RELEASE OF PROSTAGLANDINS BY POLYMORPHONUCLEAR LEUKOCYTES

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By

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ABSTRACT

The release of prostaglandins from rat polymorphonuclear leukocytes obtained from peritoneal exudates was studied. Prostaglandin release by nonphagocytizing cells was stimulated by the presence of exogenous arachidonic acid and dihomo-γ-linolenic acid, the prostaglandin precursors. Prostaglandins synthesized from exogenous precursors were not stored intracellularly.

Prostaglandin release was also stimulated during the phagocytosis of bacteria or zymosan particles, in the absence of added precursor, although the quantities released were less than when exogenous precursor was present. The release of prostaglandins by phagocytizing cells showed many similarities to release of lysosomal enzymes, particularly those originating in the azurophil granules. Cells rendered incapable of ingesting particles by treatment with cytochalasin B released prostaglandins and lysosomal enzymes when presented with a phagocytic stimulus. Both prostaglandin and β-glucuronidase release from cytochalasin B-treated cells were inhibited by dibutyryl adenosine 3':5'-cyclic monophosphate and by colchicine.

Resting cells treated with phorbol myristate acetate released lysozyme but did not release β-glucuronidase or prostaglandins. Both prostaglandins and lysosomal enzymes were released by the divalent cationic ionophore A23187 in the presence of Ca^{2+}. Maximum prostaglandin release occurred at 1 μM A23187 whereas lysosomal enzyme release occurred

in 2 stages with maxima at 1 μM and 5 μM A23187. In Ca^{2+}-free buffer the respiratory burst which normally accompanies phagocytosis could be stimulated independently of prostaglandin and lysosomal enzyme release by the cationic ionophore X537A. The release of prostaglandins during phagocytosis and by A23187 was not due to activation of prostaglandin synthetase, but appeared to be due to increased availability of prostaglandin precursors.

Preincubation of cells with exogenous prostaglandin precursors inhibited β-glucuronidase release when cells were subsequently exposed to a phagocytic stimulus. This effect was not due to prostaglandin biosynthesis but appeared to be a nonspecific fatty acid effect observed with other unsaturated fatty acids as well.

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LIST OF ABBREVIATIONS

ε-ACA	ε-aminocaproic acid
cAMP	adenosine 3':5'-cyclic monophosphate
CB	cytochalasin B
cGMP	guanosine 3':5'-cyclic monophosphate
GSH	glutathione in the reduced form
HBSS	Hank's balanced salt solution (enriched with 0.2% glucose)
HMS	hexose monophosphate shunt
IgG	immunoglobulin G
NADH	β-nicotinamide adenine dinucleotide in the reduced form
PG	prostaglandin
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
SEM	standard error of the mean

I INTRODUCTION

There is considerable evidence suggesting the involvement of prostaglandins (PGs) in inflammatory processes (Willis et al., 1972; Vane, 1973; Zurier, 1974). The injection or infusion of exogenous PGs produces symptoms of inflammation in a variety of tissues and PGs have been recovered from many types of inflammatory exudates at concentrations capable of producing these symptoms. In addition, nonsteroidal anti-inflammatory drugs have been shown to inhibit PG release (Vane, 1971; Smith and Willis, 1971; Ferriera, Moncada and Vane, 1971) and indeed it seems likely that this is the main mode of action of these drugs (reviewed by Vane, 1974). The source of PGs in inflammatory exudates and the stimulus for their release, however, remain matters of speculation. This is because PGs can be synthesized by virtually every type of animal cell and their release can be stimulated by any number of mechanical, hormonal or neurological means. Hence it is not clear whether local tissue cells, the numerous leukocytes which invade inflammatory sites, or a combination of both are responsible for PGs found in inflammatory exudates.

There is no simple model which can account for the sequence of events or the relative importance of the different mediators in all types of inflammation. One model which is widely used however is carrageenan-induced inflammation in the rat. Carrageenan is a

sulfated mucopolysaccharide which, when injected into the paw of a rat or into a subcutaneous air bleb raised on the back of a rat, elicits acute inflammatory responses. At least three distinct phases of mediator release were distinguished in this model (Willis, 1969; DiRosa et al., 1971a): an initial release of histamine and serotonin within the first hour after injection, a second phase mediated by kinins, and a third phase mediated by PGs and a second wave of histamine release. In carrageenan-induced paw edema PGs appeared 3-4 hours after injection and levels reached a maximum between 12 and 24 hours, at which time up to 80 ng of PG-like material per ml of exudate could be detected (Willis, 1969). Virtually all of the PG proved to be PGE_2 (Willis, 1970). In this model of inflammation, the PG phase was found to coincide with the migration of large numbers of polymorphonuclear leukocytes (PMNs) to the inflammatory site. A similar phenomenon was also observed in turpentine-induced pleurisy in the rat (DiRosa et al., 1971a) and in experimental uveitis (an occular inflammation) in rabbits (Eakins et al., 1972). In the latter case PG-like material was found in concentrations of up to 150 ng/ml of aqueous humour and proved to be almost exclusively PGE_1. Similarly, Higgs et al., (1974) found high levels of PGE_1 together with large numbers of PMNs in synovial fluids from patients with rheumatoid arthritis.

A number of relationships have been proposed to explain this coincidental appearance of PGs and PMNs at inflammatory sites. Kaley and Weiner (1971a and b) showed that PGE_1 was chemotactic for rabbit

peritoneal PMNs at concentrations as low as 1 μg/ml. It was suggested that the release of PGE_1 from inflamed tissues might serve as a signal to attract PMNs from the blood stream. Higgs et al., (1975), found that PGE_1 was chemotactic for rabbit PMNs at concentrations down to 10 ng/ml, well below the levels found in some inflammatory exudates. However there are a number of objections to the proposal that PGs play an important chemotactic role in vivo. The PG which is most commonly isolated from sites of acute inflammation is PGE_2 (Vane, 1974), but PGE_2 and $PGF_{2\alpha}$ have little or no chemotactic activity in vitro (Higgs et al., 1975). It appears that it is only in inflammatory states of longer duration such as in uveitis or rheumatoid arthritis that significant quantities of PGE_1 can be detected. Moreover PGE_1 is only moderately chemotactic at best. Kaley and Weiner (1971b) found that maximum chemotactic activity due to PGE_1 was only 10% of that due to activated serum. Furthermore, subcutaneous injection of PGE_1 in rats did not result in leukocyte invasion of the injected area (Arora et al., 1970). Diaz-Perez et al., (1974), while confirming that PGE_1 was moderately chemotactic for rabbit peritoneal PMNs, failed to find any chemotactic activity with human peripheral blood PMNs. A further argument against a chemotactic role for PGs is that treatment of carrageenan-injected rats with indomethacin to inhibit PG release had no effect on PMN migration to the inflammatory site (DiRosa et al., 1971b).

A second possible relationship between PMNs and PGs was proposed by Anderson et al. (1971). In the carrageenan air bleb system the appearance of PGs closely paralleled the appearance of the lysosomal

enzymes β-glucuronidase and acid phosphatase in the exudates. It was suggested that during the phagocytosis of carrageenan, PMNs inadvertently released lysosomal enzymes into the exudates and that phospholipases so released attacked membrane phospholipids releasing free fatty acids, including PG precursors. The availability of the precursors would then allow PG synthesis to take place since in most PG synthesizing systems substrate availability is the rate limiting factor, (Kunze and Bohn, 1969). There is considerable evidence that lysosomal enzymes are indeed released into the extracellular medium by phagocytizing PMNs, causing considerable tissue damage (Ignarro, 1974). Phospholipase A activity has been found in the lysosomes of PMNs (Elsbach and Rizack, 1963; Elsbach et al., 1965; Franson et al., 1974) and considerable acitivity was shown at physiological pH indicating that the enzyme(s) can function outside the acidic environment of the lysosome. Additional support for this hypothesis was provided by Whelan (1974) who showed that subcutaneous injection of a phospholipase A into rats produced an exudate containing high levels of PGE_2.

A third possible relationship between the appearance of PGs and PMNs is that PMNs themselves synthesize and release PGs into inflammatory exudates. This was first proposed by Movat et al., (1971), who showed that during phagocytosis _in vitro_ PMNs released a substance which could increase vascular permeability. On the basis of preliminary chemical characterization, the substance was proposed to be a PG. Eakins et al. (1972b) felt that in rabbit uveitis, PMNs were a likely source of PGE_1

because of their coincidental appearance and because PGE_1 could not be detected in the normal eye whereas levels of PGE_2 and $PGF_{2\alpha}$ were found.

Evidence that PMNs can indeed release PGs has come from the studies of Youlten and his coworkers (Higgs and Youlten, 1972; McCall and Youlten, 1973; Higgs et al., 1975) who showed that in vitro rabbit peritoneal PMNs phagocytizing bacteria released up to 30 ng of PG-like material after incubation for 3 hours. Sixty percent of the recoverable PG activity was PGE-like material, 25% PGF-like and the remainder unidentified. Of the PGE-like material, 39-80% behaved chromatographically like PGE_1. Resting cells or phagocytizing cells in the presence of indomethacin did not release significant amounts of PG. There was evidence to suggest that substrate availability was the limiting factor in PG release. In the absence of exogenously added substrate, cell sonicates synthesized 0.1 ng of PG/mg of protein after 30 minutes. In the presence of dihomo-γ-linolenic acid (10 μg/ml), the precursor of PGE_1, PGA_1 and $PGF_{1\alpha}$, the amount synthesized rose to 0.5 ng/mg of protein and in the presence of arachidonic acid (10 μg/ml), the precursor of PGE_2, PGA_2, and $PGF_{2\alpha}$, to 3.6 ng/mg of protein. Cells which had been preincubated with bacteria for 30 minutes prior to sonication and addition of substrate synthesized approximately twice as much PG-like material as did cells which had not been exposed to bacteria. It was concluded that the PG synthetase was activated during phagocytosis, and that this, in addition to increased substrate availability, accounted

for the increased PG release. It was further suggested that because PGE_1 was leukotactic, its release from phagocytizing PMNs might have an important controlling role in PMN migration. Once phagocytosis (and hence PGE_1 release) ceased, it was postulated, further PMN migration to the inflamed area would also cease.

In spite of the evidence for the release of PGs by PMNs in some types of inflammation, it is apparent that they are not responsible for the release in all types. In arthritis elicited in chickens by the injection of urate crystals, PGE_2 and $PGF_{2\alpha}$ levels peaked at 1 hour after injection whereas PMNs were not detected at the site of inflammation until after 2 hours (Glatt et al., 1974). Willoughby and Giroud (1969) showed that rats depleted of their leukocytes by treatment with methotrexate gave normal acute inflammatory responses to a number of stimuli. Thus the exact relationship between PMNs and PG release during inflammation is still not defined.

In an attempt to clarify this relationship, this project re-examined the release of PGs by PMNs _in vitro_. The conditions under which release takes place and the factors affecting the release were investigated. An attempt was made to determine what aspect of phagocytosis is involved; specifically if PG release is related to lysosomal enzyme release and, by implication, to release of a lysosomal phospholipase.

The phagocytic process can be divided into a number of distinct phases and metablic changes as outlined below:

(i) Particle-cell contact and interaction at the plasma

membrane: while PMNs can phagocytize 'inert' particles such as polystyrene latex beads, phagocytosis becomes much more efficient if the particles are first opsonized; that is, coated with immunoglobulins and/or components of complement. There appear to be specific receptors on the PMN cell membrane for the third component of complement (Lay and Nussenzwerg, 1968) and for portions of IgG molecules (Weissmann et al., 1974). The interaction between the particle and plasma membrane can in itself induce most of the phagocytosis-associated responses. Cells rendered incapable of ingesting particles by treatment with cytochalasin B (CB), a fungal metabolite that disrupts microfilaments (Carter, 1967), still exhibit the sequence of metabolic events that accompany phagocytosis (Zurier et al., 1973). Instead of being ingested, the particles remain adherent to the surface of the cell. Cells can be made to undergo some phagocytosis-associated responses in the absence of particles by exposure to the C5a component of complement (Goldstein et al., 1975). The importance of the interaction at the plasma membrane is further demonstrated by the ability of surfactants such as deoxycholate, digitonin (Graham et al., 1967) or saponin (Rossi and Zatti, 1968) to induce similar responses.

(ii) Particle ingestion: particle contact with the PMN induces the formation of deep invaginations and pseudopodia in the plasma membrane and the particle becomes engulfed in a phagocytic vacuole or phagosome. The vacuole then moves toward the cell interior. The ingestion of particles is inhibited by agents such as theophylline and PGs

which elevate intracellular levels of adenosine 3':5'-cyclic monophosphate (cAMP) and by dibutyryl cAMP itself (Cox and Karnovsky, 1973). As discussed above, the ingestion of particles can be completely inhibited by treatment with CB.

(iii) Lysosomal enzyme release: within PMNs are numerous lysosomal granules containing a host of hydrolytic enzymes. During phagocytosis these granules move toward the cell periphery where they fuse with the phagosomes releasing their hydrolytic enzymes into what is now referred to as a phagolysosome where they act to digest the entrapped particle. As mentioned previously, considerable quantities of these enzymes escape from the cell into the external medium. This is thought to occur when lysosomes fuse with incompletely closed phagosomes at the cell periphery (Weissmann et al., 1971). There are at least two distinct types of granules within the PMN (Bainton and Farquhar, 1968). The primary or azurophil granules resemble lysosomes of other tissues such as the liver and contain typical lysosomal acid hydrolases such as β-glucuronidase as well as peroxidase and myeloperoxidase. In rabbit PMNs these granules contain one third of the cell lysozyme content (Bainton and Farquhar, 1968) while in human PMNs they contain approximately one-half of the lysozyme (Spitznagel et al., 1974). Secondary or specific granules contain the remainder of the cell lysozyme, virtually all of the cell's lactoferrin and in rabbit, but not human PMNs, alkaline phosphatase. During degranulation the specific granules fuse with the phagosome first, followed by the azurophil granules (Bainton, 1973).

Phorbol myristate acetate (PMA), an agent which elevates intracellular levels of guanosine 3',5'-cyclic monophosphate (cGMP) (Estensen et al., 1973), has been shown to selectively release enzymes from the specific granules (Estensen et al., 1974). When phagocytic stimuli are added to CB-treated PMNs, lysosomes migrate to the cell periphery where they fuse directly with the plasma membrane and discharge their contents into the external medium (Zurier et al., 1973). The C5a component of complement induces lysosomal enzyme release from CB-treated but not untreated cells. Enzyme release from normal and CB-treated cells appears to be under cyclic nucleotide mediated control (Zurier et al., 1973; Zurier et al., 1974; Ignarro et al., 1974; Ignarro and George, 1974). Agents which elevate intracellular levels of cAMP inhibit enzyme release whereas cGMP and agents which elevate its intracellular levels enhance release. The movement of lysosomes to fuse with the phagosome or plasma membrane appears to involve microtubule assembly, and agents such as colchicine and vinblastine which disrupt microtubules inhibit degranulation (Zurier et al., 1974).

(iv) Respiratory burst: following particle interaction at the plasma membrane of the cell there is a rapid increase in O_2 consumption. Associated with this respiratory burst there is a marked increase in the production of H_2O_2 and $O_2^{\bar{}}$, both of which are thought to be involved in the killing of micro-organisms within the phagolysosome. The respiratory burst also leads to a marked stimulation of the hexose monophosphate shunt (HMS). Dibutyryl cAMP, theophylline and PGs do not inhibit the

respiratory burst or associated phenomena (Bourne et al., 1971).

(v) Increase in glycolysis: this results in intracellular accumulation of lactic acid and a lowering of the intracellular pH.

It is becoming increasingly evident that Ca^{2+} plays an important if not essential role in the phagocytic process (Stossel, 1973). Leukocytes incubated in Ca^{2+}-free buffer show greatly reduced capacity to ingest particles. As previously mentioned, particle ingestion is not essential for eliciting lysosomal enzyme release and the respiratory burst, rather these responses are induced by particle-cell contact, presumably by way of a chemical signal. It has been proposed that this chemical signal is a rapid influx of Ca^{2+} and/or other cations across the external cell membrane (Romeo et al., 1975). Evidence for this has come from the study of the effects of cationic ionophores on PMNs. One ionophore used extensively in these studies is the divalent cation-specific ionophore A23187 which equilibrates Ca^{2+} and Mg^{2+} across biological membranes (Reed and Lardy, 1972). Addition of this ionophore to PMN suspensions results in a respiratory burst similar to that observed during phagocytosis (Romeo et al., 1975; Schell-Frederick, 1974). The effect is dependent upon Ca^{2+} and is not observed at Ca^{2+} concentrations lower than 10^{-4}M. The ionophore X537A which binds and equilibrates both monovalent and divalent cations (Pressman, 1973) produces a similar respiratory burst (Romeo et al., 1975) in the presence or absence of Ca^{2+}.

