

**CRYSTAL INTERACTIONS WITH ISOLATED HUMAN NEUTROPHILS:
EFFECT OF ADSORBED PROTEINS**

by

SEEMA JAYANT GADKARI

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Seema Jayant Gadkari

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FACULTY
~~Department~~ of PHARMACEUTICAL SCIENCES

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Crystal deposition diseases such as gout and pseudogout are characterized by the deposition of monosodium urate monohydrate (MSUM) and calcium pyrophosphate dihydrate (CPPD) crystals respectively in the joints. Inflammation arises from the interaction of these crystals with the synoviocytes and polymorphonuclear leukocytes or neutrophils. We have studied the neutrophil responses to stimulation by MSUM and CPPD crystals and the effect of various protein coatings on the crystal surface on the neutrophil responses. The neutrophil responses studied were the respiratory burst accompanying phagocytosis leading to production of superoxide anion and generation of chemiluminescence. Degranulation of the neutrophil was also studied by monitoring the release of myeloperoxidase and lysozyme enzymes.

Both MSUM and CPPD induced the generation of superoxide anion to the same degree although MSUM induced a faster rate of production of superoxide anion than CPPD. The reduction of ferricytochrome c was superoxide dismutase inhibitable for CPPD/neutrophil incubations but not inhibitable for MSUM/neutrophil incubations, probably due to inactivation of superoxide dismutase by adsorption of the protein onto MSUM crystals. Precoating of MSUM and CPPD crystals with Immunoglobulin G, Bovine serum albumin

or plasma proteins did not influence the generation of superoxide significantly.

The generation of luminol enhanced neutrophil chemiluminescence induced by MSUM was of a greater magnitude and the maximal response was attained faster than for CPPD crystals. Precoating MSUM or CPPD crystals with immunoglobulin G enhanced the chemiluminescence response while plasma precoating inhibited the chemiluminescence response.

Release of myeloperoxidase induced by MSUM crystals could not be measured due to adsorption of myeloperoxidase by MSUM. Precoating MSUM crystals with proteins did not influence the adsorption of myeloperoxidase. Hence degranulation was monitored by measuring the release of lysozyme induced by MSUM and CPPD crystals. Lysozyme was also found to adsorb onto MSUM crystals although less extensively than myeloperoxidase. There was no significant effect of protein coating on MSUM and CPPD induced lysozyme release.

The particulate stimulants MSUM and CPPD produced activation of neutrophils with superoxide release, chemiluminescence generation and degranulation. Neutrophil responses, and in particular the chemiluminescence responses, to the crystals could be modulated by the nature of the protein adsorbed to the crystal surface.

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LIST OF ABBREVIATIONS

A	Change in absorbance
A ₄₅₀	Absorbance at 450 nanometers
A ₅₅₀	Absorbance at 550 nanometers
ANOVA	Analysis of Variance
AUC	Area under the curve
BSA	Bovine serum albumin
CDPP	Calcium dihydrogen pyrophosphate
CL	Chemiluminescence
CPPD	Calcium pyrophosphate dihydrate
DSC	Differential scanning calorimetry
μm	micrometers
g	Gravity
HDL	High density lipoproteins
HP	Hewlett Packard
Ig G	Immunoglobulin G
KV	Kilovolts

LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LYZ	Lysozyme
mA	milliamperes
MPO	Myeloperoxidase
MSUM	Monosodium urate monohydrate
mV	millivolts
NL	Native luminescence
nm	Nanometer
nmoles	Nanomoles
O_2^-	Superoxide anion
PC	Personal computer
PMNL	Polymorphonuclear leukocytes
rpm	Revolutions per minute
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
U/mL	Units per milliliter
UBC	University of British Columbia

UV Ultraviolet

V.s Volts.second

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BACKGROUND

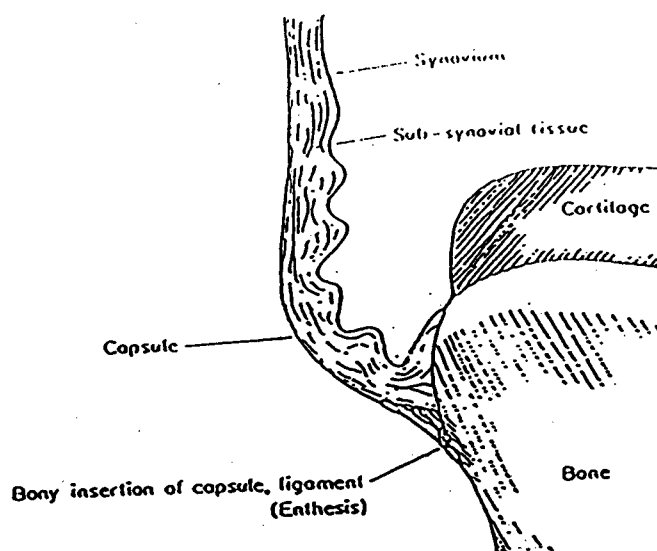
1. CRYSTAL-INDUCED ARTHRITIS

Crystal deposition disease has been defined as a pathological condition associated with the presence of crystals which then contribute to the tissue damage (Dieppe and Calvert, 1983). These crystals have been identified as being those of monosodium urate monohydrate (MSUM), calcium pyrophosphate dihydrate (CPPD) and in some cases, basic calcium phosphates (McCarty, 1989).

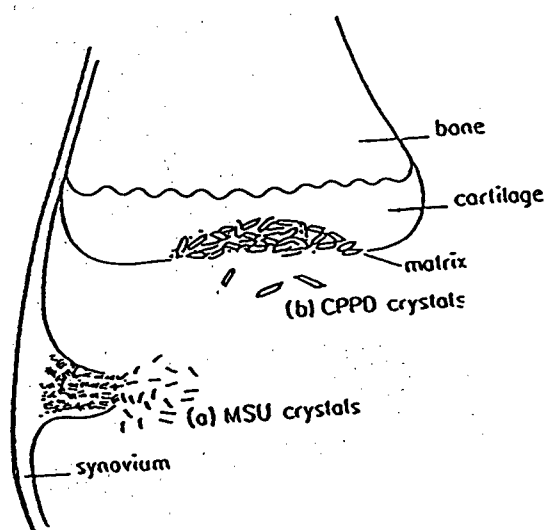
Crystal deposition diseases can be classified based on the type of crystal deposited. The condition known as gout involves deposition of MSUM crystals and pseudogout or CPPD deposition disease is characterized by the presence of CPPD, triclinic and monoclinic crystals (Ryan and McCarty, 1989).

