_1$  and  $10^{-6}\ \text{M}$  isoproterenol is shown in Fig. 5.

The cGMP response of human PMN to carbachol also appeared to be maximal within the first 5 min of incubation. An experiment with two concentrations of carbachol is shown in Fig. 6. Carbachol enhanced and maintained cGMP concentration at significantly higher levels than in suspensions of untreated control cells. Carbachol ( $10^{-6}\ \text{M}$ ) did not stimulate accumulation of

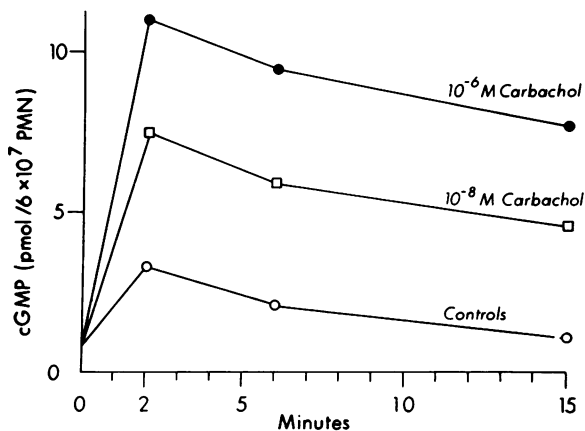


FIGURE 6 Stimulation of cGMP in suspensions of human PMN. PMN ( $6 \times 10^7$ ) incubated at  $37^\circ\text{C}$  with and without (controls) carbachol ( $10^{-6}$  and  $10^{-8}\ \text{M}$ ) for times indicated.

*Effects of  $\text{D}_2\text{O}$  and colchicine on enzyme release.* Cells were incubated with or without colchicine ( $5 \times 10^{-4}\ \text{M}$ ) for 60 min at  $37^\circ\text{C}$ , washed, and resuspended in medium containing 0 or 50%  $\text{D}_2\text{O}$ . Cytochalasin B ( $5\ \mu\text{g/ml}$ ) and autologous serum (10% vol/vol) were added, cells were incubated for 10 min and then exposed to zymosan (particle:cell ratio 30:1) for 30 min at