A23187 in the presence of external Ca^{2+} also induces degran-

ulation of lysosomes in PMNs (Zabucchi et al., 1975). In Ca^{2+} containing medium X537A induces a similar degranulation (Zabucchi and Romeo, 1976). In Ca^{2+}-free medium, however, while stimulating a respiratory burst, X537A has no effect on lysosomal enzyme release.

It has recently been shown that PMNs presented with a phagocytic stimulus (Smith and Ignarro, 1975) or chemotactic stimulus (Boucek and Snyderman, 1976) show a marked stimulation of Ca^{2+} uptake suggesting that Ca^{2+} influx does indeed play an important role in phagocytosis.

Thus lysosomal enzyme release can be distinguished from other aspects of phagocytosis in a number of ways. CB treatment of cells dissociates degranulation from the ingestion phase. By virtue of its ability to stimulate O_2 consumption but not degranulation in Ca^{2+}-free buffer, X537A provides a convenient means of distinguishing between respiratory burst-associated and lysosomal enzyme-related phenomena. In addition, lysosomal enzyme release is sensitive to cyclic nucleotide mediated control while the respiratory burst is not. With these tools at hand, PG release in rat PMNs was examined and compared to lysosomal enzyme release.

II MATERIALS AND METHODS

1. Preparations of Polymorphonuclear Leukocyte Suspensions

Peritoneal exudate PMNs were obtained from male Wistar rats weighing 150-200 g. The rats were injected intraperitoneally with 6 ml of a sterile 2% sodium caseinate solution and sacrificed 4-5 hours later. The peritoneal cavity was lavaged with 30 ml of calcium-free Hank's balanced salt solution enriched with 0.2% glucose (HBSS). The resultant cell suspension was centrifuged at 500 x g for 10 minutes to obtain a cell pellet which was resuspended and washed twice in calcium-free HBSS. Cell counts were performed with a standard hemocytometer. The total cell yield was generally $1\text{-}2 \times 10^{8}$ cells per rat. Microscopic examination of smears stained with Wright's stain showed that 90% of the cells were PMNs, the remainder being mainly macrophages along with 2-3% lymphocytes. Cell preparations contaminated with erythrocytes were not used. Phase contrast microscopy showed the cell preparations to be free of platelets. Cell viability was determined by the ability of the cells to exclude trypan blue (Phillips, 1973). Cell preparations consistently showed greater than 95% viability and maintained this viability throughout incubations of up to 3 hours. The final cell suspensions were prepared in HBSS containing 1 mM Ca^{2+} unless specified otherwise.

Cells were incubated in 12x75 mm siliconized glass tubes at

37°C and were shaken at 140 cycles per minute. Following incubation, cells were immediately placed on ice and then centrifuged in an Eppendorf Model 3200 clinical centrifuge for 2 min. at 4°C to obtain a supernatant fraction and a cell pellet.

2. Cytochalasin B-Treated Cells

A stock solution of cytochalasin B in ethanol was added to cell suspensions to give a concentration of 5 µg/ml. Cells were then incubated at 37°C for 10 min.. Stock solutions of a number of other agents were also prepared in ethanol or dimethylsulfoxide. The concentration of these solvents in samples did not exceed 0.1% and at this concentration did not affect lysosomal enzyme release, PG release or O_2 consumption.

3. Cell Sonicates

Cells were suspended in HBSS buffered with 10 mM phosphate buffer and disrupted by two 30 sec. sonications using a microprobe of a Biosonic III Sonicator (Bronwill Scientific, Rochester, New York). Reduced glutathione (GSH) (50 µg/ml) and hydroquinone (5 µg/ml) were added to sonicates before incubating.

4. Measurement of PG Levels

(a) Radioimmunoassay for PGE_1 and PGA_1

An alkaline dehydration step was used to convert the PGA_1 and PGE_1 in samples into PGB_1. A commercially available radioimmunoassay

(RIA) kit containing an anti-PGB_1 serum was then used to determine PGB_1 levels. Experimental data, representing the total PGA_1 plus PGE_1 levels are reported simply as ng of 'PG' released per 10^7 cells. The antibody used was not completely specific for PGB_1. PGB_2 (the alkaline dehydration product of PGE_2 and PGA_2) was approximately 15% as effective as PGB_1 in binding. Experimental values therefore may reflect PGE_2 and PGA_2 levels as well. $PGF_{1\alpha}$ and $PGF_{2\alpha}$ did not crossreact appreciably; neither did dihomo-γ-linolenic acid and arachidonic acid, the prostaglandin precursors.

If 10% serum was present in the samples, PGs were extracted by the method of Orczyk and Behrman (1972) before being assayed. One ml of a cell-free supernatant was shaken with 3 ml of ethyl acetate: isopropanol: 0.1 N HCl (3:3:1) for 10 min.. After the addition of 3 ml of 0.9% saline and 2 ml of ethyl acetate, the samples were mixed and centrifuged at 1,600 x g to effect a phase separation. The upper phase was then removed, the solvent evaporated under vacuum and the remaining residue dissolved in 1.0 ml of a buffer containing 0.01 M tris-HCl, 0.14 M NaCl, 5×10^{-4} $CaCl_2$, 0.1% gelatin, pH 7.4. The efficiency of this extraction step was approximately 90% and was reproducible to within a few percent as determined by the extraction of [^{3}H]-PGE_1. In the absence of serum the above extraction step was omitted and a direct assay was carried out. Cell sonicates were extracted as described above.

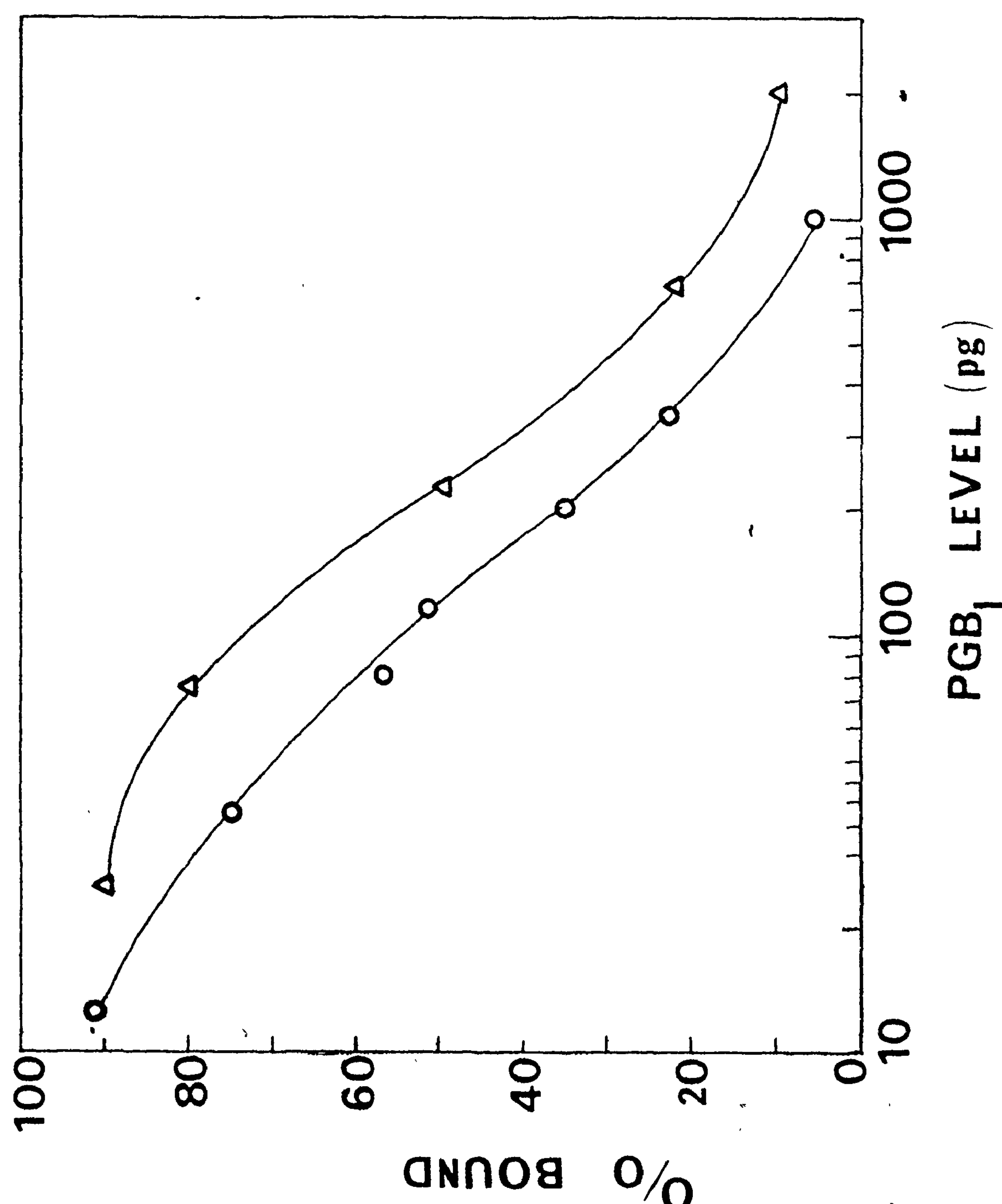
The assay was carried out as suggested by the manufacturers of the kit. Samples were adjusted to pH 12.5-12.9 with 1 N NaOH and heated

in a boiling water bath for 5 min. to effect the alkaline dehydration. The samples were allowed to cool and the pH readjusted to approximately 7.4 with 1 N acetic acid. Samples of up to 400 µl were added to the buffer described above to give a final volume of 1.0 ml. Next, 50 µl (0.01 µCi) of [^{3}H]-PGB_1 and 50 µl of PGB_1-antibody were added and the tubes incubated for 1 hour at 37°C. PGB_1 standards (25 µg - 2 ng) were routinely assayed with the unknowns. Following the 1 hour incubation, 100 µl of normal rabbit serum and 100 µl of goat antirabbit IgG were added and the samples incubated for 18-20 hours at 4°C to precipitate the PG-antibody complex. After this incubation, samples were centrifuged at 1,600 x g for 30 min., the supernatants decanted and the pellets dissolved in 0.5 ml of 0.1 N NaOH and added to 10 ml of Bray's scintillation cocktail (Bray, 1960). Samples were counted in a Nuclear Chicago Unilux III liquid scintillation system and counting efficiencies determined by the external standard method. The percentage of [^{3}H]-PGB_1 which had been precipitated was calculated. A typical standard curve is shown in Figure 1. Alternatively, assays were carried out using one-half the amount of antibody and [^{3}H]-PGB_1 described above. Other volumes and quantities were reduced accordingly. A standard curve for this assay is also shown in Figure 1.

(b) Conversion of [^{3}H]-Arachidonic acid into [^{3}H]-PGE_2 and [^{3}H]-$PGF_{2\alpha}$

A stock solution of [^{3}H]-arachidonic acid in benzene was added to siliconized glass tubes to provide 10^6 dpm per tube. Unlabeled

Figure 1. Typical standard curves for PGB_1 radioimmunoassay, Δ---Δ, regular assay; o---o, assay using one-half the usual amount of antibody.



100
80
60
40
20
0
% BOUND
10
100
1000
PGB$_1$ LEVEL (pg)

arachidonic acid was then added in a hexane stock solution to provide the required specific activity. The tubes were blown dry under N_2 and 1 ml of cell suspension or cell sonicate was added. Samples were incubated for 30 min. after which cell suspensions were spun down, the supernatants decanted and the cell pellets washed, resuspended in 1.0 ml HBSS and disrupted by sonication. PGs were extracted from the supernatants and sonicates as described above and the solvent evaporated under vacuum. Samples were then chromatographed on silica gel columns (Jaffe et al., 1971) to remove the [^{3}H]-arachidonic acid. This step was found necessary as in the subsequent thin layer chromatography of the samples there was considerable trailing of the leading [^{3}H]-arachidonic acid masking the [^{3}H]-PG peaks. [^{3}H]-arachidonic acid was washed through the columns with 6 ml of benzene:ethyl acetate (60:40). PGs were then eluted with three ml of benzene:ethyl acetate:methanol (60:40:20). This fraction was evaporated under vacuum, redissolved in 50 µl of ethanol and chromatographed on 20x20 cm plastic-backed silica gel plates in ethyl acetate:water:iso-octane: acetic acid (11:10:5:2), the AII solvent system of Green and Samuelsson (1964). The developed chromatogram was cut into 0.5 cm strips which were placed directly into scintillation vials containing 10 ml of Bray's cocktail.

5. Phagocytic Stimuli

Zymosan particles were suspended in HBSS at 10 mg/ml, boiled for 10 min., washed twice in HBSS and resuspended in pooled rat serum to

give a concentration of 10 mg/ml. This suspension was incubated at $37^{\circ}C$ for 30 min. and then added to cell suspensions to give a 10 fold dilution of the serum. This provided a particle concentration of $5x10^8$ particles/ml.

C5a-containing serum was prepared by opsonizing zymosan in the presence of 0.25 M ε-aminocaproic acid (ε-ACA), an inhibitor of the C5a inactivator (Vallota and Muller-Eberhard, 1973). Following opsonization, the zymosan particles were spun down in an Eppendorf Model 3200 clinical centrifuge and the supernatant decanted. The treated serum was added to cell suspensions to give a concentration of 10%.

Latex particles were dialyzed extensively against distilled H_2O. They were added to samples to give a particle:cell ratio of 200:1.

Micrococcus lysodeikticus cells were heat killed by autoclaving and were opsonized in pooled rat serum at a concentration of 10 mg/ml. This suspension was added to samples to give a 10 fold dilution of the serum.

Stock solutions of A23187 were prepared in ethanol or dimethylsulfoxide. X537A stock solutions were prepared in dimethylsulfoxide.

6. Enzyme Assays

Enzyme release from PMNs is expressed as the percent of the total enzyme activity released from cells incubated in the presence of 0.2% Triton X-100.

(a) β-glucuronidase

β-glucuronidase activity was determined as described by

Brittinger et al. (1968), using phenolphthalein β-glucuronide as substrate and incubating for 15 hours at 37°C. Absorbance readings at 550 nm were obtained.

(b) Lysozyme

Lysozyme activity was determined from the rate of lysis of a suspension of *Micrococcus lysodeikticus* as described by Jolles (1962). The decrease in absorbancy at 450 nm was followed.

(c) Lactic dehydrogenase

Lactic dehydrogenase activity was determined by measuring the rate of oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) (Bergmeyer et al., 1965). The decrease in absorbancy at 340 nm was followed.

7. Oxygen Consumption

Oxygen uptake by PMNs was measured with a Clark membrane oxygen electrode (Yellow Spring Instrument Co., Yellow Springs, Ohio) attached to a thermostatically controlled (37°C) plastic vessel. A 2 ml suspension of PMNs was placed in the reaction vessel and allowed to equilibrate for 5 min. The phagocytic stimulus was then added and the respiratory burst recorded.

8. Reagents and Materials

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): oleic acid, linoleic acid, stearic acid, lipid

chromatography grade silica gel (325 mesh), polystyrene latex beads (0.79 μm diameter), _Micrococcus lysodeikticus_ (dried cells), GSH, cGMP, indomethacin, NADH, colchicine, acetylcholine bromide and quinacrine HCl; dibutyryl cAMP was purchased from Calbiochem (LaJolla, CA); arachidonic acid and dihomo-γ-linolenic acid from NU Chek Prep (Elysian, MN); histamine dihydrochloride from BDH (Toronto, Ont.); phorbol myristate acetate from Midland Corp. (Brewster, NY); Triton X-100 and 5,6,8,9,11,12,14,15(N)-[^{3}H]-arachidonic acid (87 Ci/mmole) from Amersham/Searle (Arlington Heights, IL); [^{3}H]-PGE_1 from New England Nuclear (Boston, MA); "chromagram" plastic-backed thin layer plates (6060 silica gel) from Eastman Kodak Co. (Rochester, NY); and the PG radioimmunoassay kit from Clinical Assays Inc. (Cambridge, MA); PGE_1 was a generous gift of Dr. John Pike, Upjohn Co., (Kalamazoo, MI); the ionophores A23187 and X537A were kindly provided by Dr. R.L. Hamill of Eli Lilly and Co. (Indiannapolis, IN), and Dr. J. Berger of Hoffman-LaRoche Inc., (Nutley, NJ) respectively.