2. DEPOSITION OF CRYSTALS

The synovial joints of the extremities (hands, feet, elbows, knees) are particularly prone to crystal deposition diseases. The structure of a synovial joint is shown in Figure 1a. The common sites for deposition of crystals are in the articular cartilage, synovium or directly in the synovial fluid of the joint. Crystals may be released into the joint fluid by the rupture of preformed synovial



a) STRUCTURE OF SYNOVIAL JOINT



b) RELEASE OF CRYSTALS INTO THE JOINT

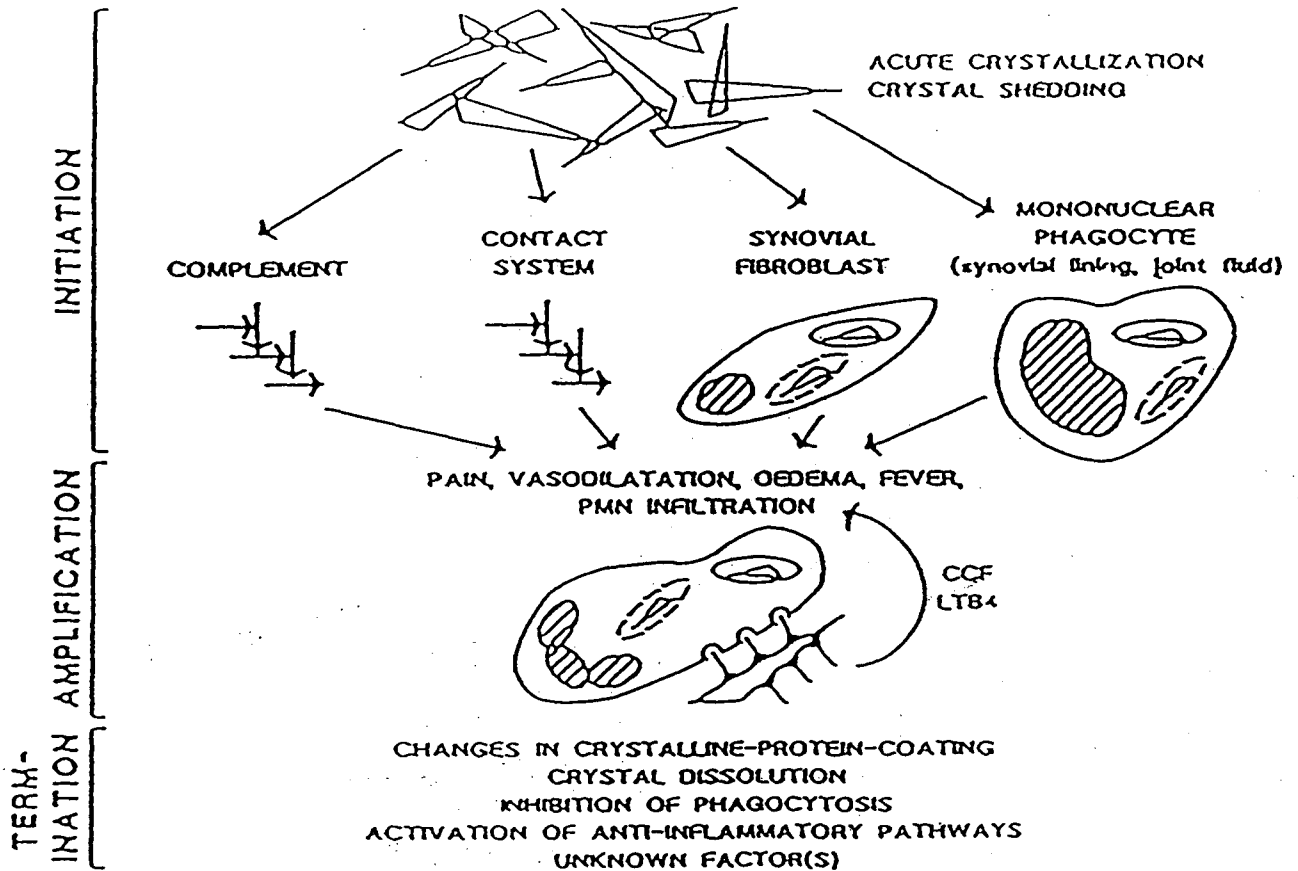
FIGURE 1

deposits, by a "crystal shedding" mechanism from the cartilage as seen in Figure 1b, or they may precipitate in the synovial fluid itself due to transient hyperuricemia in the synovium or a transient fall in temperature (McCarty, 1989).