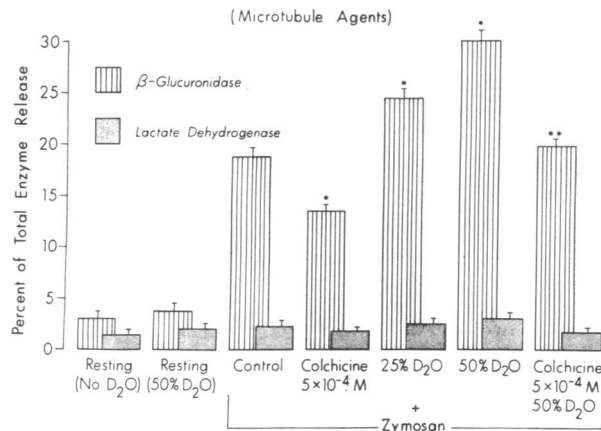
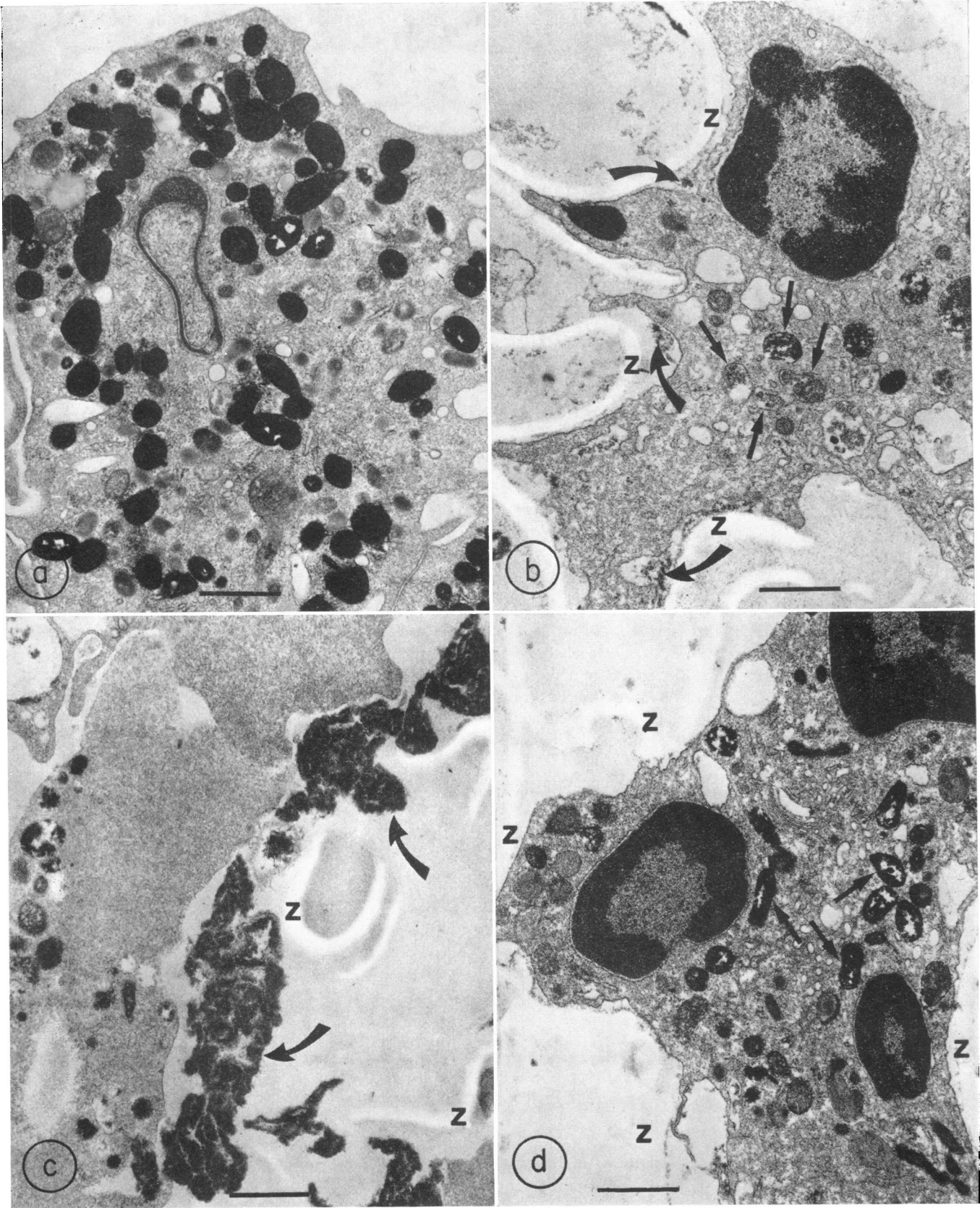


FIGURE 7 Lysosomal enzyme release from CB-treated cells. Human leukocytes ( $4 \times 10^6$ ) incubated 10 min with  $5\ \mu\text{g/ml}$  CB, incubated 60 min with or without colchicine in appropriate buffer ( $\pm\text{D}_2\text{O}$ ), and exposed to zymosan 30 min. Total (100%) activity (released by 0.2% Triton X-100) for  $\beta$ -glucuronidase =  $22.8 \pm 2.1\ \mu\text{g}$  phenolphthalein/h/4  $\times 10^6$  cells. Total activity LDH =  $462 \pm 51\ \text{AU}/4 \times 10^6$  cells. (\*) Significant at  $P < 0.01$  vs. control. (\*\*) Significant at  $P < 0.01$  vs. 50%  $\text{D}_2\text{O}$ .





37°C. D<sub>2</sub>O alone did not induce enzyme release in unstimulated cells. Colchicine, as previously reported (8, 12), reduced  $\beta$ -glucuronidase release, whereas D<sub>2</sub>O enhanced release. In the presence of both D<sub>2</sub>O and colchicine, release was intermediate between that induced by either agent alone (Fig. 7).

**Morphology of enzyme secretion.** Cells incubated with CB, then fixed and stained for endogenous peroxidase, showed many dense, peroxidase-positive, azurophilic granules as well as peroxidase negative specific granules scattered throughout the cytoplasm (Fig. 8a). Whereas large organelles such as the nucleus, centrioles, mitochondria, and granules could be seen, fine structures of the cytoplasm such as microtubules, microfilaments, or glycogen granules were not visualized by the cytochemical procedure used specifically to stain myeloperoxidase-containing azurophils (25).