III RESULTS

1. Prostaglandin Release by Resting Whole Cells

(a) PGE_1 and PGA_1 release

As reported by Higgs et al., (1975), resting PMNs did not release significant quantities of PGE_1 and PGA_1. In four experiments the amount of PG released after incubation for 2 hours ranged from less than 0.1 ng/10^7 cells to 1.3 ng/10^7 cells (mean 0.39). The presence of serum (10%) and of cofactors required by prostaglandin synthetase such as GSH (50 μl/ml) and hydroquinone (10 μg/ml) did not enhance PG release. If however the free fatty acid precursor of PGE_1 and PGA_1, dihomo-γ-linolenic acid, was added to cell suspensions, PG release was greatly enhanced. As shown in Figure 2, PG release reached a maximum after 15 min. at which time approximately 50 ng/10^7 cells was detected. In 5 experiments the average maximum release was 23±4 ng/10^7 cells. Complexing of the free fatty acid with bovine serum albumin to afford more rapid uptake by the cells, did not further increase PG release. The leveling off of PG levels after 15 min. did not appear to be due to increased PG breakdown. As shown in Figure 3, PMNs did not significantly metabolize $[^3H]$-PGE_1 with which they had been incubated. Virtually all of the $[^3H]$-PGE_1 was recovered from the sample apparently unmodified. The AII solvent system in which the samples were

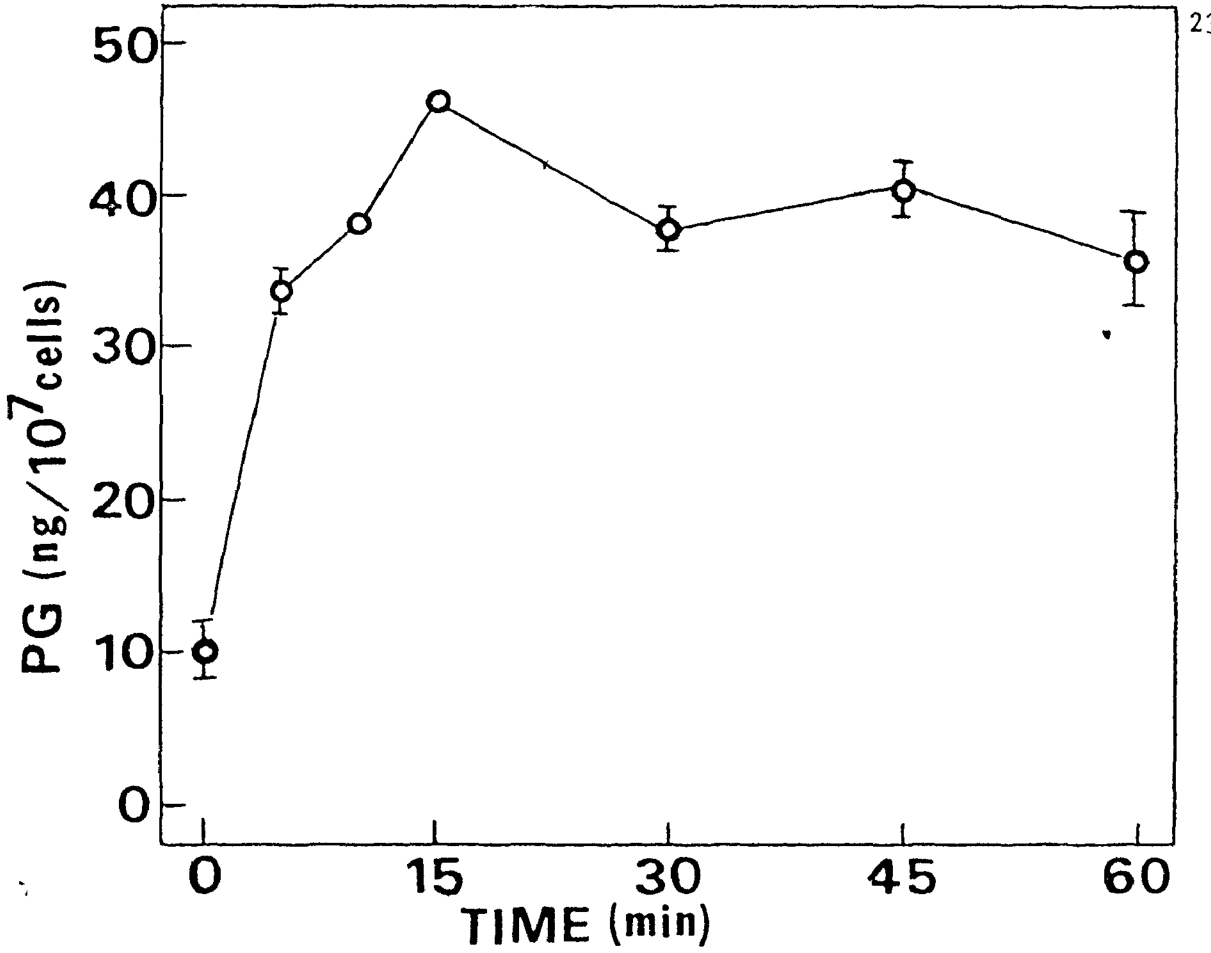
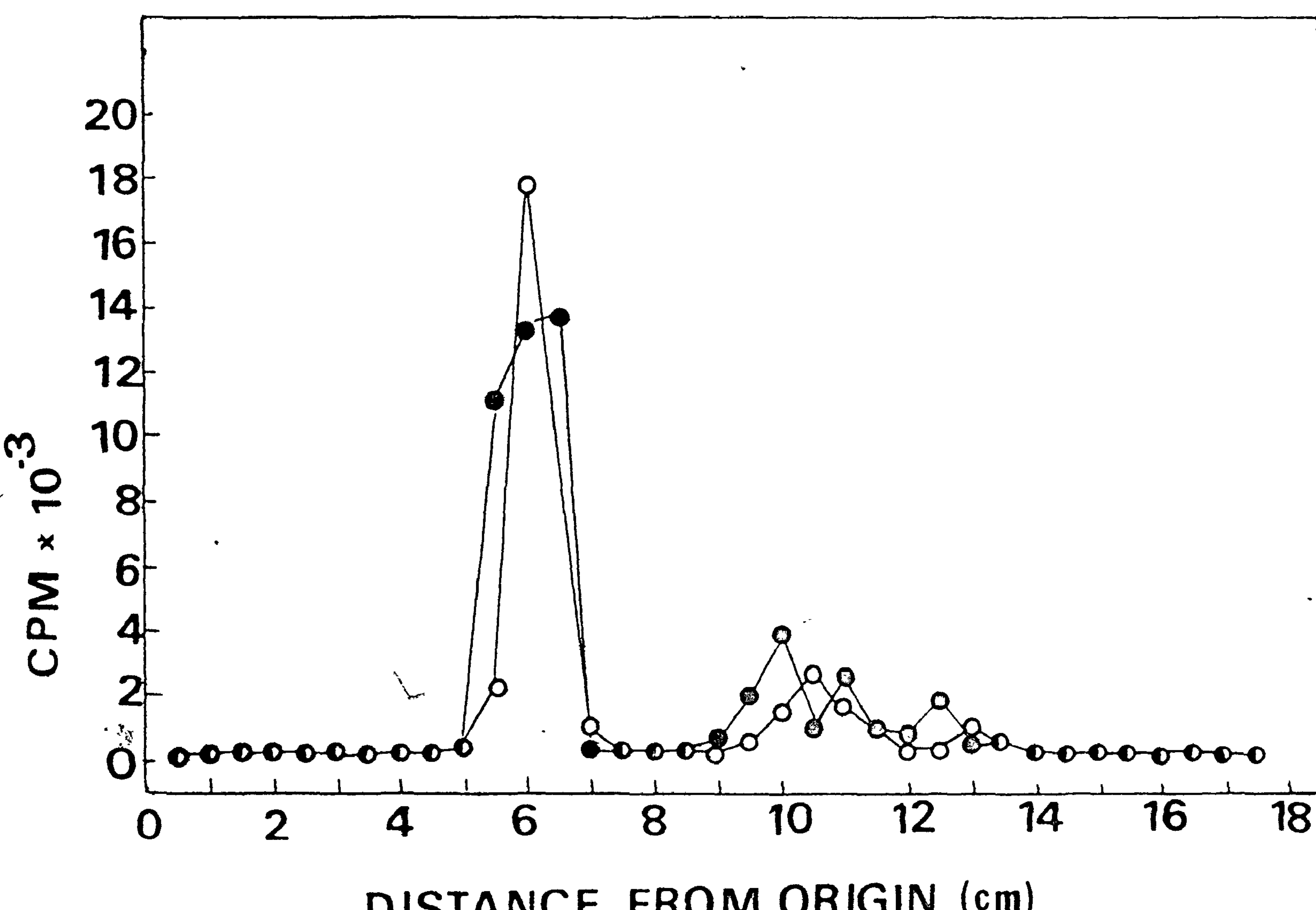


Figure 2. Time course of PG release from nonphagocytizing PMNs incubated in the presence of PG precursor. PMNs ($3.1x10^6$ cells/ml) were incubated in the presence of dihomo-γ-linolenic acid (10 μg/ml). Samples were removed at the indicated times, centrifuged and the supernatants assayed for PG content by RIA. Data represent the mean ± one-half the range for duplicate determinations. In this and in subsequent figures, error bars fall within the points when not shown.

Figure 3. Thin layer chromatogram of labeled material extracted from cell-free supernatants of cell suspensions incubated with $[^3H]$-PGE_1. $[^3H]$-PGE_1 was added to PMNs (5.2×10^6 cells/ml) to give a final concentration of 50 ng/ml. Incubations were carried out for 1 hour after which the cell-free supernatants were extracted as described in Methods and chromatographed in the AII solvent system. A control in which $[^3H]$-PGE_1 had been incubated in cell-free buffer was similarly extracted and chromatographed. (o---o) control, (●---●)+cells.



CPM × 10-3
0
2
4
6
8
10
12
14
16
18
20
DISTANCE FROM ORIGIN (cm)
0
2
4
6
8
10
12
14
16
18

chromatographed is one which has been routinely used for analysis of PG mixtures and any metabolites such as hydroxylated products should have been readily separated from the parent compound. The dependence of PG release on exogenous precursor concentration was examined. As shown in Figure 4, maximum release was achieved at concentrations of 5 μg/ml and higher.

It has been reported that unsaturated fatty acids, by acting as membrane perturbing agents, can induce a number of phagocytosis-related responses in PMNs such as a respiratory burst and degranulation of lysosomes (Kakinuma, 1974). At concentrations of dihomo-γ-linolenic acid below 20 μg/ml we did not observe such a response. The release of β-glucuronidase and lysozyme, and O_2 consumption were not affected by the addition of the free fatty acid to non-phagocytizing cells. At concentrations above 20 μg/ml however a slight increase in lysosomal enzyme release was observed, accompanied by an increase in the release of the cytoplasmic enzyme lactic-dehydrogenase, suggesting that the release was due to cell damage or lysis. $PGF_{2\alpha}$ also causes cell lysis at comparable concentrations (Zurier et al., 1973).

As expected, PG release was markedly inhibited by indomethacin (10 μg/ml) (Table 1). This provided strong evidence that the material measured by the radioimmunoassay was indeed PG. Moreover, no release was observed when fatty acids which were not PG precursors, such as oleic acid and stearic acid, were added to cell suspensions (Table 1). When arachidonic acid was used however a considerable amount of PG

Figure 4. Dependence of PGE_1 and PGA_1 release from nonphagocytizing PMNs on concentration of exogenous precursor. PMNs ($6.4x10^6$ cells/ml) were incubated in the presence of dihomo-γ-linolenic acid at the indicated concentrations for 15 min. after which PG levels in the cell-free supernatants were determined by RIA. Results represent mean ± one-half the range for duplicate determinations.

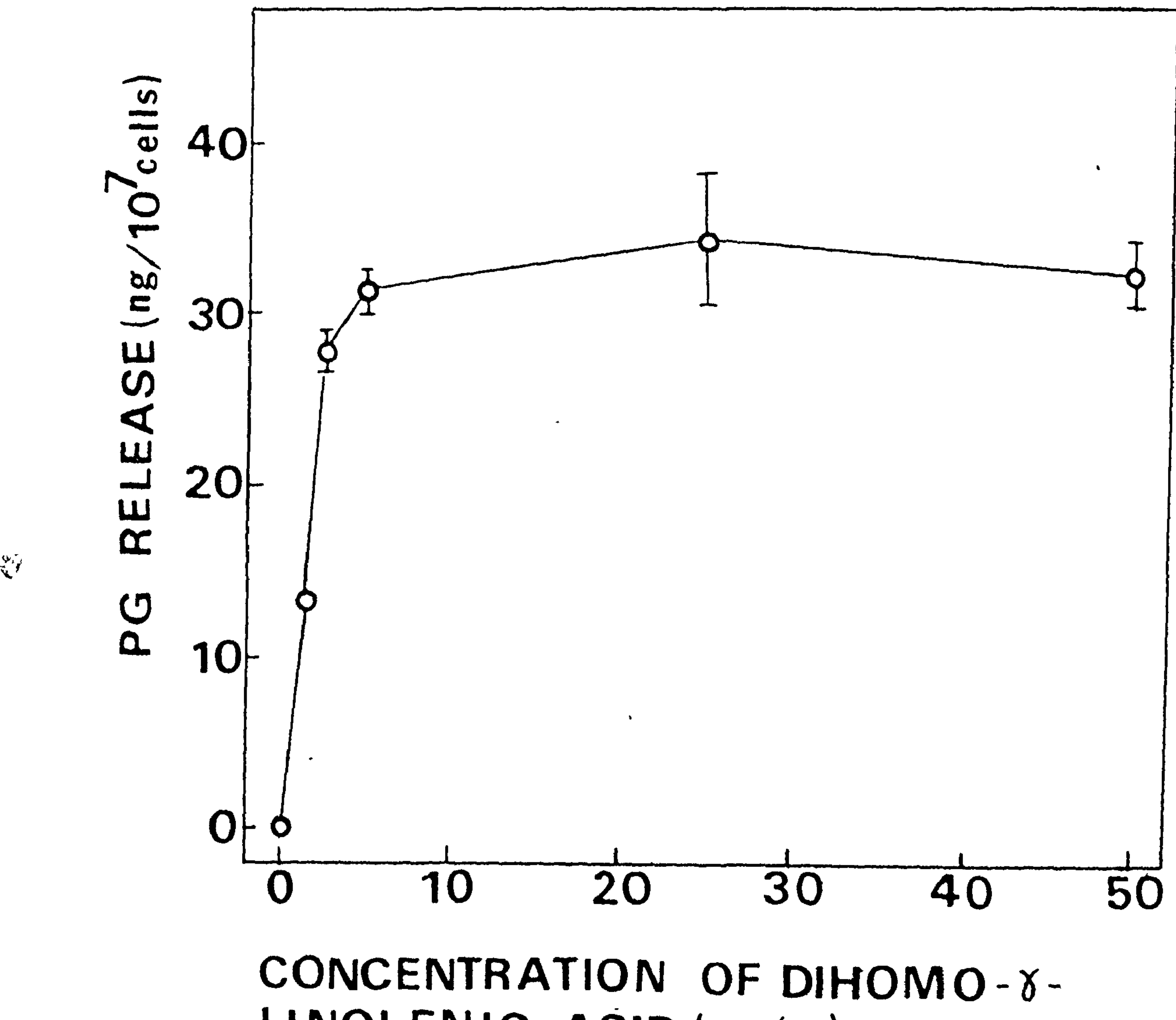

PG RELEASE (ng/10⁷cells)
40
30
20
10
0
0
10
20
30
40
50
CONCENTRATION OF DIHOMO-γ-
LINOLENIC ACID (μg/ml)

TABLE 1

Effect of fatty acids on PG release from resting PMNs.

Fatty acid	PG release (ng/10^7 cells)
none (control)	0.4±0.2
dihomo-γ-linolenic acid	25.0±2.1
dihomo-γ-linolenic acid + Indomethacin (10 μg/ml)	2.8±0.5
Arachidonic acid	22.2±0.8
oleic acid	1.6±0.4
linoleic acid	0.8±0.5
stearic acid	0.7±0.2

PMNs ($4.2x10^6$ cells/ml) were incubated at 37°C in the presence of the indicated fatty acids (10 μg/ml) for 30 min., after which PG levels in the cell-free supernatants were determined by RIA. Results represent mean ± standard error of mean (SEM) for triplicate determinations.