3. MECHANISM OF CRYSTAL-INDUCED INFLAMMATION

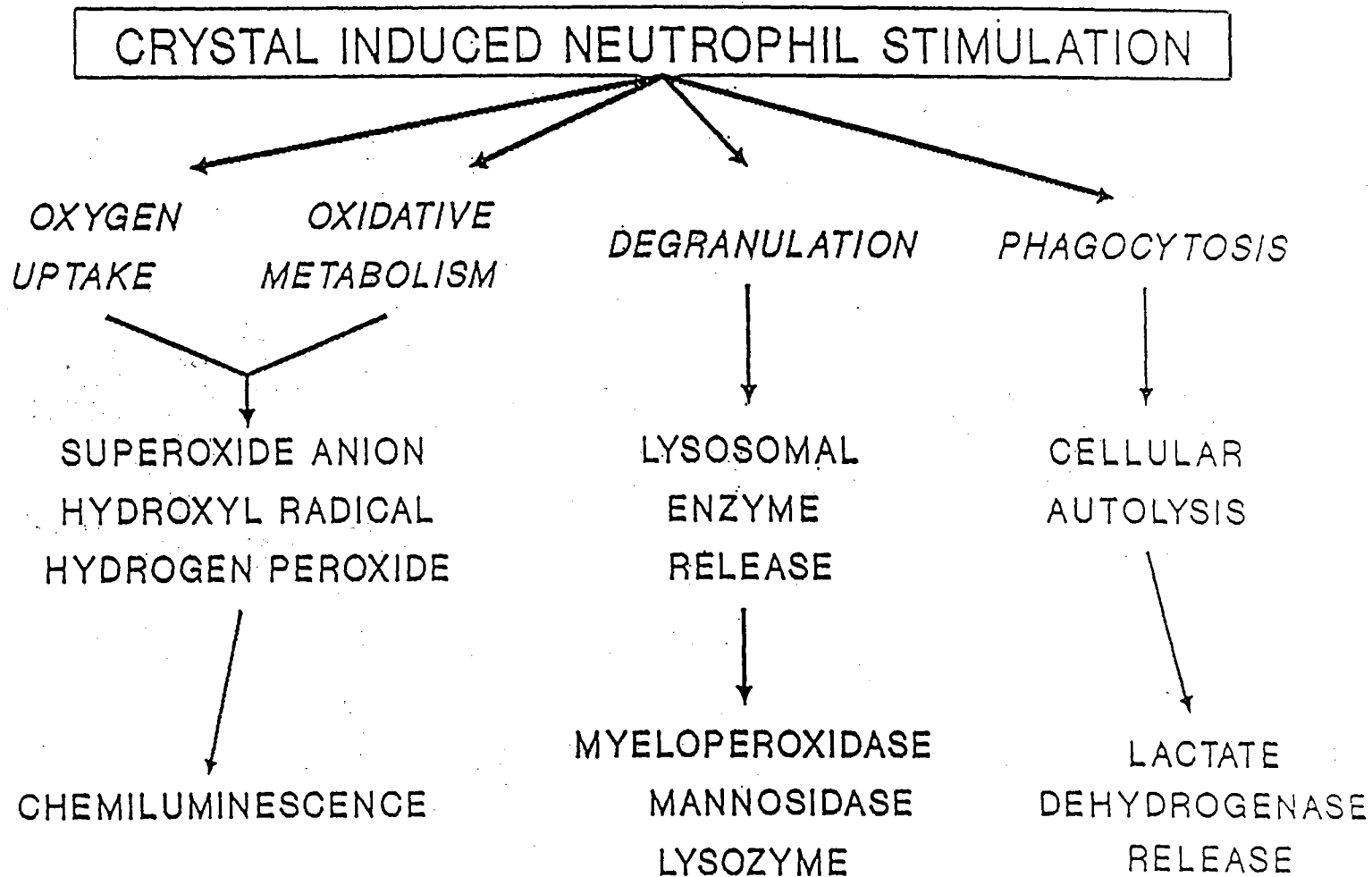
Crystal-induced inflammation involves several stages. The initial response is through interaction of the crystals with the synoviocytes or phagocytic cells lining the synovium (Wallingford and McCarty, 1971) rather than with polymorphonuclear leukocytes (PMNL). The binding of the crystals to the synoviocytes may be enhanced by adsorbed proteins such as immunoglobulin G (Ig G) (Kozin and McCarty, 1976). Crystal-induced lysis of the phagocytic synoviocytes leads to release of a crystal-induced chemotactic factor which promotes the migration of polymorphonuclear leukocytes (PMNL/neutrophils) into the area (McCarty, 1989). This chemotactic factor has been identified as a low molecular weight peptide with a molecular weight of 8,500 daltons (Phelps et al., 1981) but other reports indicate a molecular weight of 11,500 daltons (Spilberg and Mandell, 1983).

Following increased migration of PMNL into the joint, the interaction of the crystals with neutrophils plays a central role in the crystal-induced inflammatory response and is shown in Figure 2 and Scheme 1 and summarized below.