When CB-treated cells were exposed to zymosan for 30 min and subsequently fixed and stained for peroxidase, degranulation to the plasma membrane could be demonstrated morphologically (Fig. 8b). Peroxidase-positive granules were neither so numerous nor as densely stained in the cell interior when compared with resting cells. Indeed, peroxidase-positive deposits could be seen at the cell-zymosan interface (Fig. 8b).

Cells incubated with carbachol (10<sup>-6</sup> M) as well as with CB, before exposure to zymosan, underwent extensive degranulation. Large, dense masses of peroxidase-positive material were observed to lie between the cells and the adherent zymosan particles, whereas relatively few lysosomes remained intact in the cell cytoplasm. Furthermore, in cells treated with carbachol and CB, fewer lysosomal residua remained visible in the cell interior. Large areas of featureless cytoplasm appeared which were free of granules and vacuoles (Fig. 8c). Similar extensive degranulation was observed when CB-treated PMN were incubated with cGMP before their exposure to zymosan (not shown). In contrast, when PMN were incubated with PGE<sub>1</sub> (not shown), isoproterenol

TABLE IV  
*Controls of Lysosomal Enzyme Secretion from Human PMN*

Control	Secretion↓	Secretion↑
(a) Autonomic agonist	$\beta$ -Adrenergic	Cholinergic
(b) Agonist blocked by	Propranolol	Atropine
(c) Exogenous cyclic nucleotide	cAMP	cGMP
(d) Endogenous cyclic nucleotide accumulated	cAMP	cGMP
(e) Enzyme activated by cyclic nucleotide	Protein kinase	Unknown
(f) State of microtubules	Disassembled = colchicine, vinblastine effect	Assembled D <sub>2</sub> O effect

(Fig. 8d), or cAMP (not shown) before exposure to zymosan, degranulation was retarded and the cells were intermediate in appearance between resting cells and untreated cells exposed to zymosan. This inhibition of degranulation has been quantitated and is presented in detail elsewhere (37).

## DISCUSSION

It has been demonstrated (6-8, 38, 39) that the CB-treated leukocyte resembles a secretory cell in which the influence of various compounds on membrane fusion and enzyme extrusion may be measured directly. These cells, upon contact with a phagocytic stimulus (e.g., zymosan), selectively merge lysosomes with the plasma membrane as if the latter were a phagocytic vacuole. Selective secretion of lysosomal materials after this merger occurs in the absence of cell death and despite absence of particle ingestion. The results of experiments presented in this communication suggest that the secretion of lysosomal hydrolases from human PMN can be modified by agents which affect cyclic nucleotides and microtubules. Control of lysosomal enzyme release, summarized in Table IV, may be compared with the regulation of release of mediators of inflammation and secretion of exportable proteins from other cell types:

FIGURE 8 (a) A human peripheral blood neutrophil treated with CB (5  $\mu$ g/ml) for 5 min then fixed and stained for endogenous peroxidase. Numerous black, peroxidase-positive azurophilic granules as well as many peroxidase-negative specific granules can be seen in the cytoplasm of this cell. Magnification  $\times$  14,500. Line equals 1  $\mu$ m. (b) Another neutrophil, pretreated with CB and then exposed to zymosan (Z) for 30 min before fixing and staining for peroxidase. Only two intact azurophilic granules are visible in this cell although many lysosomal residua can be seen with some peroxidase-positive material persisting within them (arrows). Some peroxidase-positive material can also be seen at the cell-zymosan interface (curved arrows). Magnification  $\times$  14,500. Line equals 1  $\mu$ m. (c) A neutrophil pretreated with CB, incubated (37°C) with 10<sup>-6</sup> M carbachol for 3 min, and exposed to zymosan for 30 min. Masses of peroxidase-positive material are visible (arrows) between the cell surface and the zymosan (Z) with large expanses of featureless cytoplasm adjacent to these masses. Magnification  $\times$  14,500. Line equals 1  $\mu$ m. (d) CB-treated neutrophil incubated (37°C) 15 min with 10<sup>-6</sup> M isoproterenol and 5  $\times$  10<sup>-4</sup> M theophylline, then exposed to zymosan for 30 min. Although four zymosan particles are seen in contact with the cell, many peroxidase-positive azurophilic granules and peroxidase-negative specific granules, remain in the cytoplasm. Magnification  $\times$  14,500. Line equals 1  $\mu$ m.