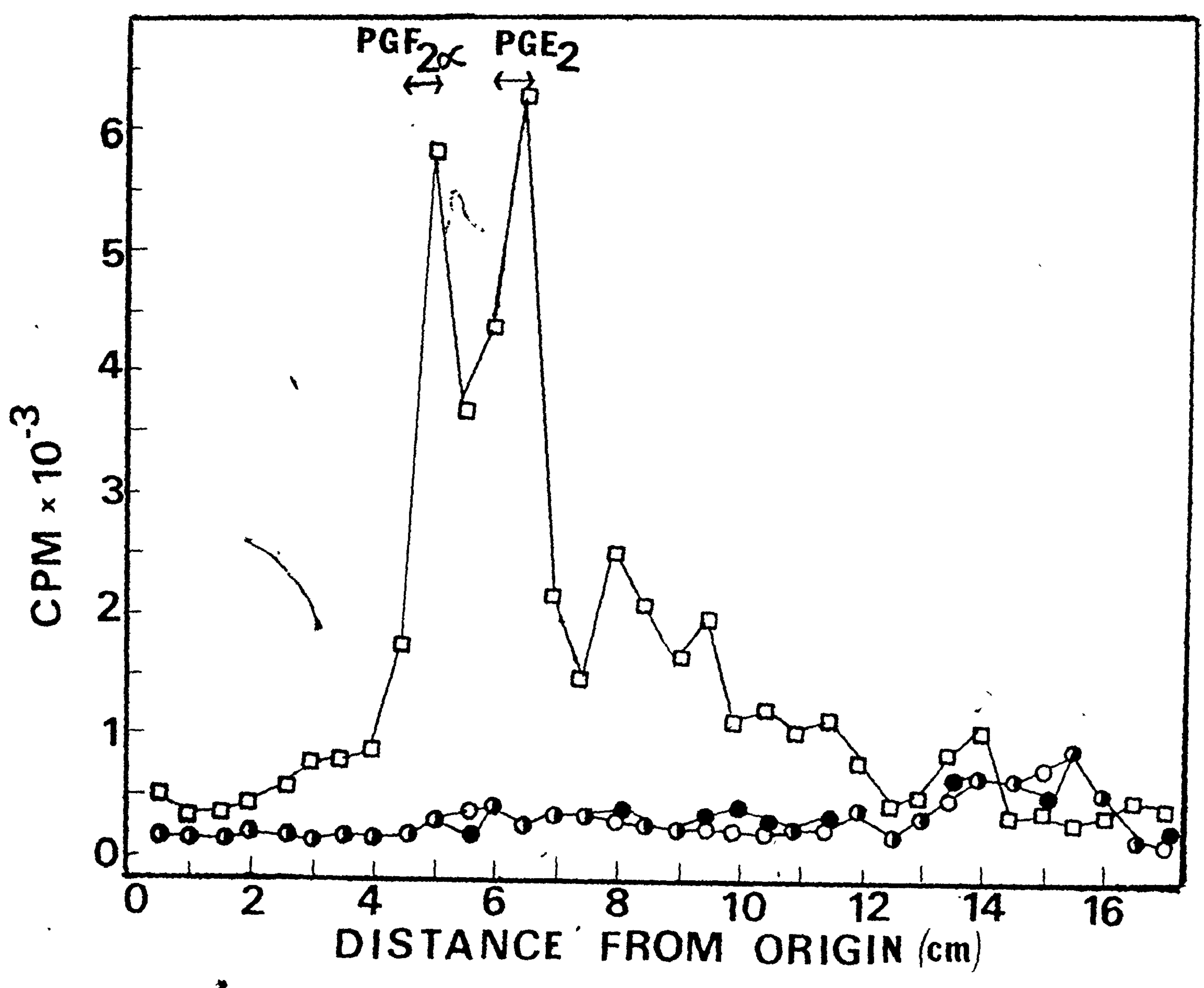
immunoreactive substance was released. As discussed in methods the anti-PGB_1 serum crossreacted with PGB_2 the alkaline dehydration product of PGE_2 and PGA_2. Since the crossreactivity was approximately 15%, the actual amount of PGE_2 and PGA_2 could have been 6 or 7 times higher than that listed in Table 1, suggesting that the cells are capable of releasing considerably more PGE_2 and PGA_2 than PGE_1 and PGA_1. Linoleic acid, which is a precursor of arachidonic acid and of dihomo-γ-linolenic acid, did not induce a significant release.

Prostaglandin release in the presence of exogenous precursor did not appear to be under hormonal or cyclic nucleotide-mediated control. Cells which were preincubated for 30 min. with 10^{-3} M theophylline, 10^{-3} M dibutyryl cAMP, 10^{-4} M histamine, 10^{-4} M cGMP or 10^{-4} M acetylcholine prior to the addition of 10 μg of dihomo-γ-linolenic acid /ml released quantities of PGs which did not differ significantly from those released by controls. Similarly the presence of GSH (50 μg/ml) and hydroquinone (10 μg/ml) did not enhance release.

(b) <u>Synthesis of $[^3H]$-PGE_2 and $[^3H]$-$PGF_{2\alpha}$ from $[^3H]$-arachidonic acid</u>

Figure 5 shows the chromatogram of labeled material extracted from the cell-free supernatant and the sonicated cell pellet obtained from a cell suspension which had been incubated with $[^3H]$-arachidonic acid. Essentially all of the radioactivity was located in the cell-free supernatant fraction. The R_f values of the two major peaks of radioactivity found in the supernatant fraction corresponded to those of

Figure 5. [^{3}H]-PG release from PMNs incubated with [^{3}H]-arachidonic acid. PMNs ($3.5x10^{7}$ cells/ml) were incubated in the presence of 5 μg/ml [^{3}H]-arachidonic acid ($1x10^{6}$ dpm/ml) for 30 minutes. Extraction and chromatography carried out as described in methods. (□--□) cell-free supernatant, (o--o) cell pellet, (●—●) cell-free supernatant from sample containing indomethacin (10 μg/ml).



PGF2α
PGE2
CPM × 10-3
DISTANCE FROM ORIGIN (cm)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16

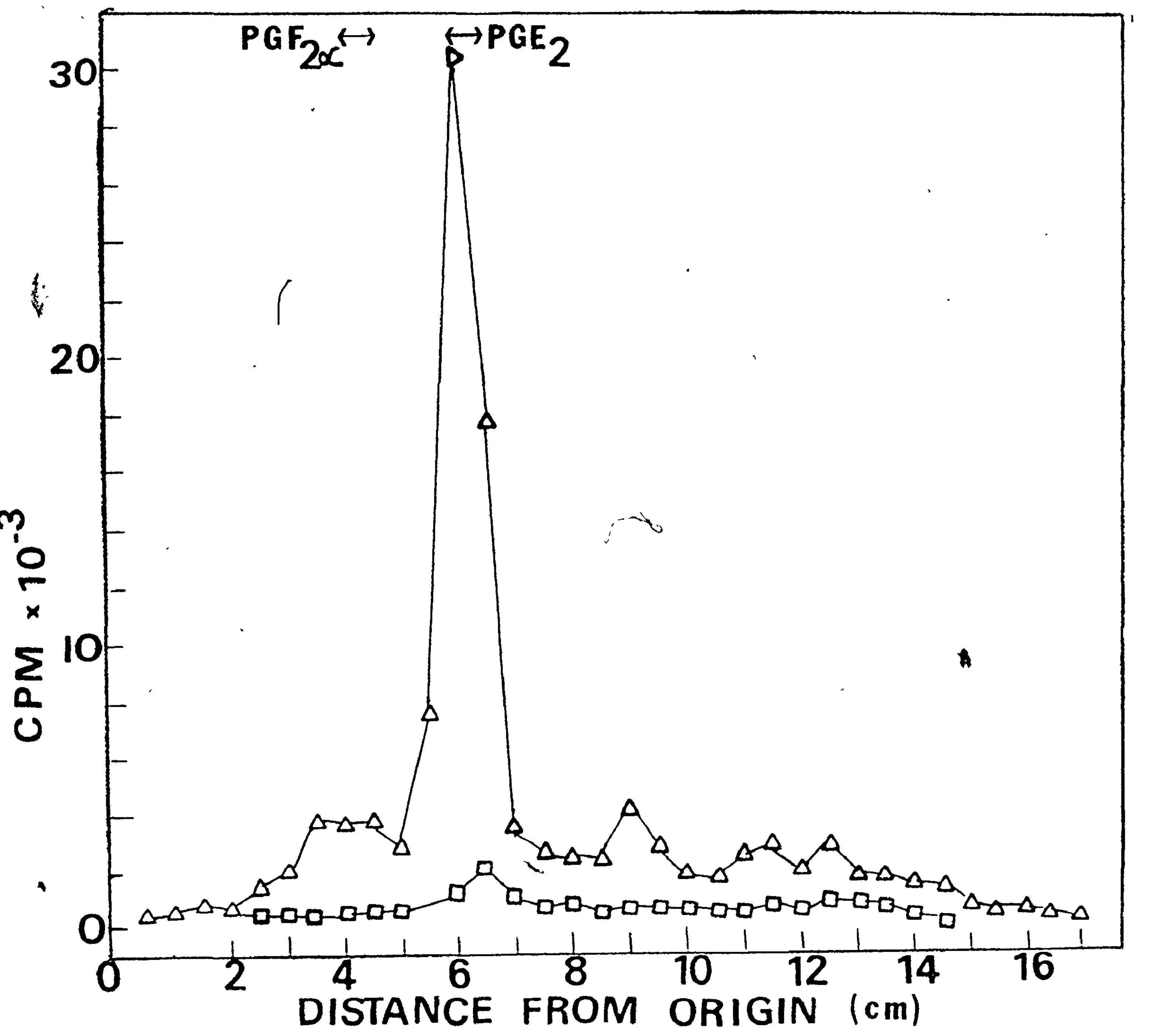
authentic PGE_2 and $PGF_{2\alpha}$. In this and in other experiments roughly comparable amounts of PGE_2 and $PGF_{2\alpha}$ were formed. As the two PGs did not give well resolved peaks over a flat baseline (Figure 5), the amounts of PGE_2 and $PGF_{2\alpha}$ formed can only be calculated approximately to be 35-40 ng/10^7 cells by integration. Figure 5 also shows that in the presence of 10 μg of indomethacin per ml there was no PG release.

Figure 6 shows the chromatogram of labeled material extracted from cell sonicates incubated in the presence of [^{3}H]-arachidonic acid. In contrast to the case with whole cells, sonicates synthesized much more PGE_2 than $PGF_{2\alpha}$. The amount of PGE_2 synthesized by the homogenate was approximately 5 times greater than that synthesized by whole cells. However the total amount of PGE_2 plus $PGF_{2\alpha}$ was less than 3 times that synthesized by whole cells.

2. Release of PGs by Phagocytizing Cells

Figure 7 shows the time course of PG release from PMNs exposed to two phagocytic stimuli - a suspension of *Micrococcus lysodeikticus* and polystyrene latex beads. With the bacteria as the stimulus, maximum PG release was not attained until after 2 hours, in marked contrast to PG release from resting cells incubated in the presence of PG precursor (Figure 2). The levels of PG released were markedly lower than those obtained when exogenous precursor was present. Latex particles proved to be a relatively weak stimulus; they were however correspondingly weak in inducing other phagocytic responses such as lysosomal enzyme

Figure 6. [^{3}H]-PG release from cell sonicates incubated in the presence of [^{3}H]-arachidonic acid. PMNs (3.5×10^7 cells/ml) were sonicated and incubated in the presence of 5 μg/ml [^{3}H]-arachidonic acid (1×10^6 dpm/ml) for 30 min. (Δ—Δ) control, (□--□) indomethacin (10 μg/ml)



PGF2α ↔
↔ PGE2
CPM × 10-3
30
20
10
0
0
2
4
6
8
10
12
14
16
DISTANCE FROM ORIGIN (cm)

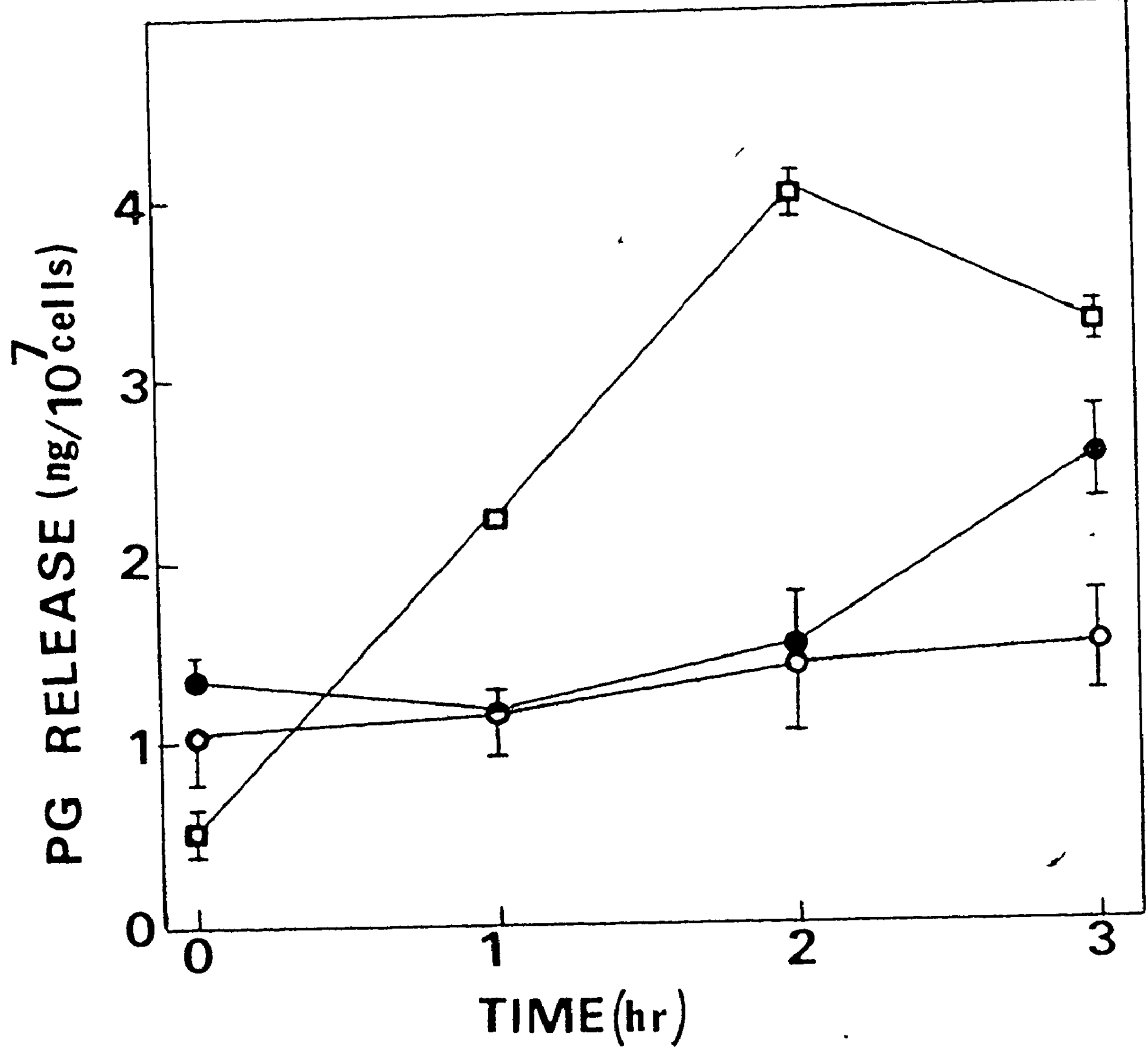


Figure 7. Release of PG by phagocytizing PMNs. PMNs (3.9×10^6 cells/ml) were exposed to 10% serum (o—o), opsonized Micrococcus lysodeikticus (□--□), and latex particles (●--●). Cell suspensions were incubated for the indicated times after which the PGs were extracted from the cell-free supernatants and measured by RIA. Results represent mean ± one-half the range for duplicate determinations.

release. Opsonized zymosan particles proved to be comparable to *Micrococcus lysodeikticus* in inducing PG release.

A comparison was made between the release of PGs and the release of the lysosomal enzyme β-glucuronidase from both CB-treated and normal cells. As shown in Figure 8, CB treatment by itself induced a slight release of β-glucuronidase. CB-treated cells exposed to opsonized zymosan for 15 min. released significantly more β-glucuronidase than did normal cells. After exposure to zymosan for 2 hours however the release from normal and treated cells was comparable. Zymosan-treated serum containing 0.25 M ε-ACA induced a significant β-glucuronidase release from CB-treated cells, although the total release was considerably less than that obtained with opsonized zymosan.

Figure 9 shows the corresponding release of PGE_1 plus PGA_1 under the same conditions. Significantly, after a 15 min. incubation, CB-treated cells released considerably less PG than untreated cells, in marked contrast to β-glucuronidase release. Moreover zymosan-treated serum did not induce a significant release of PG within the first 15 min., at which time most of the β-glucuronidase release had taken place. Thus, under certain conditions, there appeared to be differences between lysosomal enzyme and PG release.

3. Control of PG and Lysosomal Enzyme Release

The effects of dibutyryl cAMP (10^{-3}M), and colchicine (10^{-3}M) on the release of PGs and lysosomal enzymes by CB-treated cells were studied.