CRYSTAL NEUTROPHIL INTERACTION

FIGURE 2



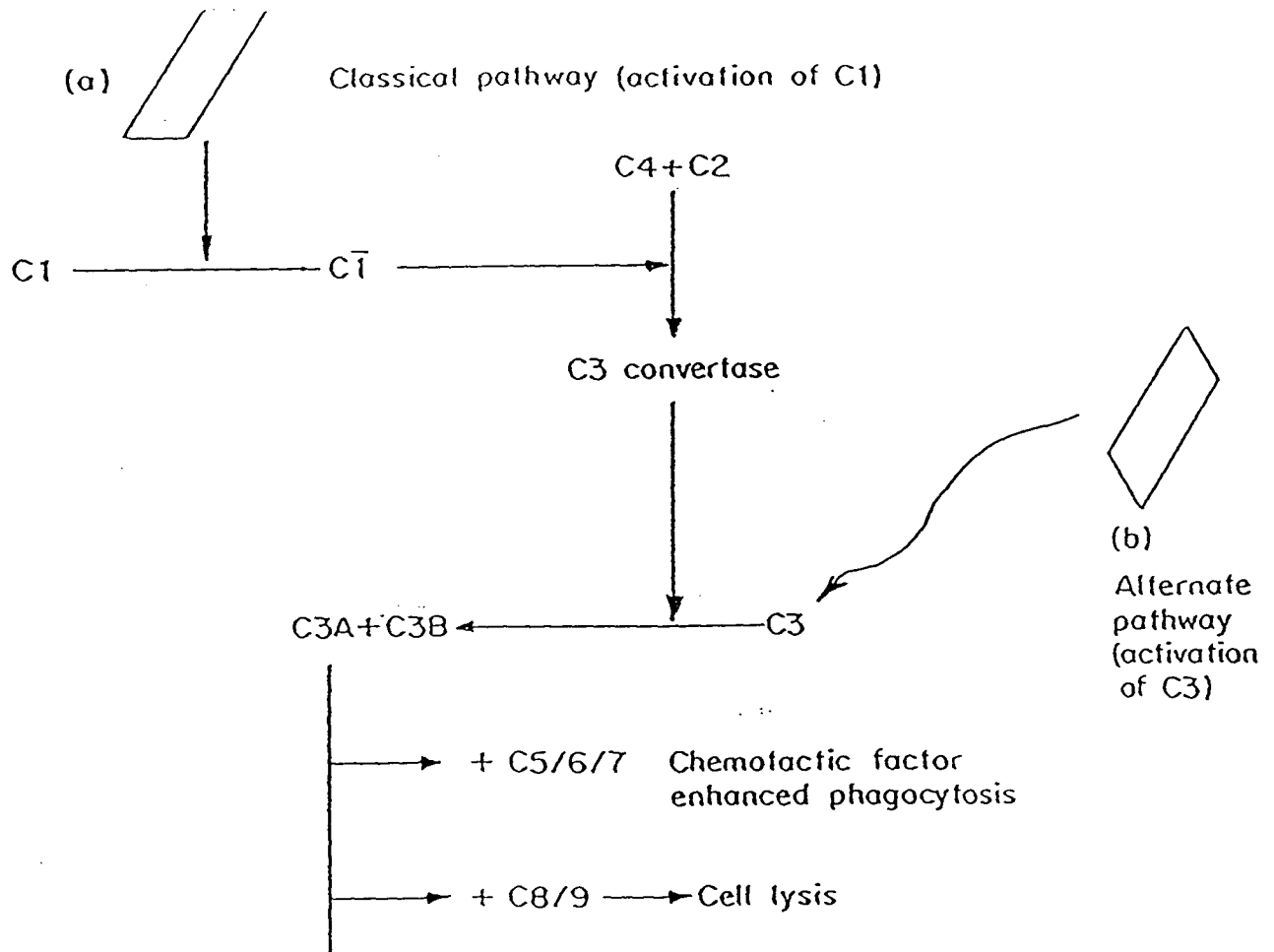
SCHEME 1

The naked crystal upon release into the joint fluid adsorbs several proteins such as immunoglobulin G (Ig G), albumin, complement fragments, clotting factors and lipoproteins from the synovial fluid (Kozin and McCarty, 1976; Hasselbacher and Schumacher, 1978; Hasselbacher, 1979a, 1979b; Terkeltaub et al., 1983). The protein coated crystal may interact with receptors on the macrophage/neutrophil plasma membrane through the mediation of the adsorbed proteins e.g. Ig G (Kozin and McCarty, 1976) and this surface stimulation of neutrophils by MSUM/CPPD crystals results in enhanced oxygen uptake and an increase in oxidative metabolism within the macrophage/neutrophil (Simchowitz et al., 1982). The crystal is phagocytosed by the PMNL and lies within the cytoplasm in a sac known as a phagosome (McCarty, 1962; Schumacher and Phelps, 1971). This is followed by fusion of the lysosomes of the PMNL with the phagosome producing a phagolysosome. Degranulation then occurs and the contents of the neutrophilic granules of the lysosomes are emptied into the phagolysosome (Shirahama and Cohen, 1974). It has been proposed that the release of the lysosomal contents within this phagolysosome results in digestion of the proteins adsorbed onto the crystal, leaving the membrane of the phagolysosome exposed to the "naked" crystal thereby allowing a crystal-membrane interaction to take place (McCarty, 1989; Gordon et al., 1988). The crystal-membrane interaction is thought to occur in two consecutive steps, crystal membrane binding followed by membrane rupture or

membranolysis (Burt and Jackson, 1988). Rupture of the phagolysosomal membrane and release of the lysosomal enzymes into the cytoplasm follows, leading to cellular autolysis and release of the crystal into the synovial fluid. The release of the cytosolic contents of the PMNL acts as a powerful stimulus for the migration of more neutrophils into the joint leading to further interaction with the crystals and potentiation of the inflammatory response (Gordon et al., 1988). This mechanism of crystal-induced cytotoxicity is known as the "perforation from within" hypothesis and was first described by Allison in his work on the toxicity of silica particles (Allison et al., 1966). Wallingford and McCarty (1971) later extended this hypothesis to the mechanism of crystal-induced inflammation.

3.1. INFLAMMATORY MEDIATORS

- a) MSUM activates the complement system by the classical and alternate pathways *in vitro* (Naff and Byers, 1973; Byers et al., 1973; Hasselbacher, 1979a, 1979b; Tenner and Cooper, 1982). *In vivo*, these activated complement factors may trigger a cascade of events eventually producing factors that enhance the chemotactic migration of neutrophils, increase phagocytosis and cause cell lysis (Scheme 2).
- b) Activation of the Hageman factor, which is factor XII of the clotting cascade, by crystals has been reported



MSUM INDUCED COMPLEMENT ACTIVATION

SCHEME 2

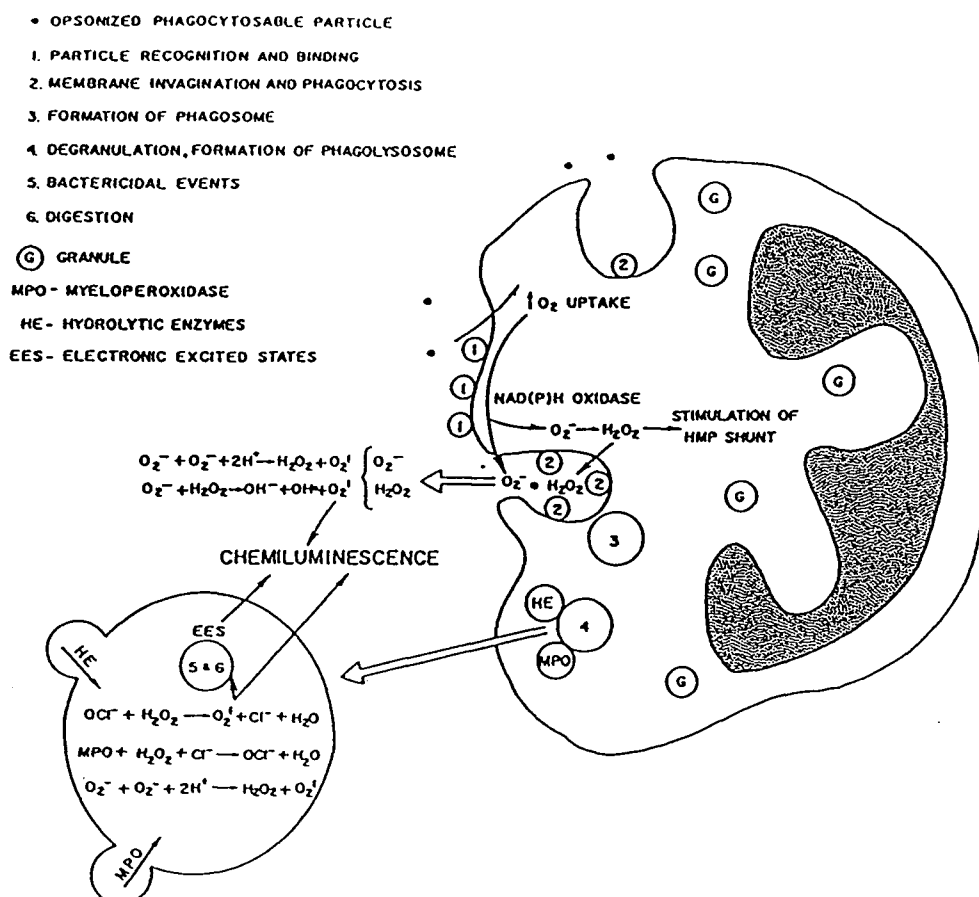
(Kellermeyer, 1967). The activated factor has been shown to activate the clotting cascade and the kinin system in vitro, the latter resulting in the formation of potent vasodilators which may enhance the inflammatory response (Ginsberg et al., 1980) but the significance of this finding in vivo has not been established (Spilberg, 1974; Green et al., 1982).

- c) MSUM is initially phagocytosed by the synoviocytes and there is an associated synthesis and release of **prostaglandins and interleukins** which cause vascular changes and which are mediators of the pain stimulus (Alwan et al., 1989; Guerne et al., 1989; Woolf and Dieppe, 1987). The release of superoxide, lysosomal enzymes and other products of the polymorphonuclear cell injury into the synovial fluid may also influence the inflammatory process (Rosen et al., 1986).