(a)  $\beta$ -Adrenergic catecholamines reduce selective extrusion of lysosomal enzymes from human PMN exposed to zymosan. Similarly,  $\beta$ -adrenergic stimulation reduces immunologically induced histamine release from sensitized basophils and lung tissue (14, 16, 17). In contrast, the cholinergic agonist carbachol enhances lysosomal enzyme release from leukocytes, augments histamine and SRS-A release from lung fragments (18, 22), evokes secretion of lysosomal acid hydrolases and catecholamines from the bovine adrenal gland (40), and induces gastric secretion (41) and mucin secretion from submaxillary gland (42).

(b) Appropriate blockade of neurohumoral receptors (by propranolol or atropine) inhibits actions of the autonomic agonists upon lysosomal enzyme extrusion from human leukocytes. Release of histamine and SRS-A from human lung fragments is similarly controlled (16, 17, 22).

(c) Human leukocytes possess specific and separate receptors for endogenous hormones including  $\beta$ -adrenergic catecholamines, histamine, and prostaglandins (43). Each of these agents stimulates accumulation of cAMP in suspensions of mixed human leukocytes (15, 44, 46). Our experiments demonstrate that each of these agents, as well as exogenous cAMP (and its dibutyryl analogue), added directly to leukocyte suspensions, increases cAMP levels and reduces selective extrusion of lysosomal enzymes from human leukocytes exposed to zymosan. Consequently, it is reasonable to suppose that it is accumulation of cAMP which causes inhibition of enzyme secretion. Similarly, exogenously added cAMP inhibits the release of lymphocytotoxins (47), histamine, and SRS-A (16, 17). Although  $\beta$ -adrenergic agents, prostaglandin  $E_1$ , histamine, and cholera enterotoxin cause accumulation of cAMP in neutrophil suspensions, far greater increments of cAMP are found in mononuclear fractions (35, 36). Moreover, when neutrophils were separated from mixed suspensions, after incubation with isoproterenol, it was not possible to demonstrate increments in cAMP (36) within cells.

In the experiments we report, as well as in those of others (35), small but significant increments of cAMP were detected after stimulation by isoproterenol. In such experiments, the measured cyclic nucleotide represents the sum of intracellular cAMP and that released into the medium. Since cAMP leaks from stimulated cells (48), it is important not only to measure intracellular, but also extracellular cAMP.

Our experiments with purified preparations of PMN indicate that isoproterenol,  $PGE_1$ , histamine, and cholera enterotoxin act to increase cAMP and to inhibit release of lysosomal enzymes in the absence of mononuclear cells. However, reduction of enzyme release is usually more marked in suspensions of mixed leukocytes than

in suspensions of purified PMN. This would suggest that mononuclear cells, perhaps by releasing their cAMP, may further reduce enzyme release from PMN. It will be necessary to define more precisely the nature and extent of intercellular cAMP fluxes in this system.

(d) After incubation with polystyrene latex beads for 5 min the cAMP content of human peripheral blood leukocytes is increased severalfold (49). This increase, however, has been held to be due to the accumulation of the cyclic nucleotide mainly in mononuclear cells (50). CB-treated human PMN exposed to zymosan respond with only modest increments in cAMP. However, when the cells are incubated with compounds which produce these modest increases in cAMP levels and are then exposed to zymosan, the cAMP burst is enhanced and sustained. The reason for this is not apparent. It is possible that adenylyl cyclase stimulated by zymosan contact with the plasma membrane acts synergistically with adenylyl cyclase stimulated by the test compound to produce the striking elevation in cAMP. Additionally, or alternatively, the test compound may interfere with the mechanisms responsible for regulating the return of cAMP to basal levels after zymosan contact.