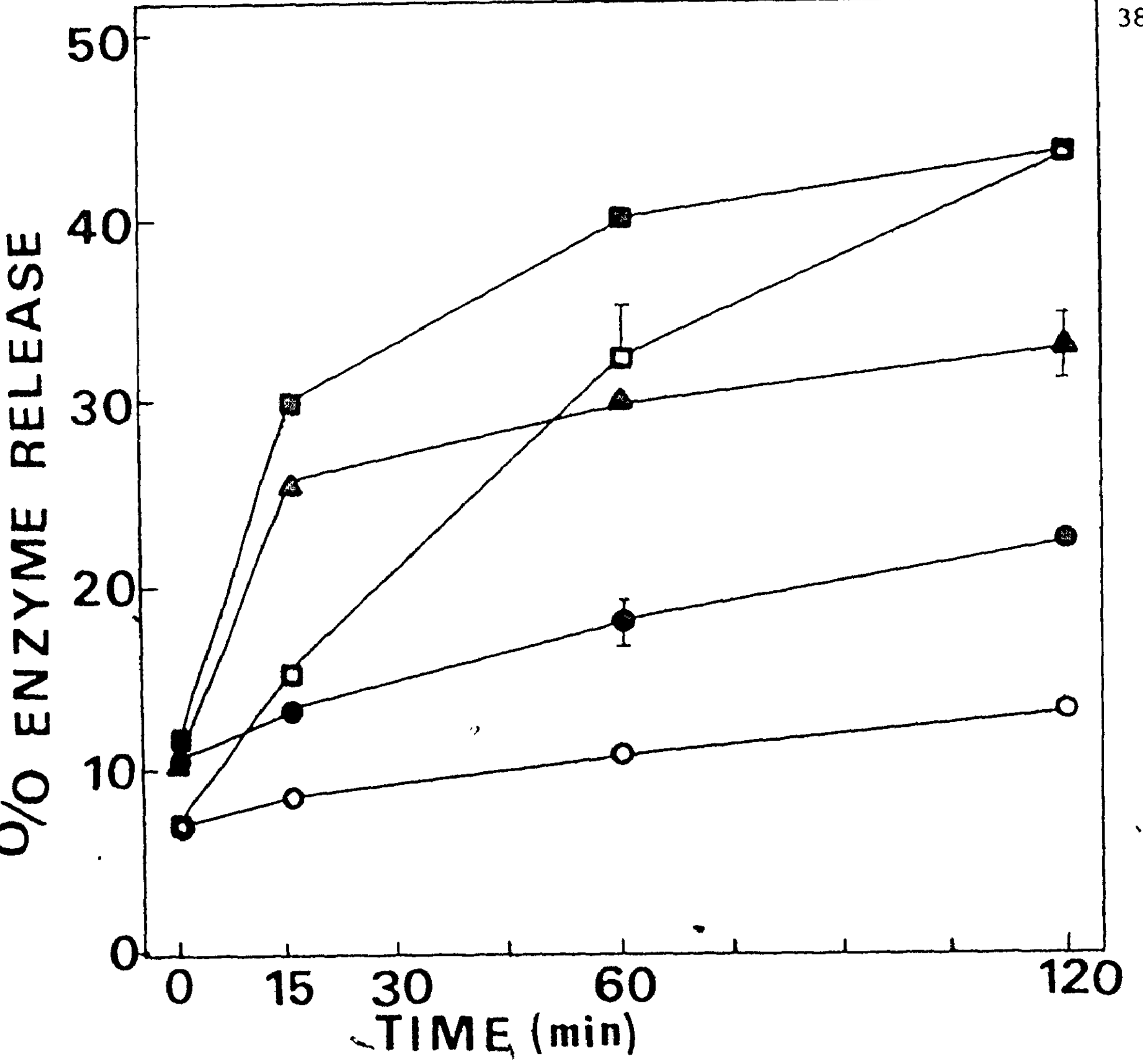


Figure 8. β-glucuronidase release from normal and CB-treated PMNs. PMNs were suspended to give a concentration of 6.0×10^6 cells/ml. A portion of the suspension was treated with CB. Untreated cells were exposed to 10% serum (o—o) and opsonized zymosan (□--□); CB-treated cells exposed to 10% serum (●—●), opsonized zymosan (■--■) or to zymosan treated serum containing 0.25 M ε-ACA (▲--▲). Samples were removed at the indicated times and β-glucuronidase release determined. Results represent the mean ± one-half the range for duplicate determinations.

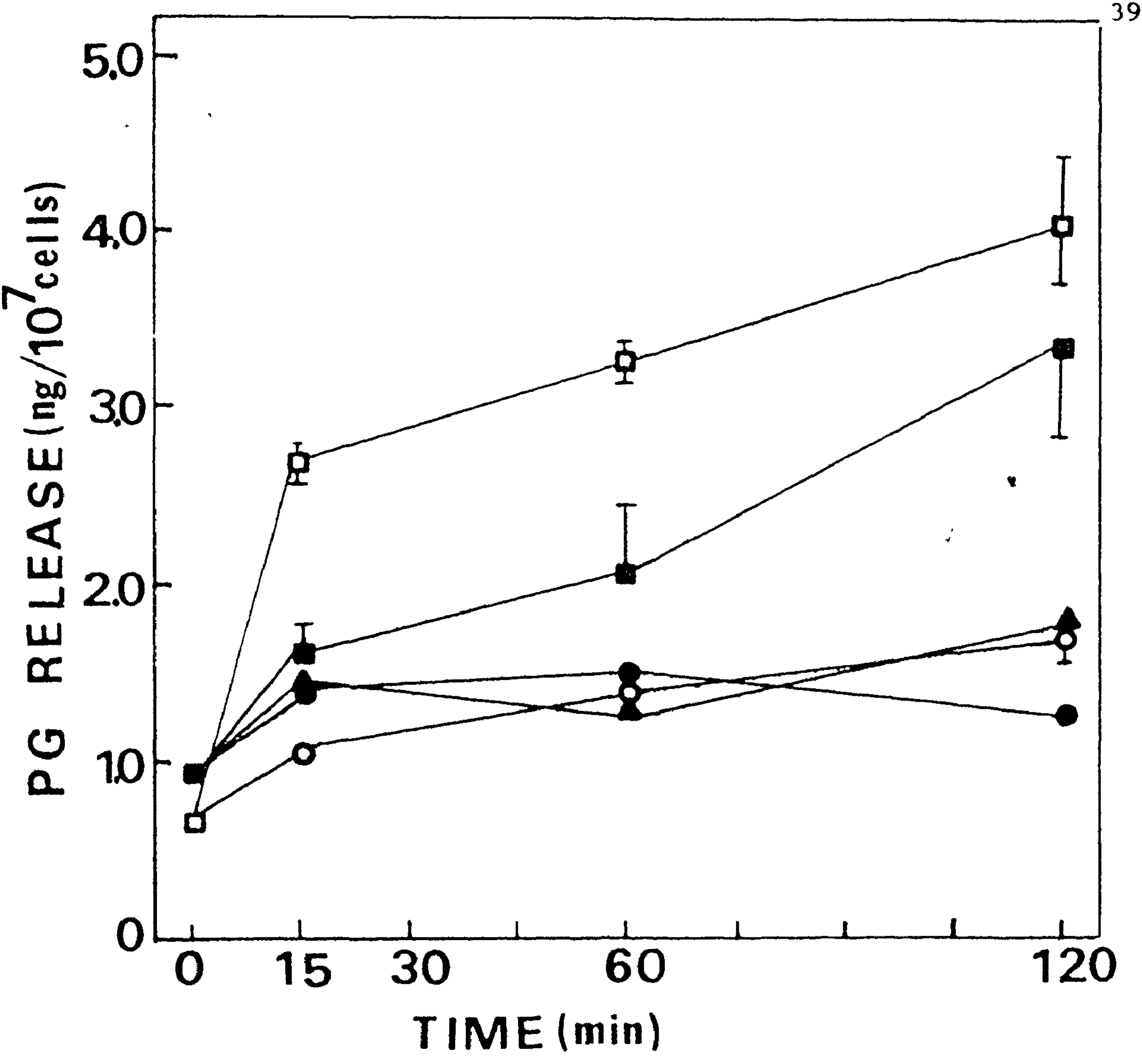


Figure 9. PG release from normal and CB-treated PMNs. PG levels determined in samples described in Figure 8. Legend the same as in Figure 8.

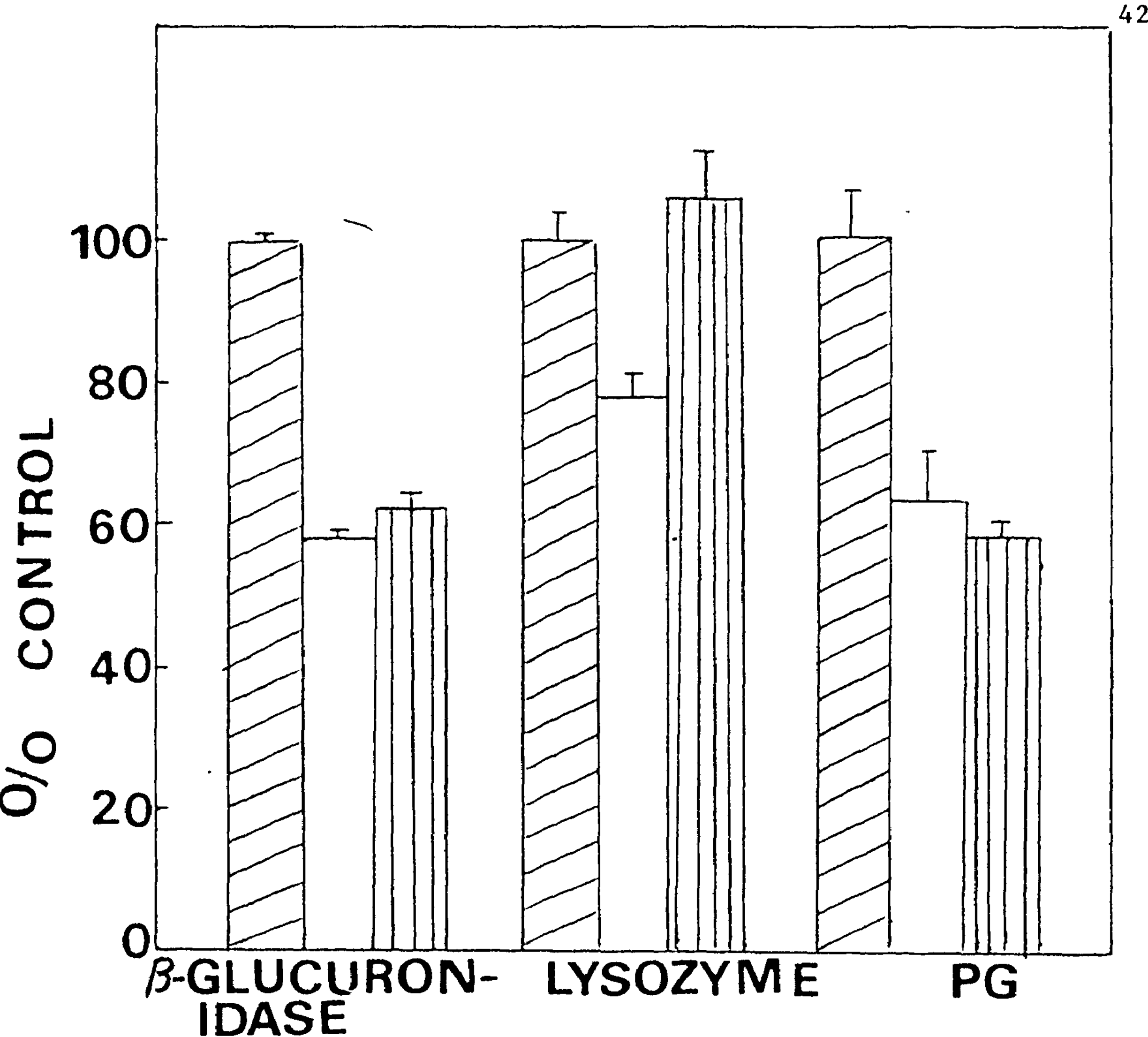
Figure 10 shows that dibutyryl cAMP inhibited the release of both lysosomal enzymes and PGs. The inhibition of lysozyme release was significantly less than for β-glucuronidase and PG release. While colchicine markedly inhibited β-glucuronidase and PG release, it had no significant effect on lysozyme release. This emphasized the presence of the two major types of granules and suggested that PG release was more closely related to azurophil granule release than to specific granule release. This was further substantiated by treatment of cells with PMA (20 ng/ml). Figure 11 shows that while PMA induced the release of lysozyme, it had no effect on β-glucuronidase and PG release.

4. <u>Ionophore Mediated Release of PGs</u>

(a) <u>Release with A23187</u>

The effect of the divalent ionophore A23187 on PG release was examined. As shown in Figure 12, the ionophore induced a PG release whose time course closely paralleled that of the accompanying lysosomal enzyme release. The levels of PG and lysosomal enzymes released were comparable to those released during phagocytosis of zymosan particles (Figures 8 and 9). However, release appeared to be much more rapid with A23187. The cell suspension used in this experiment contained 1 mM Ca^{2+} and 0.7 mM Mg^{2+}. The dependence of the release reaction on Ca^{2+} was examined. Figure 13 shows that PG release was dependent on Ca^{2+} concentration in a manner similar to lysosomal release. Release was observed at Ca^{2+} concentrations of $5x10^{-5}$ M and higher and reached a

Figure 10. Pharmocological control of lysosomal enzyme and PG release from CB-treated PMNs. PMNs (4.1×10^6 cells/ml) were treated with CB and preincubated at 37°C for 30 min. with the following agents: none (control) ▨, dibutyryl cAMP (10^{-3}M) ☐ and colchicine (10^{-3}M) ▥. Samples were then exposed to opsonized zymosan for 1 hour and β-glucuronidase, lysozyme and PG levels determined in cell-free supernatants. Results are expressed as the percent of control values and represent the mean ± SEM for 5 determinations. The control values were: β-glucuronidase, 59.1 ± 0.5% of the total activity released by 0.2% Triton X-100; lysozyme, 51.7 ± 4.5% of the activity released by Triton X-100; and PG, 4.57 ± 0.34 ng PG/10^7 cells.