4. CRYSTAL-NEUTROPHIL INTERACTIONS

4.1. PHAGOCYTOSIS

Figure 3 is a schematic representation of phagocytosis of particles by neutrophils (Trush et al., 1978). The PMNL is stimulated on contact with the opsonized crystal through the process of particle recognition. The proteins adsorbed onto the crystal surface are believed to play an important role, in particular adsorbed Ig G on the crystal surface opsonizes the crystal thereby enhancing the neutrophil response (Roos



Schematic representation of the endocytic events and accompanying metabolic processes that occur during phagocytosis by polymorphonuclear leukocytes (PMNs). For more detailed discussions of these processes see: (a) B. D. Cheson, J. T. Curnutte, and B. M. Babior, in "Progress in Clinical Immunology" (B. S. Schwartz, ed.), Vol. 3, p. 1. Grune & Stratton, New York, 1977. PMN biochemistry. (b) S. J. Klebanoff, *Semin. Hematol.* 12, 117 (1975). PMN antimicrobial mechanisms. (c) T. P. Stossel, *N. Engl. J. Med.* 290, 717, 774, 833 (1974). General review on phagocytosis.

FIGURE 3

et al., 1981). The stimulus provided by the opsonized crystal causes invagination of the plasma membrane of the neutrophil. The lysosomes also migrate towards the invaginated areas of the plasma membrane and some of the lysosomes fuse with the plasma membrane before the particle is completely phagocytosed and discharge their contents into the extracellular medium (Weissmann et al., 1972; Hawkins, 1972; Baggiolini and Dewald, 1984).

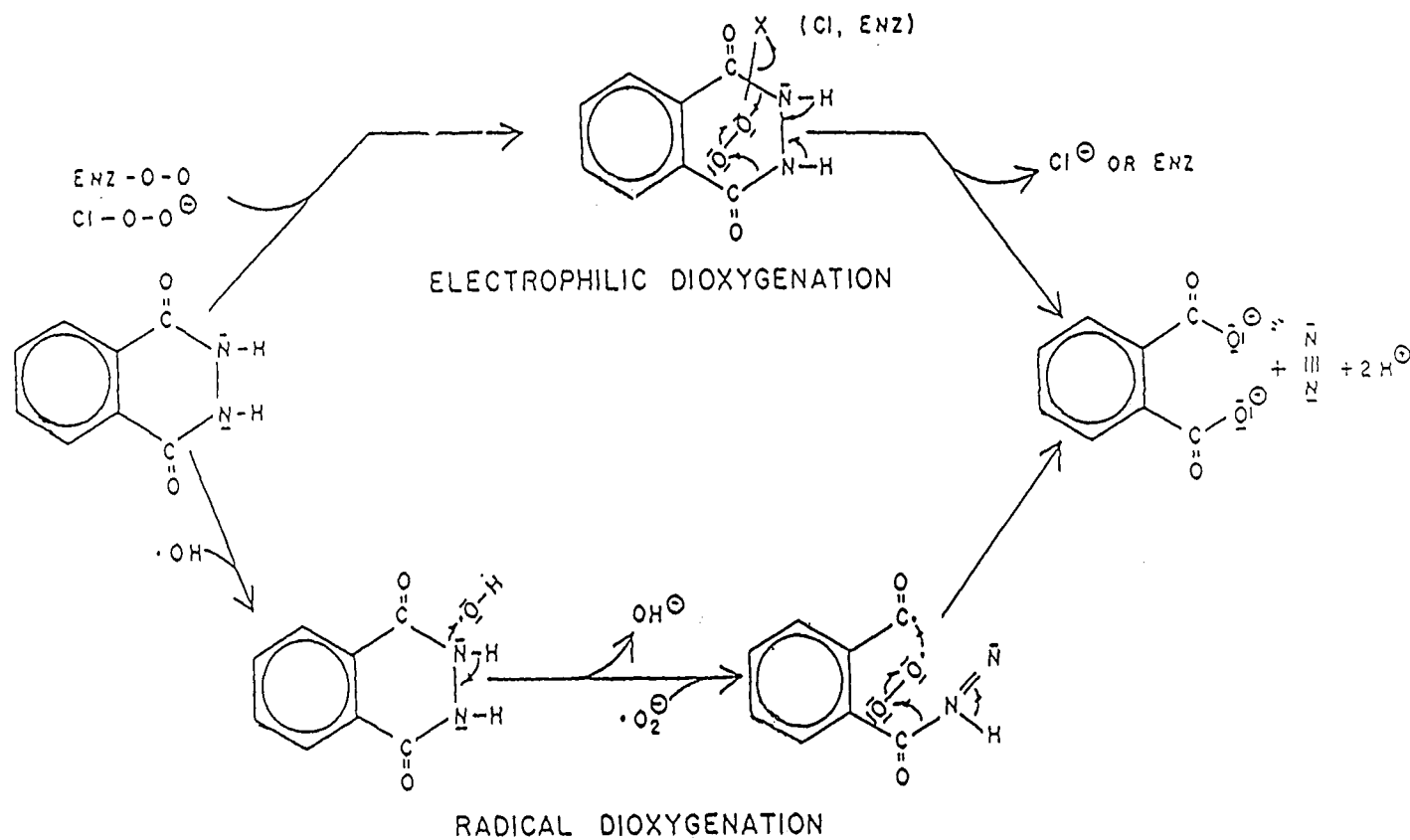
Polymorphonuclear leukocytes are capable of responding to stimulation by either particulate or soluble stimuli which produce distinct metabolic changes in the cells. PMNL normally exhibit anaerobic metabolism. However, on exposure to particulate or soluble stimuli the metabolic pattern changes and PMNL exhibit increased oxidative metabolism (Babior et al., 1973). This is manifested in terms of increased oxygen uptake and the generation of oxidation by-products and highly reactive species such as the superoxide anion, the hydroxyl radical and strong oxidizing agents such as hydrogen peroxide (Simchowitz et al., 1982). Phagocytosis of particulate stimuli by the PMNL is accompanied by this increase in oxidative metabolism which is termed the 'respiratory burst'.

4.2. COMPONENTS OF THE RESPIRATORY BURST

4.2.1. CHEMILUMINESCENCE

Chemiluminescence (CL) is the production of light as a result of a chemical reaction and PMNL exhibit CL on stimulation by particulate and non-particulate stimuli (Allen et al., 1972; Cheson et al., 1976; Westrick et al., 1980; Roschger et al., 1988; Harber and Topley, 1986). This luminescence can be measured through the use of light amplifying compounds such as luminol and lucigenin. CL reflects the excitation state of the neutrophil and thus serves as an indicator of neutrophil function (Trush et al., 1978).