Exogenous cGMP enhances secretion of lysosomal hydrolases from human leukocytes, results which are concordant with studies in which the introduction of 8-bromo-cGMP to sensitized lung tissue was associated with enhancement of antigen-induced release of histamine and SRS-A. There is evidence, in other tissues, that cholinergic agents stimulate the accumulation of endogenous cGMP (22, 23). Similarly, the results of the studies presented here indicate that the cholinergic agonist carbachol increases the cGMP level in suspensions of human PMN and increases zymosan-induced release of lysosomal enzymes. This reciprocal relationship between cGMP and cAMP, whether exogenously added or endogenously accumulated after appropriate stimulation, has been observed in other biological systems. Thus, exogenous cAMP inhibits the uptake of uridine, leucine, and 2-deoxyglucose by cultured mouse fibroblasts, an effect counteracted by cGMP (51). In addition, exogenous cAMP as well as agents that elevate intracellular concentrations of cAMP, prevent the transformation of lymphocytes by phytohemagglutinin (52, 53), and prevent the cytotoxic actions of lymphocytes upon cells bearing alloantigens to which they are sensitized (47), whereas cholinergic agents enhance the cytotoxicity of lymphocytes (54). Indeed, mitogenic concentrations of phytohemagglutinin produced 10 to 50-fold increments in the concentration of lymphocyte cGMP whereas lymphocyte cAMP was not elevated (55).

(e) A unique protein kinase, active upon histone, has been isolated from purified human PMN (56). This enzyme phosphorylates a number of substrates when ex-

posed to cAMP and cyclic inosine 5'-monophosphate (cIMP) ( $> 10^{-8}$  M) but not to cGMP. At present no enzyme has been isolated, the activity of which is uniquely susceptible to stimulation of cGMP.

(f)  $D_2O$  favors the assembly of microtubules (18, 19) and enhanced the selective extrusion of lysosomal enzymes from human leukocytes.  $D_2O$  also favors the secretion of histamine from appropriately sensitized cells and appears to do so by virtue of an effect upon microtubule assembly as evidenced by the fact that colchicine, in histamine-rich cells, seems to reverse the effect of  $D_2O$  (20, 21). Colchicine, which binds to tubulin subunits (57), leads to disassembly of microtubules and reduces selective enzyme release. Moreover, prior incubation of cells with colchicine reduced the potentiating effect of  $D_2O$ , suggesting that granule movement and hydrolase release both depend upon intact microtubules. Similarly, vinblastine, which causes the disassembly of microtubules by forming dense precipitates (58), also inhibits release of lysosomal enzyme (8). Louie and Dixon (59) have shown that phosphorylation of histone, by introducing surface-negative charges, promotes disassembly of histones: such experiments provide a precedent for disassembly of tubulin after protein kinase-mediated phosphorylation.

It is therefore reasonable to suspect that each of these agents acts at the same, final, site to prevent or enhance the secretion of previously stored lysosomal hydrolases to the outside. Our ultrastructural studies have demonstrated that appearance of lysosomal enzymes in the supernate appears to be due to the direct secretion of the contents of lysosomes from a granule that has merged with the plasma membrane as if it were a phagocytic vacuole. Consequently, one explanation for our findings is that the cyclic nucleotides, cAMP and cGMP, effect the state of assembly of microtubules in PMN and that their state of assembly regulates the flow of lysosomes and the secretion of enzymes to the outside. This, of course, need not be the only site at which these agents act. The reciprocal relationships between cAMP and cGMP can be demonstrated not only when these agents are added exogenously, but also when compounds which influence their endogenous accumulation are exhibited.

Only studies in which it is not unlikely that inactive cholera enterotoxin was employed (35) appear to contradict the hypothesis presented above. We have now shown that freshly prepared enterotoxin protected by serum protein from inactivation stimulates accumulation of cAMP and inhibits hydrolase release. However, the results presented in this paper have been obtained with CB-treated cells and cannot be unequivocally demonstrated in cells not treated with this agent. The explanation may lie in the fact that, in cells not treated with CB, lysosomes merge with phagocytic vacuoles in the

interior of the cell and enzyme release to the periphery only occurs by the process of "regurgitation during feeding" (3). Thus, direct quantitation of fusion is not afforded by measurements of lysosomal hydrolases in the suspending medium. The bulk of these enter phagocytic vacuoles intracellularly and drug effects may indicate actions upon phagocytosis per se and not upon the fusion process. It is only in cells in which phagocytosis is completely inhibited by CB that it becomes possible to quantitatively monitor this intracellular event by measurements of the extracellular release of lysosomal hydrolases. However, our previous studies (37) have indicated that intracellular degranulation (measured morphometrically) is inhibited by exogenous cAMP and agents which elevate endogenous cAMP. Moreover, preliminary studies indicate that carbachol, which stimulates endogenous cGMP causes enhanced intracellular degranulation in cells not treated with CB.