100
80
60
40
20
0
% CONTROL
β-GLUCURON-
IDASE
LYSOZYME
PG

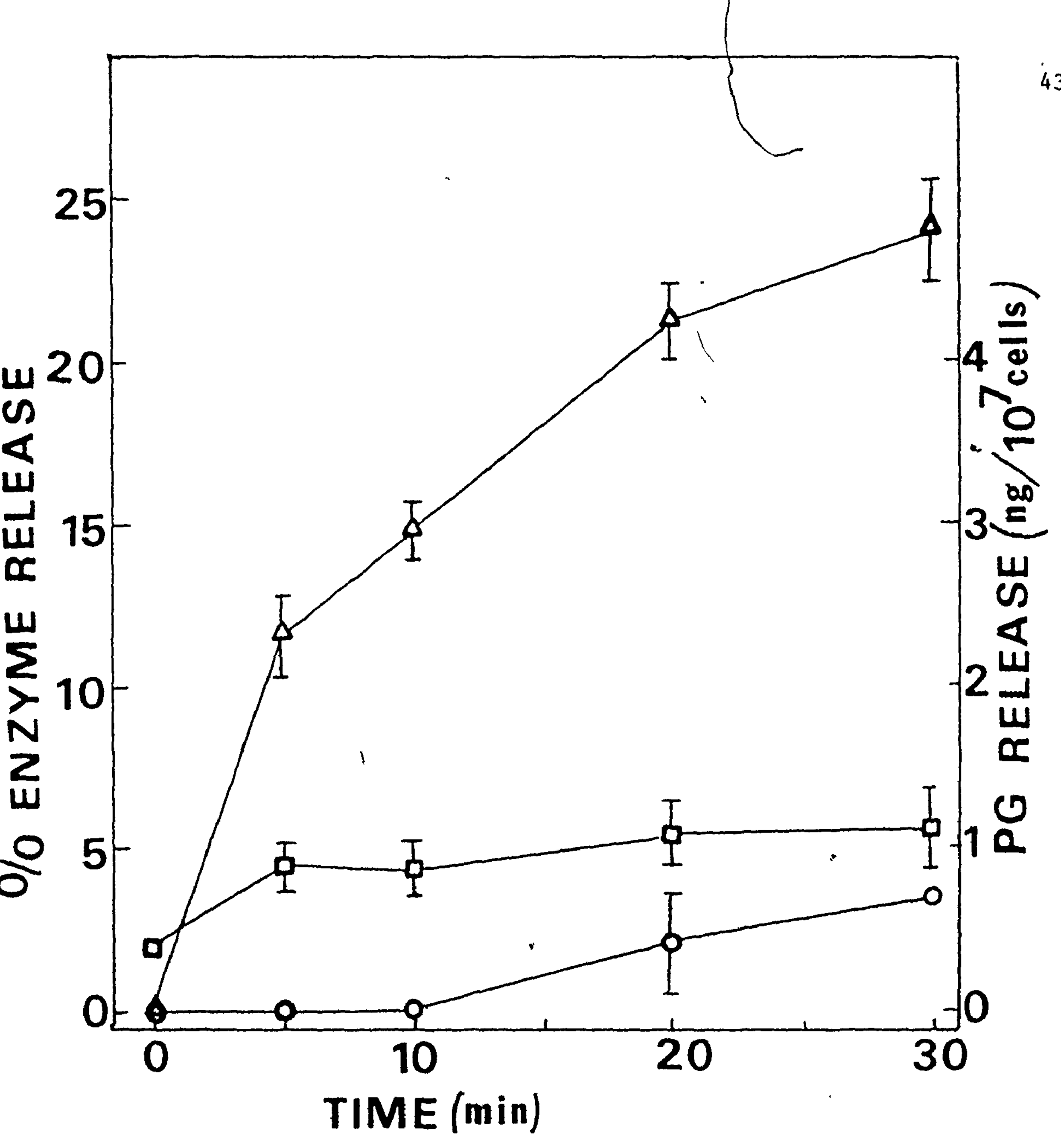
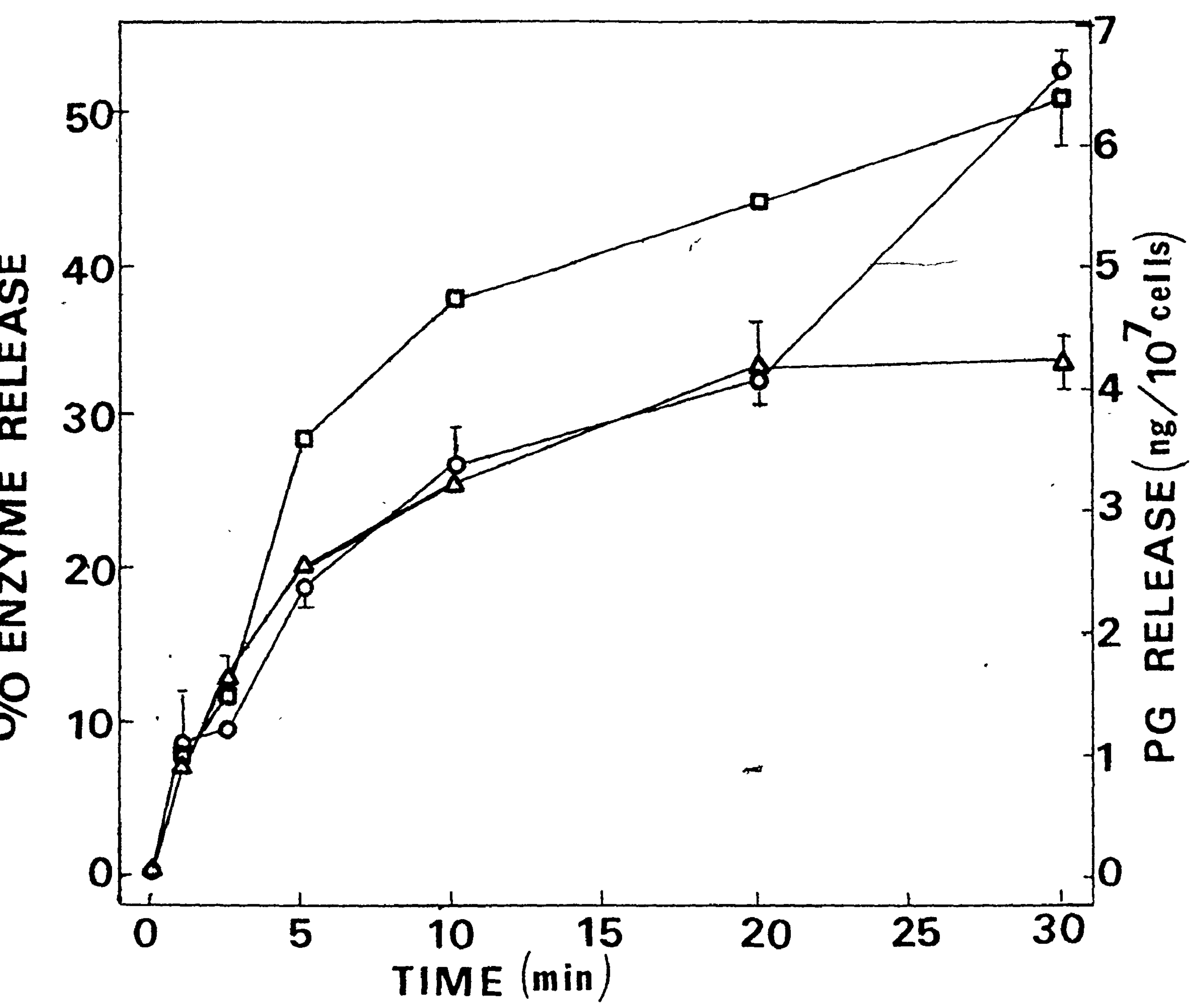


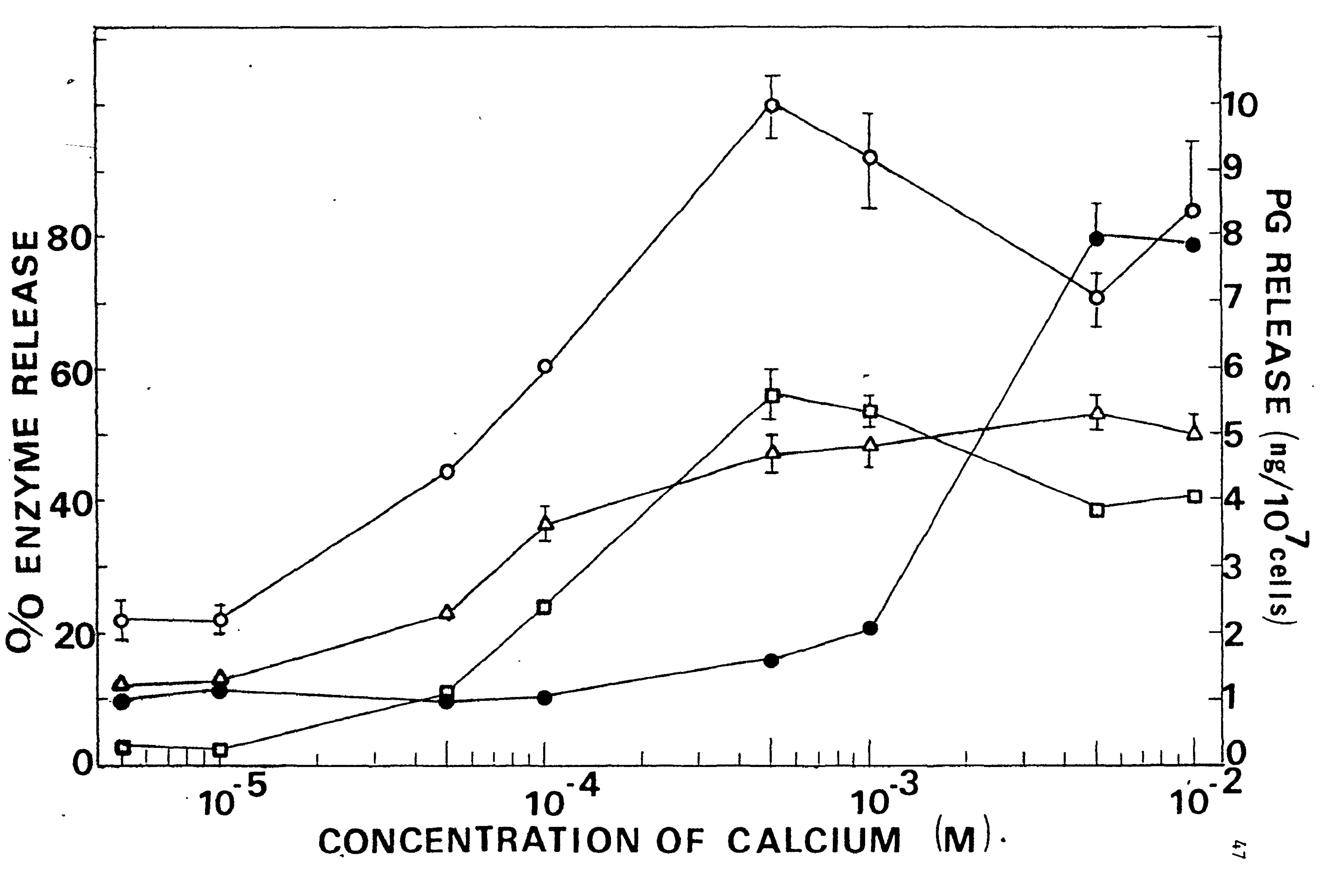
Figure 11. Effects of PMA on lysosomal enzyme and PG release from PMNs. PMNs (4.1×10^6 cells/ml) were incubated in the presence of PMA (20 ng/ml) for the indicated times after which the cell-free supernatants were assayed for PG (o—o), β-glucuronidase (□—□), and lysozyme (Δ—Δ) levels. Results represent the mean ± one-half the range for duplicate determinations.

Figure 12. Time course of PG and lysosomal enzyme release from PMNs induced by A23187. PMNs (4.6×10^6 cells/ml) were incubated in HBSS containing 1 mM Ca^{2+} and 0.7 mM Mg^{2+} in the presence of 10 μM A23187. Samples were removed at the indicated times and the cell-free supernatants assayed for PG (o--o), β-glucuronidase (Δ--Δ), and lysozyme (□--□) content. Results represent the mean ± one-half the range for duplicate determinations.



O/O ENZYME RELEASE
PG RELEASE (ng/10^7 cells)
TIME (min)
0
5
10
15
20
25
30
10
20
30
40
50
1
2
3
4
5
6
7

Figure 13. Dependence of A23187-induced PG and lysosomal enzyme release on Ca^{2+} concentration. PMNs (3.4×10^6 cells/ml) were exposed to 10 μM A23187 in the presence of the indicated concentrations of Ca^{2+} for 20 minutes. (o—o) PG release, (□—□) β-glucuronidase release, (Δ--Δ) lysozyme release, (●—●) lactic dehydrogenase release. Results represent mean ± SEM for triplicate determinations.



% ENZYME RELEASE
0
20
40
60
80
PG RELEASE (ng/10^7 cells)
0
1
2
3
4
5
6
7
8
9
10
10^-5
10^-4
10^-3
10^-2
CONCENTRATION OF CALCIUM (M)

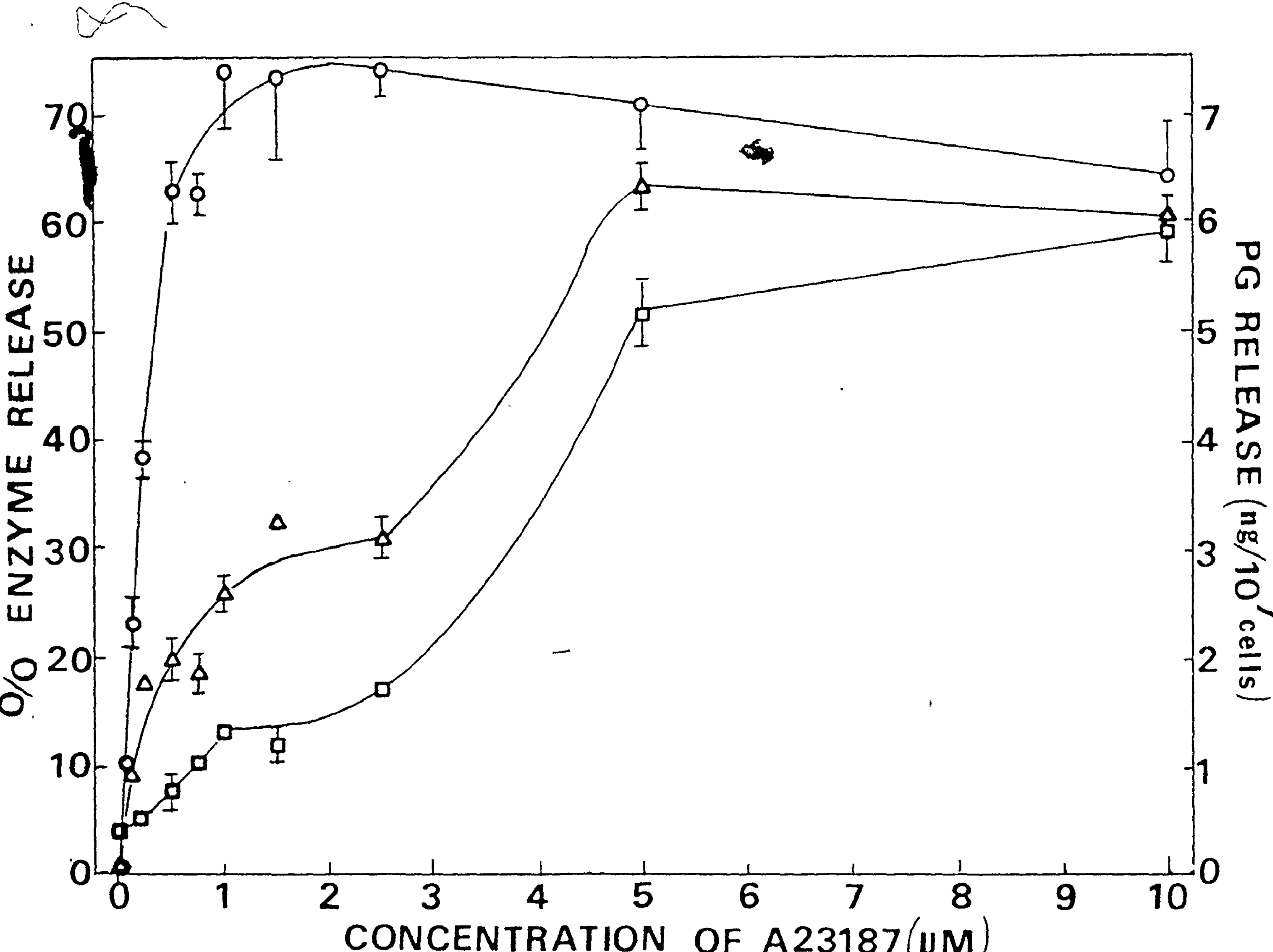
maximum at 5×10^{-4} M Ca^{2+}. Above this concentration there was no significant change in lysozyme release whereas PG and β-glucuronidase release decreased. This decrease was apparently due to loss of cell viability as evidenced by the dramatic increase in lactic dehydrogenase release.

The release of PGs and lysosomal enzymes as a function of A23187 concentration was also studied. As shown in Figure 14, there appeared to be major differences between PG release and lysosomal enzyme release. PG release was maximal at an ionophore concentration of 1.0-1.5 μM and decreased slightly at higher ionophore concentrations. Lysosomal enzyme release, however, took place in two stages. At low ionophore concentrations (less than 2.5 μM) there was a modest enzyme release, reaching a maximum of approximately 15% of the total β-glucuronidase content of the cell and approximately 30% of the lysozyme content of the cell. At ionophore concentrations greater than 2.5 μM there was a much greater release of enzyme reaching a maximum of approximately 70% of the total lysosomal enzyme content of the cell at A23187 concentration greater than 5.0 μM. In a second experiment release of β-glucuronidase and PGs at low ionophore concentrations was examined more closely. As shown in Figure 15, this phase of β-glucuronidase release closely paralleled PG release.