Luminol, (5-amino-2,3-dihydro-1,4-phthalazinedione), has been routinely used to amplify the CL produced by various chemiluminescent reactions (Trush et al., 1978). The nature of luminol activation is shown in Scheme 3 (Allen, 1982). Luminol exhibits a high CL quantum yield i.e. there is an increase in the number of photons released per electronically excited species generated, thereby increasing the sensitivity of the CL measurement. The CL of luminol depends on dioxygenation of luminol and hence it can be used in the study of biological oxygenation reactions such as phagocyte oxidative metabolism. Luminol has been shown not to alter the respiratory burst in neutrophils (Allen, 1982).



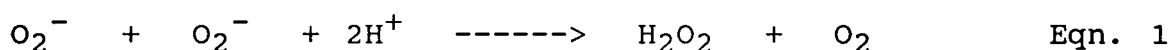
Proposed biochemical mechanism for cyclic hydrazide chemiluminescence at neutral to acid pH.

ACTIVATION OF LUMINOL

SCHEME 3

Scheme 4 depicts the reactions occurring in the cell that may possibly result in CL (adapted from Wilson et al., 1978). The oxidative products produced during the respiratory burst may react with components of the neutrophil to yield luminescent products.

The generation of luminol-amplified CL was shown to follow two pathways (Dahlgren, 1988), one dependent on MPO and hydrogen peroxide and the other independent of hydrogen peroxide, possibly dependent on superoxide since luminol may be oxygenated by reaction with superoxide or hydrogen peroxide. The contribution of superoxide to CL has been studied using superoxide dismutase (SOD) (Webb et al., 1974; Allen and Loose, 1976), an enzyme normally present in PMNL which catalyses the dismutation of superoxide to hydrogen peroxide and oxygen as shown in equation 1 below (McCord et al., 1971).



The role of MPO in the generation of CL has been studied using sodium azide as an inhibitor of MPO (Nurcombe and Edwards, 1989).

Luminol-dependent CL was found to be affected by factors such as opsonization of the particulate stimuli, incubation temperature and the presence of calcium and albumin in the buffer (Harber and Topley, 1986; Roschger et al., 1988).

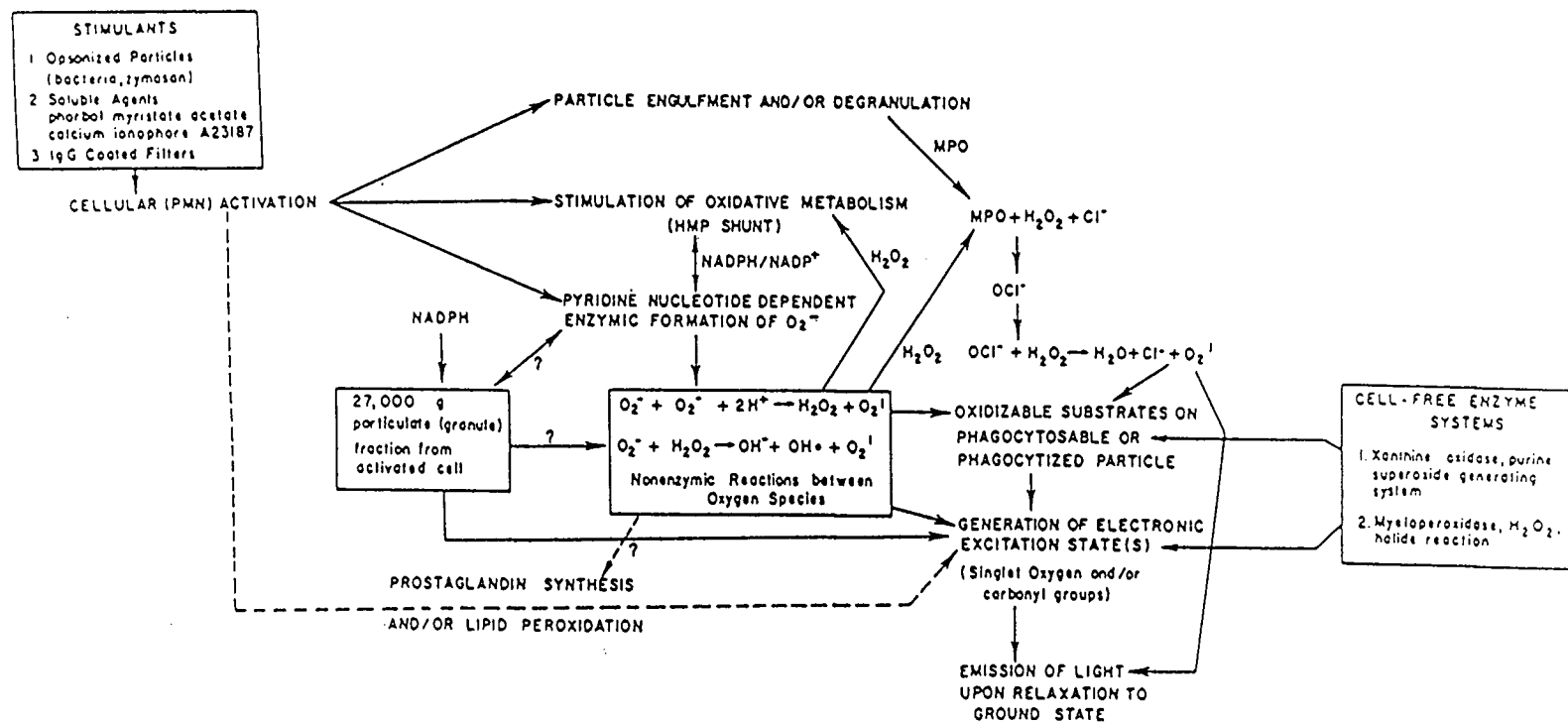


FIG. 2. Representation of the enzymic and nonenzymic mechanisms involved in the generation of chemiluminescence (CL) by polymorphonuclear leukocytes (PMNs) following cellular activation by both particulate and soluble stimulants. Dashed line represents two processes, prostaglandin synthesis and lipid peroxidation, known to generate CL; however, the contribution, if any, of these processes to the CL response of PMNs has yet to be defined.