#### ACKNOWLEDGMENTS

The authors thank Ms. Nancy Oakman and Mr. Joel Ross for their expert technical assistance.

This study was aided by grants from the National Institutes of Health (AM-11949 and HL-15140), National Institutes of Health Special Fellowship (AM-50489), Population Council of New York (M-72.120), The New York Heart Association, and The Whitehall Foundation.

#### REFERENCES

1. Metchnikoff, E. 1905. *Immunity in Infective Diseases*. Cambridge University Press, London. 540.
2. Weissmann, G., P. Dukor, and R. B. Zurier. 1971. Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nat. New Biol.* 231: 131.
3. Weissmann, G., R. B. Zurier, P. J. Spieler, and I. M. Goldstein. 1971. Mechanisms of lysosomal enzyme release from leucocytes exposed to immune complexes and other particles. *J. Exp. Med.* 134: 149s.
4. Henson, P. 1971. The immunological release of constituents from neutrophil leucocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *J. Immunol.* 107: 1535.
5. Hawkins, D., and S. Peeters. 1971. The response of polymorphonuclear leukocytes to immune complexes *in vitro*. *Lab. Invest.* 24: 483.
6. Weissmann, G., R. B. Zurier, and S. Hoffstein. 1972. Leucocytic proteases and the immunologic release of lysosomal enzymes. *Am. J. Pathol.* 68: 539.
7. Henson, P. 1972. Pathologic mechanisms in neutrophil-mediated injury. *Am. J. Pathol.* 68: 593.
8. Zurier, R. B., S. Hoffstein, and G. Weissmann. 1973. Cytochalasin B: Effect on lysosomal enzyme release from human leucocytes. *Proc. Natl. Acad. Sci. U. S. A.* 70: 844.
9. Weissmann, G., and P. Dukor. 1970. The role of lysosomes in immune responses. *Adv. Immunol.* 12: 283.
10. Weisenberg, R. C., C. G. Borisy, and E. W. Taylor. 1968. The colchicine-binding protein of mammalian brain and its relation to microtubules. *Biochemistry.* 7: 4466.