(b) PG release with X537A

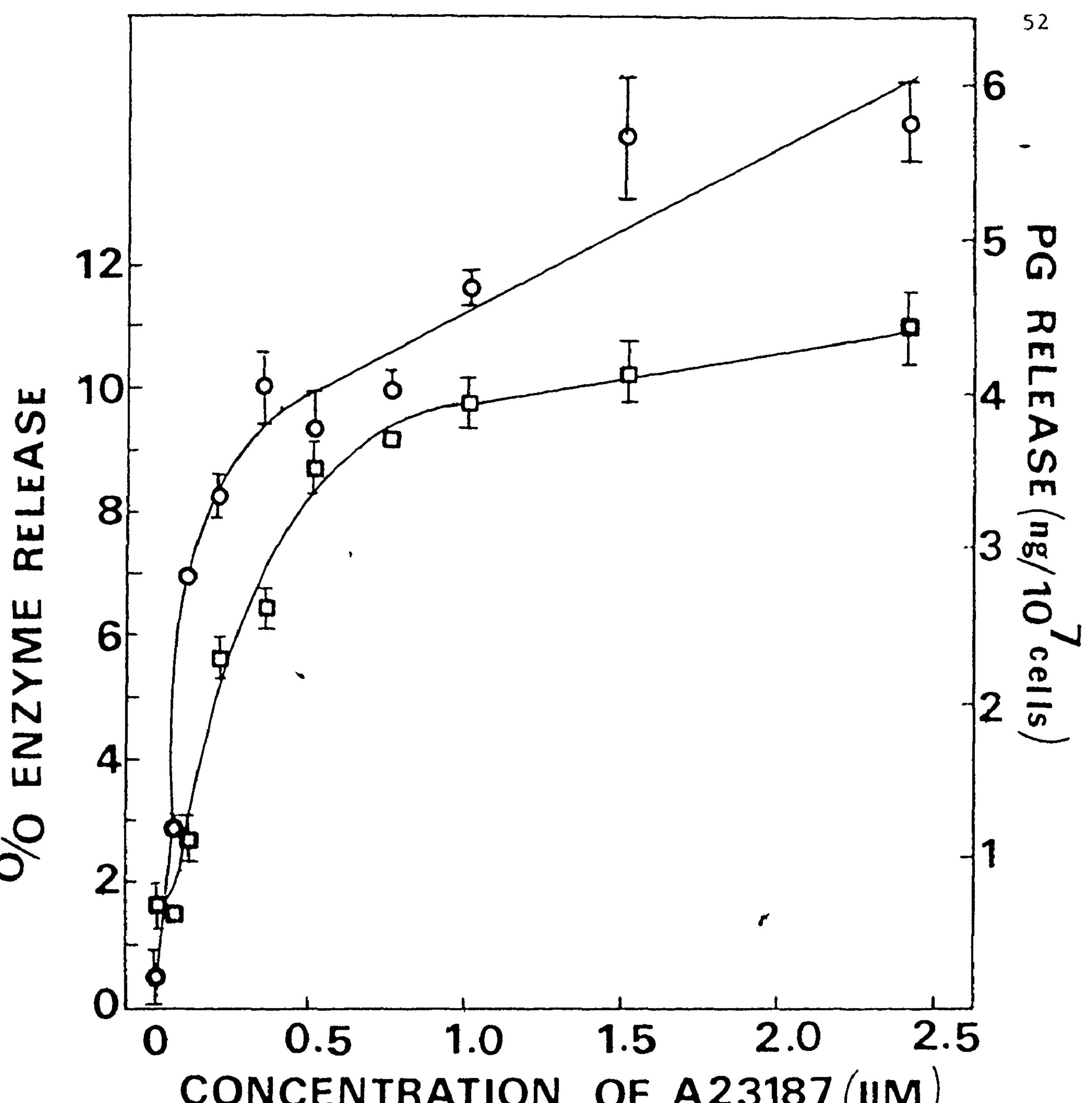
Figure 16 shows the effects of the ionophore X537A on PG and lysosomal enzyme release from PMNs. The experimental detail was similar

Figure 14. Dependence of PG and lysosomal enzyme release on A23187 concentration. PMNs (4.4×10^6 cells/ml) were incubated with the indicated concentrations of A23187 and 1 mM Ca^{2+} for 20 min. after which cell-free supernatants were assayed for PG (○--○), β-glucuronidase (□--□), and lysozyme (Δ--Δ) levels. Results represent the mean ± SEM for triplicate determinations.



% ENZYME RELEASE
0
10
20
30
40
50
60
70
PG RELEASE (ng/10^7 cells)
0
1
2
3
4
5
6
7
CONCENTRATION OF A23187 (µM)
0
1
2
3
4
5
6
7
8
9
10

Figure 15. Correlation between PG and β-glucuronidase release from PMNs by low concentrations of A23187. PMNs ($3.5x10^6$ cells/ml) were incubated in the presence of the indicated concentrations of A23187 and 1 mM Ca^{2+} for 20 min. PG (o--o), and β-glucuronidase (□--□), levels were determined in cell-free supernatants. Results represent mean ± SEM for triplicate determinations.


% ENZYME RELEASE
PG RELEASE (ng/10⁷ cells)
CONCENTRATION OF A23187 (μM)
0
2
4
6
8
10
12
1
2
3
4
5
6
0
0.5
1.0
1.5
2.0
2.5

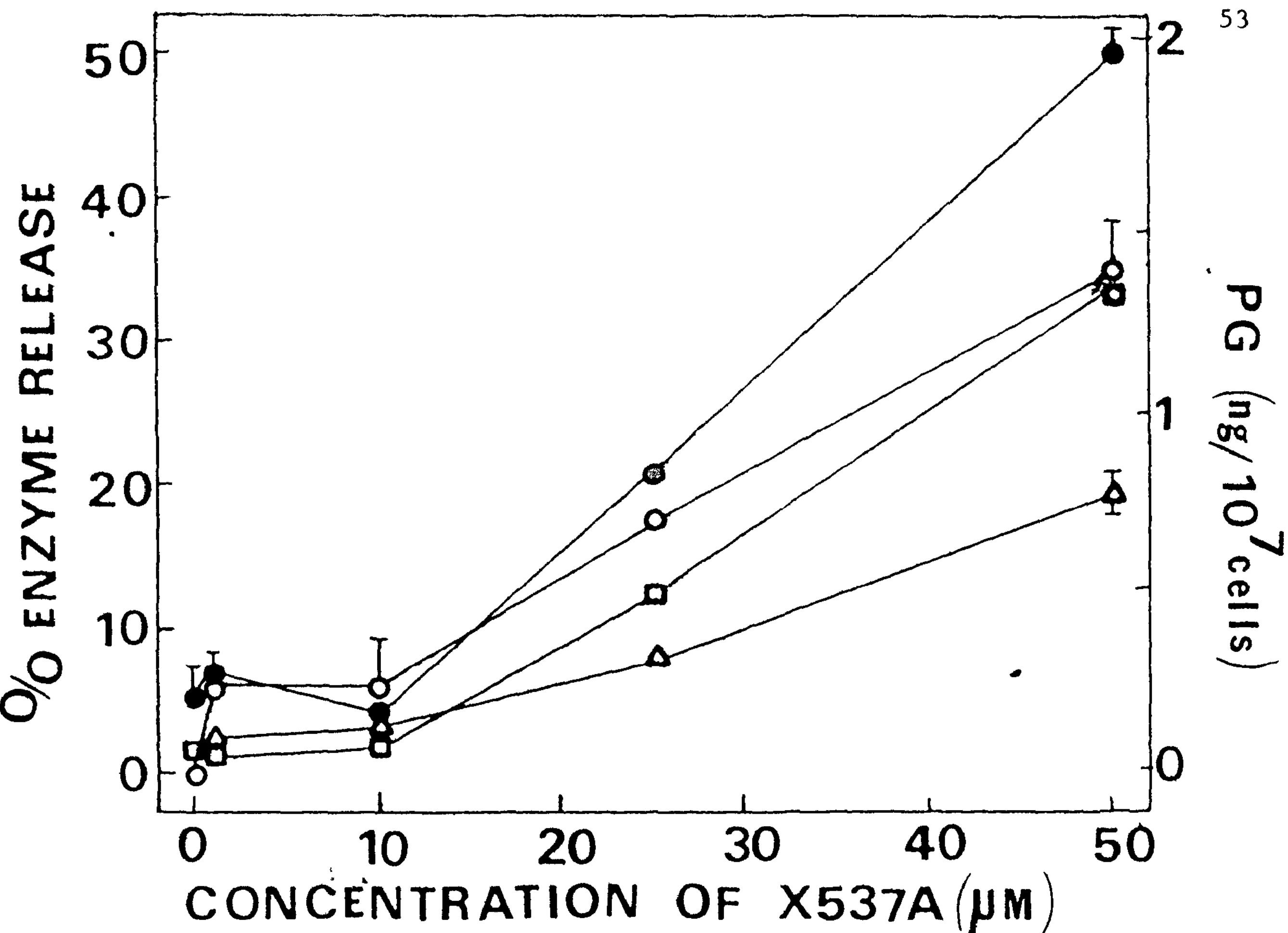


Figure 16. PG and lysosomal enzyme release induced by X537A. PMNs (2.85×10^6 cells/ml) were incubated in Ca^{2+}-free HBSS with the indicated concentrations of X537A for 30 min. Supernatants were then assayed for PG (o—o), β-glucuronidase (□--□), lysozyme (Δ--Δ) and lactic dehydrogenase (•—•) levels. Results represent the mean ± SEM for triplicate determinations.

to that in previous experiments with A23187 except that Ca^{2+}-free buffer was used. Release of both PGs and lysosomal enzymes occurred only at relatively high ionophore concentration, and this release appeared to be due to cell and lysosome lysis.

5. Ionophore Induced Respiratory Burst

While X537A (20 μM) was ineffective in inducing lysosomal enzyme release, it did however induce a respiratory burst (Figure 17) comparable to that induced by 10 μM A23187. Similar results have been reported by others (Romeo et al., 1975 ; Zabucchi and Romeo, 1976). Both ionophores were less effective than zymosan in stimulating respiration.

6. Activation of PG Synthetase

Higgs et al. (1973) suggested that PG synthetase was activated during phagocytosis. We tested this hypothesis on our system using whole cells and assaying for PGE_1 and PGA_1 release. Cells were incubated in the presence of a phagocytic stimulus with or without PG precursor. As shown in Figure 18, in the presence of zymosan particles or 10 μM A23187, 7-8 ng of $PG/10^7$ cells were released while resting cells in the presence of dihomo-γ-linolenic acid released 13 ng $PG/10^7$ cells. In the presence of both zymosan and precursor or A23187 and precursor, release was only slightly enhanced over release with precursor alone.

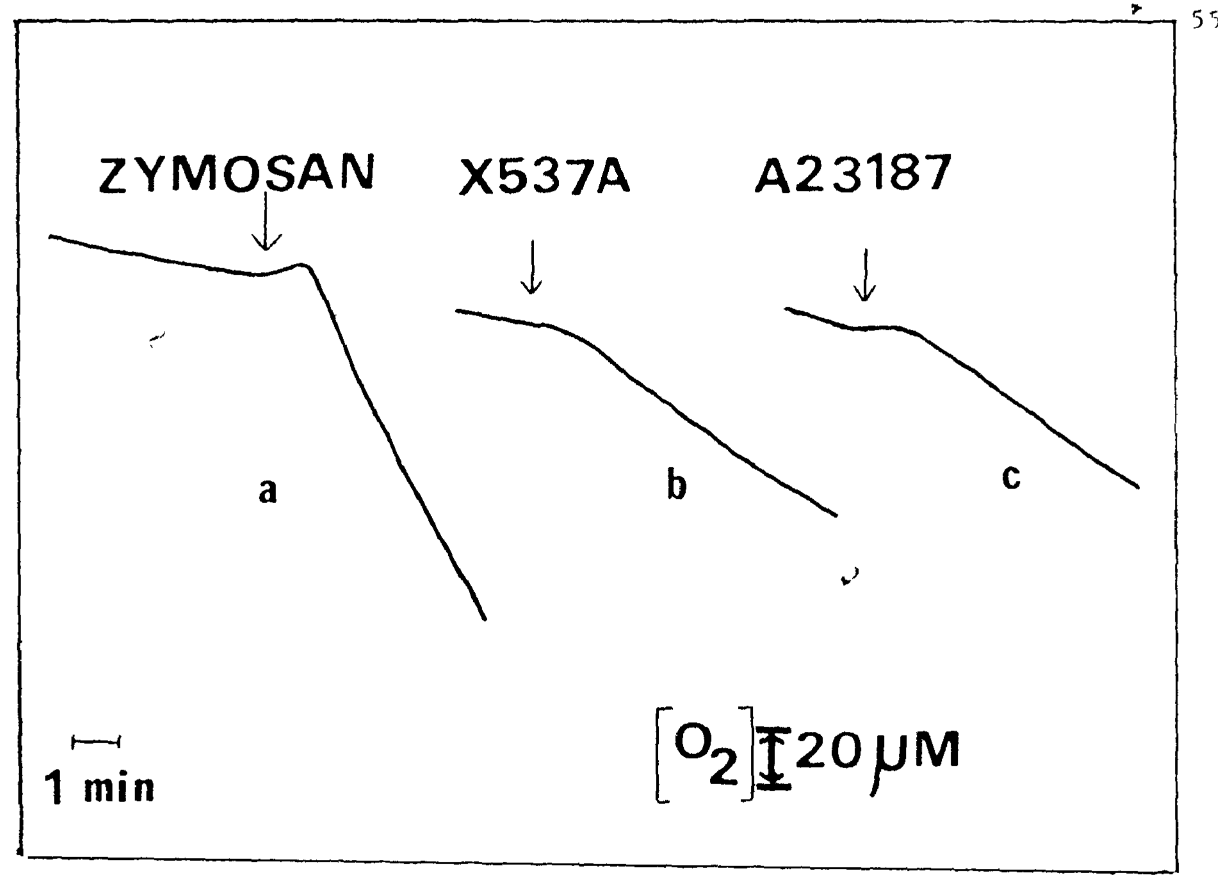


Figure 17. Polarographic traces of oxygen consumption by PMNs exposed to opsonized zymosan and ionophores. PMNs (2.3×10^7 cells) were suspended in 2 ml of HBSS containing 1 mM Ca^{2+} (a and b) or in 2 ml of Ca^{2+}-free HBSS (b). After incubating for 5 min. 0.2 ml of opsonized zymosan suspension was added to (a), 40 nmoles of X537A in 0.2 ml of Ca^{2+}-free HBSS to (b), and 20 nmoles of A23187 in 0.2 ml of HBSS to (c). The oxygen uptake was monitored with a Clark oxygen electrode.

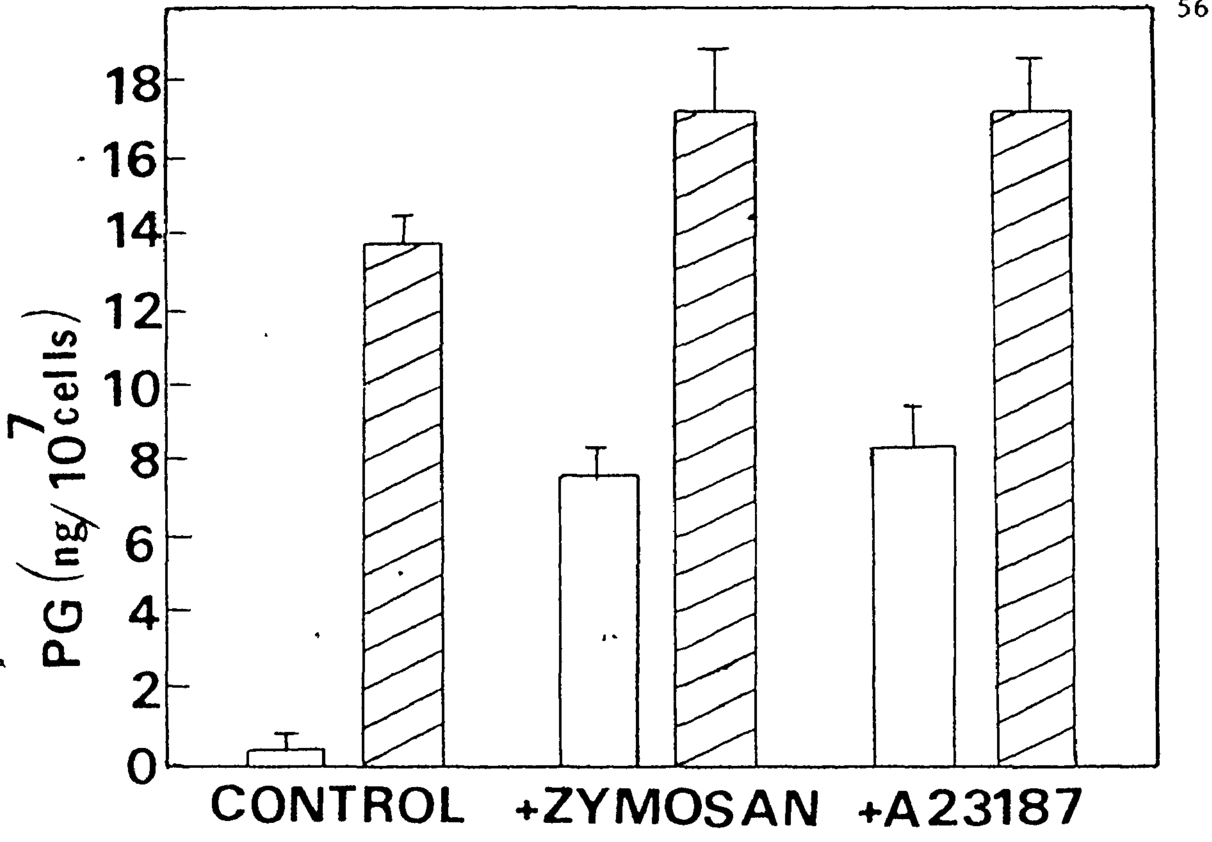


Figure 18. PG release induced by phagocytic stimuli in the presence and absence of exogenous PG precursor. PMNs (3.25×10^6 cells/ml) were incubated in the presence [hatched] or absence [open] of 10 μg/ml dihomo-γ-linolenic acid and without phagocytic stimulus (control), in the presence of opsonized zymosan or in the presence of 5 μM A23187. Results represent mean ± SEM for triplicate determinations.