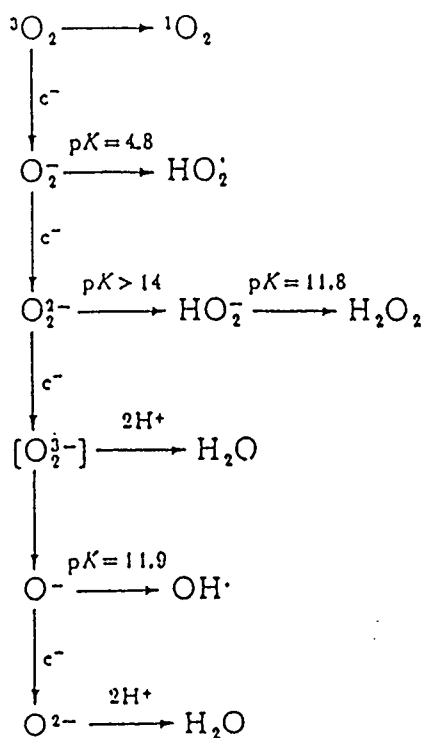
The concentrations of luminol and PMNL as well as stimuli all affect the observed response (Wilson et al., 1978). Other factors such as diet may also affect the CL response of PMNL (Magaro et al., 1988).

4.2.2. PRODUCTION OF SUPEROXIDE ANION

Superoxide anion is the term used to describe the electronically excited species arising from singlet molecular oxygen following a one electron reduction process. Scheme 5 illustrates the generation of superoxide and other electronically excited states of oxygen (Green and Hill, 1984).

Molecular oxygen (O_2) contains two unpaired electrons in the outermost orbital and hence can exist in either of two spin states, triplet and singlet states. In the triplet state the unpaired electrons possess parallel spin while in the singlet state one of the electrons is flipped so that the spin is anti-parallel. Energy must be supplied to cause the electron to flip; thus the singlet state of oxygen is an electronically excited state. This can undergo further reduction, i.e. addition of an electron, leading to formation of superoxide anion, O_2^- . Further addition of electrons leads to the formation of other oxidative products such as hydrogen peroxide and hydroxyl radical.

Neutrophils generate the electronically excited species of oxygen during the respiratory burst (Allen et al., 1972).



The products derived from the successive one-electron reductions of dioxygen.

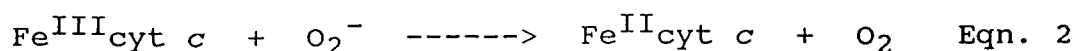
GENERATION OF SUPEROXIDE

SCHEME 5

The production of superoxide in neutrophils involves a membrane bound NADPH-dependent oxidase (Figure 3) believed to be located on the plasma membrane of the PMNL (Babior and Peters, 1981; Goldstein et al., 1977; Badwey and Karnovsky, 1979; Dewald et al., 1979).

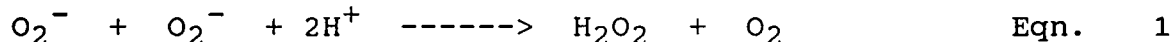
The superoxide anion is responsible for the bactericidal action of phagocytes (Babior et al., 1973) and may also be responsible for damage to the host tissue (Hsie et al., 1986). MSUM and CPPD crystal-induced superoxide release has been studied and MSUM was found to be a stronger stimulus than CPPD (Nagase et al., 1989; Naccache et al., 1991).

Methods have been developed for the measurement of superoxide generation by neutrophils such as the nitroblue tetrazolium reduction assay (NBT) and reduction of ferricytochrome c. Measurement of oxygen uptake and consumption by manometric methods have also been reported (Babior and Cohen, 1981). Manometry requires high concentrations of cells and is not a very sensitive method while potentiometry is more sensitive but unsuitable for long-term incubations. The method most commonly used in the study of neutrophil respiratory burst is based on the reduction of ferricytochrome c to ferrocytochrome c by superoxide as described in equation 2 (McCord and Fridovich, 1969).



This reduction is accompanied by a shift in UV absorption maximum from 520 nm to 550 nm and hence can be readily monitored.

The reduction of ferricytochrome c can be made specific for superoxide through the use of superoxide dismutase (SOD), a Cu-Zn metalloenzyme normally present within the PMNL as part of the protective mechanisms against superoxide-inflicted damage. SOD catalyses the dismutation of superoxide as described previously in equation 1 (McCord et al., 1971).



The reduction of ferricytochrome c by superoxide is inhibited by the presence of SOD in the reaction mixture and this can be used to confer specificity on the assay (Babior and Cohen, 1981).

Ferricytochrome c being a high molecular weight compound is excluded from the cell and hence this assay can only measure changes in the level of extracellular superoxide. However, it has been found that a large proportion of superoxide is released extracellularly by PMNL (Roos and Weening, 1979).

4.3. DEGRANULATION

Degranulation of the neutrophil occurs into the extracellular medium during phagocytosis and into the phagosome after phagocytosis (Baggiolini and Dewald, 1984; Weissmann et al., 1972; Hawkins, 1972). Degranulation can occur even in the absence of phagocytosis (Weissmann et al., 1972; Hawkins, 1972; Blackburn et al., 1987). The granules release their contents, including several enzymes such as lysozyme, myeloperoxidase and alpha mannosidase (Jones and Cross, 1988), into the phagosome containing the opsonized crystal. It has been hypothesized that proteases such as lysozyme strip the proteins adsorbed on the crystal surface to expose the "naked" crystal to the phagolysosomal membrane. Myeloperoxidase (MPO) catalyzes a hydrogen peroxide dependent oxidation reaction forming part of the respiratory burst.

The group of compounds known as the cytochalasins are useful in the study of degranulation since such agents inhibit phagocytosis by interfering with the polymerization of the actin-myosin network (Davis et al., 1971; Malawista et al., 1971). This results in degranulation and release of granule contents into the extracellular fluid, thereby facilitating measurement of the contents.

Degranulation can be monitored by measuring the release of any of the enzymes released during this process. The

presence of MPO in the extracellular fluid is useful as an indicator of neutrophil degranulation since it is present exclusively in the azurophilic granules of the neutrophil (Andrews and Krinsky, 1985). MPO can be measured by the hydrogen peroxide dependent MPO-catalyzed oxidation of o-dianisidine. Lysozyme (LYZ) released from the lysosomes of the PMNL can be assayed by monitoring the decrease in turbidity of a suspension of Micrococcus lysodeikticus on addition of LYZ (Babior and Cohen, 1981). LYZ hydrolyzes the muramic acid-containing mucopeptide in the polysaccharide of the cell walls of the Micrococcus thus converting the spores to protoplasts resulting in a decrease in turbidity.

5. EFFECT OF CRYSTAL HISTORY ON INFLAMMATORY POTENTIAL OF CRYSTALS

5.1. CRYSTAL SIZE

The size of individual crystals is known to be important in acute inflammation. Amorphous deposits and large crystals of monosodium urate were found to be relatively ineffective, whereas crystals of about 5 μm in length were the most reactive (Schumacher et al., 1975; Burt et al., 1989).