11. Gillespie, E. 1971. Colchicine binding in tissue slices. Decrease by calcium and biphasic effect of adenosine 3'5'-monophosphate. *J. Cell Biol.* **50**: 544.
12. Zurier, R. B., S. Hoffstein, and G. Weissmann. 1973. Mechanisms of lysosomal enzyme release from human leucocytes. I. Effects of cyclic nucleotides and colchicine. *J. Cell Biol.* **58**: 27.
13. Wright, D. G., and S. E. Malawista. 1971. The mobilization and extracellular release of granular enzymes from phagocytosing human leucocytes; inhibition by colchicine and cortisol but not salicylate. *Arthritis Rheum.* **14**: 425. (Abstr.)
14. Lichtenstein, L. M., and S. Margolis. 1968. Histamine release *in vitro*: inhibition by catecholamines and methylxanthines. *Science (Wash. D. C.)*. **161**: 902.
15. 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.
16. Orange, R. P., M. A. Kaliner, P. J. Laraia, and K. F. Austen. 1971. Immunological release of histamine and slow reacting substance of anaphylaxis from human lung. II. Influence of cellular levels of cyclic AMP. *Fed. Proc.* **30**: 1725.
17. Orange, R. P., W. G. Austen, and K. F. Austen. 1971. Immunological release of histamine and slow-reacting substance of anaphylaxis from human lung. I. Modulation by agents influencing cellular levels of cyclic 3'5'-adenosine monophosphate. *J. Exp. Med.* **134**: 136s.
18. Sato, H., S. Inoué, J. Bryan, N. E. Barclay, and C. Platt. 1966. The effect of D<sub>2</sub>O on the mitotic spindle. *Biol. Bull.* **131**: 405. (Abstr.)
19. Marsland, D., L. G. Tilney, and M. Hirshfield. 1971. Stabilizing effects of D<sub>2</sub>O on the microtubular components and needle-like form of heliozoan axopods—a pressure-temperature analysis. *J. Cell. Physiol.* **77**: 187.
20. Gillespie, E., and L. M. Lichtenstein. 1972. Heavy water enhances IgE-mediated histamine release from human leukocytes: evidence for microtubule involvement. *Proc. Soc. Exp. Biol. Med.* **140**: 1228.
21. Gillespie, E., and L. M. Lichtenstein. 1972. Histamine release from human leucocytes. Studies with deuterium oxide, colchicine and cytochalasin B. *J. Clin. Invest.* **51**: 2941.
22. Kaliner, M., R. P. Orange, and K. F. Austen. 1972. Immunologic release of histamine and slow reacting substance of anaphylaxis from human lung. IV. Enhancement by cholinergic and alpha adrenergic stimulation. *J. Exp. Med.* **136**: 556.
23. Kuo, J-F., T-P. Lee, P. L. Reyes, K. G. Walton, T. E. Donnelly, Jr., and P. Greengard. 1972. Cyclic nucleotide dependent protein kinases. X. An assay method of the measurements of guanosine 3'5'-monophosphate in various biological materials and a study of agents regulating its level in heart and brain. *J. Biol. Chem.* **247**: 16.
24. Böyum, A. 1968. Isolation of leucocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97): 9.
25. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**: 291.
26. Brittinger, G., R. Hirschhorn, S. D. Douglas, and G. Weissmann, 1968. Studies on lysosomes. XI. Characterization of a hydrolase-rich fraction from human lymphocytes. *J. Cell Biol.* **37**: 394.
27. Wacker, W. E. C., D. D. Ulmer, and B. L. Vallee. 1956. Metalloenzymes and myocardial infarction. II. Malic and lactic dehydrogenase activities and zinc concentration in serum. *N. Engl. J. Med.* **255**: 449.
28. Gilman, A. G. 1970. A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 305.
29. Miyamoto, E., J. F. Kuo, and P. Greengard. 1969. Cyclic nucleotide-dependent protein kinases. III. Purification and properties of adenosine 3',5'-monophosphate-dependent protein kinase from bovine brain. *J. Biol. Chem.* **244**: 6395.
30. Mashiter, K., G. D. Mashiter, R. L. Hauger, and J. B. Field. 1973. Effects of cholera and *E. coli* enterotoxins on cyclic adenosine 3'5'-monophosphate levels and intermediary metabolism in the thyroid. *Endocrinology.* **92**: 541.
31. Tsung, P.-K., and G. Weissmann. 1973. Inhibitor of adenosine 3'5'-monophosphate binding and protein kinase activity in leucocyte lysosomes. *Biochem. Biophys. Res. Commun.* **51**: 836.
32. Steiner, A. L., C. W. Parker, and D. M. Kipnis. 1970. The measurement of cyclic nucleotides by radioimmunoassay. In *Role of cyclic AMP in Cell Function*. P. Greengard and E. Costa, editors. Raven Press, New York. 89.
33. Burrows, W. 1970. Toward an effective prophylactic immunity to cholera. *J. Infect. Dis.* **121**(Suppl.): S58.
34. Finkelstein, R. A., K. Fujita, and J. J. LoSpalluto. 1971. Procholeragenoid: an aggregated intermediate in the formation of cholera toxin. *J. Immunol.* **107**: 1043.
35. Bourne, H. R., R. I. Lehrer, L. M. Lichtenstein, G. Weissmann, and R. B. Zurier. 1973. Effects of cholera enterotoxin on adenosine 3',5'-monophosphate and neutrophil function. Comparison with other compounds which stimulate leucocyte adenyl cyclase. *J. Clin. Invest.* **52**: 698.
36. Parker, C. W., and J. W. Smith. 1973. Alterations in cyclic adenosine monophosphate metabolism in human bronchial asthma. I. Leukocyte responsiveness to  $\beta$ -adrenergic agents. *J. Clin. Invest.* **52**: 48.
37. Hoffstein, S., R. B. Zurier, and G. Weissmann. 1974. Mechanisms of lysosomal enzyme release from human leucocytes. III. Factors influencing fusion with phagocytic vacuoles and the plasma membrane. Submitted for publication.
38. Henson, P. M., and Z. G. Oades. 1973. Enhancement of immunologically induced granule exocytosis from neutrophils by cytochalasin B. *J. Immunol.* **110**: 290.
39. Hawkins, D. 1973. Neutrophilic leucocytes in immunologic reactions *in vitro*: effect of cytochalasin B. *J. Immunol.* **110**: 294.
40. Schneider, F. H. 1970. Secretion from the bovine adrenal gland. Release of lysosomal enzymes. *Biochem. Pharmacol.* **19**: 833.
41. Amure, B. O., and A. A. Omole. 1970. Sex hormones and acid gastric secretion induced with carbachol histamine and gastrin. *Gut.* **11**: 641.
42. Rossignol, B., G. Herman, and G. Keryer. 1972. Inhibition by colchicine of carbamylcholine induced glycoprotein secretion by the submaxillary gland. A possible mechanism of cholinergic induced protein secretion. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **21**: 189.
43. Melmon, K. L., H. R. Bourne, J. Weinstein, and M. Sela. 1972. Receptors for histamine can be detected on