7. Effects of PG Synthesis on Cell Function

Table II shows the effects of a number of concentrations of exogenously added PGE_1 on β-glucuronidase release from PMNs phagocytizing zymosan. As reported elsewhere (Zurier et al., 1973), PGE_1 inhibits lysosomal enzyme release. However, below a concentration of 5 μg/ml the inhibition is modest and at a concentration of 0.05 μg/ml it is negligible. PGE_2 shows comparable effects while $PGF_{2\alpha}$ does not appreciably affect PG release at concentrations below 25 μg/ml. The maximum PG release from PMNs detected was less than 25 ng/ml at cell concentrations of $5x10^6$/ml or less. Thus, it is unlikely that PG released by PMNs has any significant inhibitory effect on lysosomal enzyme release under physiological conditions.

It has recently become evident that in some cell types PG intermediates, such as the endoperoxides, and products other than PGs derived from the endoperoxides, such as thromboxanes, have more marked effects on cell function than PGs themselves (Samuelsson et al., 1975). Therefore it was reasoned that while PMNs released PGs at concentrations too low to affect cell function, they may have released endoperoxides and thromboxanes at high enough levels to have major effects on cell activities. However, as shown in Table III, indomethacin which inhibits the formation of the endoperoxides had no effect on β-glucuronidase release from PMNs phagocytizing zymosan.

To allow greater synthesis of endoperoxides and thromboxanes, PMNs were preincubated with precursors prior to the addition of zymosan.

TABLE II

Inhibition of β-glucuronidase release from PMNs by PGE_1.

PGE_1 conc. (μg/ml)	% Inhibition
0.05	0.6±0.5
0.10	3.5±1.0
1.0	7.8±1.4
5.0	7.1±1.9
10.0	21.9±1.7
25.0	33.2±1.1

PMNs (5.2×10^6 cells/ml) were pretreated with CB, preincubated with the indicated amounts of PGE_1 for 30 min. and then exposed to zymosan for 60 min. Results represent the mean ± SEM for triplicate determinations.

TABLE III

Inhibition of lysosomal enzyme release by fatty acids.

Agent	β-glucuronidase Release (% control)	Lysozyme Release (% control)
none (control)	100.0±0.9	100.0±4.4
indomethacin (10 μg/ml)	99.3±4.2	100.7±3.9
PGE_1 (10 μg/ml)	74.5±2.3	86.6±3.5
PGE_1 (25 μg/ml)	79.1±2.6	84.8±3.4
dihomo-γ-linolenic acid (25 μg/ml)	79.1±2.6	109.2±2.4
dihomo-γ-linolenic acid (25 μg/ml) + indomethacin (10 μg/ml)	81.8±3.9	104.6±4.1
Arachidonic acid (25 μg/ml)	64.4±2.2	108.1±5.4
Arachidonic acid (25 μg/ml) + indomethacin (10 μg/ml)	67.3±5.3	112.7±4.7
oleic acid (25 μg/ml)	87.8±3.8	111.4±4.5
linoleic acid (25 μg/ml)	72.1±2.2	104.4±6.4
palmitic acid (25 μg/ml)	102.0±2.9	119.3±4.1

PMNs (4.37×10^6 cells/ml) were treated with CB and preincubated with the indicated agents for 15 min. Zymosan was then added and the incubations carried out for 1 hour. Results represent mean ± SEM for triplicate determinations.

As shown in Table III arachidonic acid and dihomo-γ-linolenic acid markedly inhibited β-glucuronidase release; the inhibition was comparable to that achieved with equivalent concentrations of PGE_1. However, while PGE_1 inhibited both β-glucuronidase and lysozyme release, the PG precursors had no effect on lysozyme release. Oleic and linoleic acid similarly inhibited β-glucuronidase release even though oleic acid is not a PG precursor and linoleic acid had been shown not to induce PG release (Table I). This suggested that the effect was a nonspecific effect not related to PG biosynthesis. Further evidence for this was provided by the fact that indomethacin did not inhibit the fatty acid effect.

IV DISCUSSION

This study has shown that resting whole cell preparations of PMNs release significant quantities of PGs if provided with exogenous precursor (Figures 2 and 4). Previous studies (McCall and Youlten, 1973) have demonstrated that sonicates of PMNs incubated in the presence of exogenous precursor and cofactors also synthesize PGs. It is therefore possible that the synthesis which we attributed to whole cells was due to contamination with broken or damaged cells. Indeed as shown in Figures 5 and 6, cell sonicates synthesized approximately 3 times more PG than did whole cells. It seems unlikely though that the few non-viable cells in our whole cell preparations (less than 5%) could account for one third of the synthesis seen with cell sonicates.

As previously mentioned, fatty acids can induce phagocytosis-like responses in PMNs; however at the concentrations used in this study, there was no evidence of lysosomal enzyme release or increased oxygen consumption, indicating that the PG release occurred independently of phagocytosis. Mason et al. (1972) have shown that PMNs phagocytizing unsaturated fatty acids incorporated into paraffin oil emulsions readily peroxidized the fatty acids, forming a number of products, including malonyldialdehyde, a product produced during PG biosynthesis. Indeed, a colorimetric assay for malonyldialdehyde has been used as an indication of PG biosynthesis in a number of systems (Flower et al., 1973).

It is possible that PG-like oxidation products capable of reacting with the PGB_1-antibody used in our study are also formed during this non-enzymatic peroxidation of fatty acids. However in our system, indomethacin, a known PG synthetase inhibitor, strongly inhibited the release of PG, providing strong evidence that the material measured by the radioimmunoassay was indeed PG. Moreover, release was not observed with fatty acids which are not PG precursors (Table 1) and as shown in Figure 5, the PG-like material behaved chromatographically like authentic PGE_2 and $PGF_{2\alpha}$. Thus it appears likely that exogenous PG precursors readily gain access to PMNs where they stimulate PG synthesis. In several other whole cell systems, the introduction of exogenous precursor has also led to increased PG release. The infusion of arachidonic acid through frog intestine (Bartels et al., 1970) or through guinea pig lung (Vargaftig and Dao Hai, 1972) and the addition of arachidonic acid to platelet suspensions (Silver et al., 1973) stimulated PGE_2 release.

The release of PGs by intact resting PMNs provides a convenient system in which to study PG synthesis. It allows one to examine PG release independently of phagocytosis and to look at the effects of pharmacological agents on PG release per se rather than on the phagocytic process as a whole. In addition it does not necessitate the disruption of the cells by sonication or the addition of exogenous cofactors but allows one to examine synthesis under close to physiological conditions.

PGs synthesized from exogenous precursors were not stored within the cell but appeared to be released into the medium immediately (Figure 5). This is consistent with results found for other tissues; in general PG release can be equated with PG synthesis (Piper and Vane, 1971).

As shown in Figures 5 and 6 synthesis by PMN sonicates resulted in a markedly different distribution of products compared to the synthesis by whole cells. This may have been due to the availability of cofactors. Takeguchi et al. (1971) found that in microsomal preparations of bovine seminal vesicles the $PGE_2:PGF_{2\alpha}$ ratio synthesized using hydroquinone as a cofactor was 11:1 whereas using epinephrine as a cofactor the ratio was 13:12. Since in our experiments hydroquinone was added as a cofactor to cell sonicates, the high $PGE_2:PGF_{2\alpha}$ ratio observed is consistent with these findings. Exogenous cofactors were not added to the whole cell preparations in the experiment illustrated in Figure 5. The natural intracellular cofactors are not known, but the catecholamines are considered likely candidates (Sih and Takeguchi, 1973). The greater PG synthesis by cell sonicates compared to synthesis by whole cells may have been due to the readily available supply of exogenous cofactors. The addition of GSH and hydroquinone to whole cell suspensions did not increase PG synthesis from exogenous dihomo-γ-linolenic acid as measured by RIA (data not shown) suggesting that cofactor availability was not a limiting factor. However it was not known if these cofactors were taken up by the cells. The release of PGs from resting cells was also

not affected by cyclic nucleotides or hormones known to elevate their intracellular levels.

The time course of PG release from whole cells in the presence of exogenous precursor (Figure 2) is similar to that observed with microsomal preparations of bovine seminal vesicles (Takeguchi et al., 1971) and with acetone powder preparations of sheep vesicular glands (Smith and Lands, 1972). In both of these systems PG synthesis in the presence of exogenous precursor and cofactors occurred for less than 10 min. In the latter case the cessation of synthesis was not due to product inhibition, but rather to a self-catalyzed destruction of the PG synthetase itself. This may be a universal property of PG synthetases.

The release of PGs by cells in the presence of exogenous precursor indicates that substrate availability is a rate limiting factor in PG synthesis. This implies that PG release during phagocytosis is due to increased substrate availability, probably through release or activation of a phospholipase or lipase. Considerably more PG was released and at a much faster rate by resting cells in the presence of added precursor than from phagocytizing cells without added precursor (Figures 2 and 7). The levels of PG precursors in PMN phospholipids are relatively low. Stossel et al. (1974) found that the molar fractions of arachidonic acid and dihomo-γ-linolenic acid in human PMN phospholipids were 12.4% and less than 1% respectively, while in guinea pig PMN phospholipids the molar fraction of arachidonic acid was less than 3%. This may account for the low yield of PGs by phagocytizing cells.

There was no evidence to suggest that PG synthetase was activated during phagocytosis. As shown in Figure 16, the addition of a phagocytic stimulus to cell suspensions containing exogenous precursor did not result in greatly enhanced release of PG. This is in disagreement with McCall and Youlten (1973) who found that the PG synthetase of PMNs was activated during phagocytosis. The major difference between their experiments and ours was that they measured synthesis by cell sonicates containing exogenous cofactors as opposed to synthesis by whole cells. This may have accounted for the conflicting results. Synthesis by whole cells would appear to be closer to *in vivo* conditions. Other differences between their experiments and ours included use of rabbit PMNs rather than rat PMNs, measurement of PGE_2 synthesis rather than PGE_1 and PGA_1 synthesis, use of bioassay rather than RIA to measure PG levels, and use of bacteria rather than zymosan or A23187 as the phagocytic stimulus. It seems unlikely that any of these factors would be responsible for the conflicting results.

PG release by phagocytizing cells showed many similarities to lysosomal enzyme release. Both were released over similar periods of time (Figure 8); both were released by CB-treated cells indicating that particle ingestion was not essential; both lysosomal enzyme release and PG release were inhibited to a comparable degree by dibutyryl cAMP. PG release and β-glucuronidase release were inhibited by colchicine indicating that microtubule assembly was involved. The fact that lysozyme release was not inhibited by colchicine suggests

that most, if not all, of this enzyme is located in the specific granules and that microtubule assembly is not involved (or at least not as critically) in the release of these granules. It would appear that specific granules are not involved in PG release since PMA did not induce PG release (Figure 11). One apparent difference between PG release and lysosomal enzyme release was observed when activated serum containing ε-ACA, an inhibitor of the C5a inactivator, was added to CB-treated cells. Under these conditions there was not a significant release of PG whereas β-glucuronidase was released. This would suggest that PG release was not associated with the release of the azurophil granules. The release of β-glucuronidase however was only one-half that observed when cells were exposed to opsonized zymosan so that this may not be a clear cut distinction between PG and lysosomal enzyme release.

There is considerable evidence supporting the concept that Ca^{2+} has a central role in stimulus-secretion coupling in secretory cells. It appears that Ca^{2+} influx from the cell exterior or possibly Ca^{2+} mobilization within the cell induces exocytosis by promoting the fusion of secretory granules with the plasma membrane (Douglas, 1974). The divalent ionophores X537A and A23187 have been found to promote exocytosis in a variety of secretory cells (Pressman, 1976). It is not surprising therefore that these ionophores induce degranulation of lysosomes in PMNs. The release of PGs, like the release of lysosomal enzymes appears to involve the influx of Ca^{2+}. Unlike the respiratory

burst which could be induced in Ca^{2+}-free buffer with X537A, PG release was dependent upon the presence of extracellular Ca^{2+}. This suggests that PG release was probably associated with a Ca^{2+}-induced degranulation of the lysosomes. Other explanations are possible though. For example, Ca^{2+} influx might activate a cytoplasmic phospholipase since Ca^{2+} is essential for phospholipase A activity (Hanahan, 1971). However PG and lysosomal enzyme release by A23187 showed similar time courses and dependency on Ca^{2+} concentration, suggesting that the two events were closely related.

One possible explanation for the biphasic nature of the curve showing the dependency of lysosomal enzyme release on A23187 concentration (Figure 14) is that there may have been two types of cells present, one of which was responsible for PG release and for the release of lysosomal enzymes at low ionophore concentrations, and the other responsible for enzyme release at higher concentrations. However this would imply that PMNs were not responsible for the PG release because the bulk of the enzyme release occurred at higher ionophore concentrations and must have been due to PMNs since they were the predominant cell type and they are known to be rich in lysosomal enzymes. Moreover, lysosomal enzyme release at higher ionophore concentrations was very similar to PG release with respect to time course and dependency on Ca^{2+} concentration. If two types of cells had been involved, one would not have expected this close relationship.

The major contaminating cells in our PMN preparations were macrophages and lymphocytes. Cultured macrophages have been shown to release PGs (Bray et al., 1974) and they contain numerous lysosomal granules. Our preliminary studies on rat macrophages (obtained from 4 day peritoneal exudates) showed that during phagocytosis of opsonized zymosan, cells released only 2 ng PG/10^7 cells, which would not be sufficient to account for the PG release from PMN preparations. The effect of A23187 on PG release in the macrophage was not examined however. Cultured lymphocytes have also been shown to release PGs (Ferraris and DeRubertis, 1974), but because the amounts released were relatively small and because there were so few lymphocytes in our PMN preparations they are not a likely source. Platelets contain lysosomal enzymes and can release relatively large quantities of PGs. Moreover, the ionophore A23187 has been shown to induce a release reaction in platelets (Feinstein and Fraser, 1975). However, platelets were not detected in our cell preparations.

It seems likely therefore that PGs were indeed released by the PMNs. The question of why PG release, which resembled lysosomal enzyme release in many other ways, was not biphasic then arises. One possible explanation is that PG release had reached its maximum at low ionophore concentrations. Because of the limited amount of dihomo-γ-linolenic acid present in PMN phospholipids, substrate availability may have become limiting.

Because of the close relationship between lysosomal enzyme and

PG release it would appear that release of a lysosomal phospholipase is responsible for the increased PG synthesis. However there is no direct evidence for the involvement of such a phospholipase. Experiments with quinacrine, which is purported to be a phospholipase A inhibitor (Vargaftig and Dao Hai, 1972), failed to show inhibition of PG release from phagocytizing PMNs or cells exposed to A23187. However, while quinacrine appeared to be readily taken up by the cells, its effect on PMN phospholipase was not determined. Hence the experiments were inconclusive. Since PMA, which causes degranulation of specific granules, did not induce PG release, this would imply that lysosomal phospholipase activity is associated entirely with the azurophil granules. In rabbit peritoneal PMNs, phospholipase A activity was found to be equally distributed between the two types of granules (Franson et al., 1974). However, in light of the apparent differences among species in alkaline phosphatase and lysozyme distribution (Spitznagel et al., 1974) it is possible that in rat PMNs phospholipase A activity is indeed restricted to the azurophil granules. In order to satisfactorily answer the question of whether a phospholipase is involved, phospholipase activity itself would have to be assayed and the effects of its activators, inhibitors etc. on PG release determined.

The release of PGs by PMNs does not appear to have major effects on PMNs themselves. Although Bourne et al.(1970) showed that PGs at concentrations as low as 10^{-7} M (approximately 35 ng/ml) led to increased intracellular levels of cAMP in human blood PMNs, considerably

higher concentrations appear to be necessary to affect lysosomal enzyme release (Table II and Zurier et al., 1973) and particle uptake (Cox and Karnovsky, 1973). In our in vitro system, using approximately $5x10^6$ cells/ml, these levels were not attained even in the presence of exogenous precursor. While addition of PG precursors to cell suspensions did inhibit lysosomal enzyme release when the cells were subsequently exposed to a phagocytic stimulus, the inhibition appeared to be a nonspecific fatty acid effect. It is of interest that unsaturated fatty acids were as effective as PGs in inhibiting β-glucuronidase release. It is unlikely that they were acting via a common mechanism since only PGs inhibited lysozyme release. The PGs were presumably acting via stimulation of adenylate cyclase through specific receptors while the fatty acids were probably involved in a nonspecific membrane interaction.

It would appear that phagocytizing PMNs release sufficient quantities of PGs to account for the levels found in some inflammatory exudates such as in uveitis (Eakins et al., 1972) or in carrageenan-induced inflammation (Willis, 1969). As shown in this study the levels released can be greatly enhanced by the presence of exogenous precursors. This could be physiologically important if, as suggested by Anderson et al. (1971), lysosomal phospholipases escaping from phagocytizing cells at inflammatory sites release fatty acids from local tissue membranes. PG precursors released into the inflammatory exudate in this way could stimulate PG release from PMNs. Moreover, since

the levels of arachidonic acid found in blood plasma are comparable to those used to stimulate PG release in our studies, PMNs may also play an essential role in maintaining plasma levels of PGs.

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