- the surface of selected leucocytes. *Science (Wash. D. C.)*. 177: 707.
44. Scott, R. E. 1970. Effects of prostaglandins, epinephrine, and NaF on human leucocyte, platelet and liver adenylyl cyclase. *Blood*. 35: 514.
  45. Bourne, H. R., and K. L. Melmon. 1971. Adenylyl cyclase in human leucocytes: evidence for activation by separate beta adrenergic and prostaglandin receptors. *J. Pharmacol. Exp. Ther.* 178: 1.
  46. Bourne, H. R., R. I. Lehrer, M. J. Cline, and K. L. Melmon. 1971. Cyclic 3',5'-adenosine monophosphate in the human leucocyte: synthesis, degradation and effects on neutrophil candidacidal activity. *J. Clin. Invest.* 50: 920.
  47. 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.
  48. Robison, G. A., R. W. Butcher, and E. W. Sutherland. 1971. *In Cyclic AMP*. Academic Press, Inc., New York. 418.
  49. Park, B. H., R. A. Good, N. P. Beck, and A. B. Davis. 1971. Concentration of cyclic adenosine 3',5'-monophosphate in human leucocytes during phagocytosis. *Nature (Lond.)*. 229: 27.
  50. Mangianello, 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 leucocytes. *J. Clin. Invest.* 50: 2741.
  51. Kram, R., and G. M. Tomkins. 1973. Pleiotypic control by cyclic AMP. Interaction with cyclic GMP and possible role of microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 70: 1659.
  52. Hirschhorn, R., J. Grossman, and G. Weissmann. 1970. Effect of cyclic 3',5'-adenosine monophosphate and theophylline on lymphocyte transformation. *Proc. Soc. Exp. Biol. Med.* 133: 1361.
  53. 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.
  54. Strom, T. B., A. Deisseroth, J. Morganroth, C. B. Carpenter, and J. P. Merrill. 1972. Alteration of the cytotoxic action of sensitizer lymphocytes by cholinergic agents and activators of adenylyl cyclase. *Proc. Natl. Acad. Sci. U. S. A.* 69: 2995.
  55. Hadden, J. W., E. M. Hadden, M. K. Haddox, and N. D. Goldberg. 1972. Guanosine 3':5'-cyclic monophosphate: a possible intracellular mediator of mitogenic influences in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 69: 3024.
  56. Tsung, P.-K., N. Hermina, and G. Weissmann. 1972. Inosine 3',5'-monophosphate and adenosine 3',5'-monophosphate-dependent protein kinase from human PMN leucocytes. *Biochem. Biophys. Res. Commun.* 49: 1657.
  57. Borisy, G. G., and E. W. Taylor. 1967. The mechanism of action of colchicine. Binding of colchicine-<sup>3</sup>H to cellular protein. *J. Cell Biol.* 34: 525.
  58. Bensch, K. G., and S. E. Malawista. 1968. Microtubule crystals: a new biophysical phenomenon induced by Vinca alkaloids. *Nature (Lond.)*. 218: 1176.
  59. Louie, A. J., and G. H. Dixon. 1973. Kinetics of phosphorylation and dephosphorylation of testis histones and their possible role in determining chromosomal structure. *Nat. New Biol.* 243: 164.