5.2. CRYSTAL PRETREATMENT

Grinding of crystals to reduce particle size, a practice reported by earlier workers, has now been demonstrated to

effect a change in the crystal structure and hence alter the inflammatory potential of the crystals (Burt et al., 1986). Similarly, depyrogenation of the crystals by heating at 200 - 250°C for 2 hr was found to produce a change in the crystal structure leading to alteration of its inflammatory potential (Hasselbacher, 1979; Mandel, 1980; Cheng and Pritzker, 1981).

6. EFFECT OF PROTEIN ADSORPTION

MSUM and CPPD crystals have a highly reactive surface that can adsorb many different proteins and other molecules from the synovial fluid. It is now well recognized that proteins bound to the inflammatory crystals play an important role in determining the biological activity of the crystals.

6.1. PLASMA PROTEINS

MSUM crystals have been shown to adsorb several proteins such as fibronectin, fibrinogen, Hageman factor, which are part of the clotting cascade, proteins of the complement system, immunoglobulins and lipoproteins (Wallingford and McCarty, 1974; Terkeltaub et al., 1983). It has been suggested that the spontaneous remission which is characteristic of gout and pseudogout may be due to the proteins adsorbed on the crystal surface (Kozin and McCarty, 1976). As the inflammatory episode proceeds, the accompanying increase in vascular permeability alters the composition of the synovial fluid such that it closely

resembles that of plasma (Swan, 1978). The lipoproteins and other proteins present in synovial fluid are adsorbed onto the crystal surface and may interfere with the crystal-neutrophil interaction to bring about spontaneous remission of the inflammatory episode (Terkeltaub et al., 1984). Recently α_1 -acid glycoprotein has been shown to inhibit neutrophil responses such as chemotaxis, aggregation and superoxide anion generation (Laine et al., 1990). Serum α_2 -HS glycoprotein had earlier been shown to be a potent and specific inhibitor of neutrophil stimulation by hydroxyapatite crystals (Terkeltaub et al., 1988).

6.2. IMMUNOGLOBULIN G (Ig G)

Ig G is strongly adsorbed to MSUM but less so to CPPD crystals (Hasselbacher and Schumacher, 1978). The binding of Ig G to MSUM has been shown to be a function of the charge density of the Ig G molecule (Hasselbacher, 1979) and the binding may, therefore be electrostatic in nature. The F_{ab} portion of the Ig G molecule is more positive than the F_c strands and hence it is the F_{ab} part that binds to the crystal leaving the F_c fragments exposed. Ig G-coated MSUM has been shown to enhance neutrophil responses to the crystals with increases in the extent of phagocytosis of the crystals, elevated levels of superoxide and enhanced lysosomal enzyme release (Kozin et al., 1979; Hasselbacher et al., 1978; Abramson et al., 1982; Rosen et al., 1986). The enhanced response of the neutrophil to the crystals is

apparently through interaction of the exposed F_C fragments with the cellular receptors present on the PMNL (Hasselbacher, 1979).

6.3. LIPOPROTEINS

MSUM and CPPD crystals bind low density lipoprotein (LDL) and high density lipoprotein (HDL) (Terkeltaub et al., 1986; Burt et al., 1989). The protein moiety of LDL is mostly apolipoprotein B and that of HDL is apolipoprotein A1 and A2. Terkeltaub et al. (1983, 1984, 1986) monitored the extent of phagocytosis, CL and degranulation produced by neutrophils on exposure to HDL and LDL coated MSUM crystals. LDL coated MSUM crystals inhibited all MSUM induced neutrophil responses while HDL coated MSUM crystals apparently did not have any significant inhibitory effect. Burt et al., (1989) studied the effects of LDL and HDL bound to MSUM and CPPD on crystal-induced neutrophil cytolysis measured by lactate dehydrogenase release (LDH). Both HDL and LDL strongly inhibited CPPD and MSUM induced neutrophil cytolysis (Burt et al., 1989).

6.4. ALBUMIN

The synovial fluid is an ultrafiltrate of plasma (Swan, 1978). Albumin, a major constituent of plasma is among the proteins present in the synovial fluid. MSUM and CPPD crystals have been reported to bind albumin (Kozin and McCarty, 1976). The effect of adsorbed albumin on the

particle stimulated luminol dependent luminescence and native luminescence (NL) has been reported by Roschger et al. (1988). Their results indicate that while luminol dependent luminescence is inhibited, generation of NL is enhanced by albumin. In their studies of the inhibitory effects of lipoproteins on MSUM induced neutrophil stimulation, Terkeltaub et al. (1983, 1984, 1986) included albumin in the buffers used for neutrophil/crystal incubations. They stated that the purpose of the albumin was to "to eliminate non specific inhibition". However in similar studies by Burt et al. (1989) of the inhibitory effect of lipoproteins on neutrophil cytolysis, albumin was not added to the buffers.

7. HYPOTHESIS AND OBJECTIVE

It is our hypothesis that the presence of proteins on the crystal surface can influence the crystal-neutrophil interaction. The objective of our work is to study the neutrophil responses (CL, superoxide ion production and degranulation) induced by uncoated and protein coated MSUM and CPPD crystals as a function of incubation time.

Proteins used in these studies were bovine serum albumin, immunoglobulin g and plasma proteins.

EXPERIMENTAL

MATERIALS

Albumin, Fraction V, fatty acid free, from bovine serum,
Boehringer Mannheim GmbH, W. Germany.

Calcium tetrahydrogen di-orthophosphate, $\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$, BDH
Chemicals Ltd., Poole, England.

Catalase, from bovine liver, Sigma Chemical Co., St. Louis,
MO. U.S.A.

Cytochalasin B, from *Helminthosporium dematioideum*, Sigma
Chemical Co., St. Louis, MO. U.S.A.

Cytochrome c, Type III, from horse heart, Sigma Chemical
Co., St. Louis, MO. U.S.A.

Dextran T70, Pharmacia LKB, Biotechnology AB, Uppsala,
Sweden.

o-Dianisidine dihydrochloride, Sigma Chemical Co., St.
Louis, MO. U.S.A.

Ficoll-Paque (R), Pharmacia LKB, Biotechnology AB, Uppsala,
Sweden.

Hydrogen Peroxide, 30%, Sigma Chemical Co., St. Louis, MO.
U.S.A.

Immunoglobulin G, Human, Sigma Chemical Co., St. Louis, MO.
U.S.A.

Lactate Dehydrogenase Assay Kit, Sigma Chemical Co., St.
Louis, MO. U.S.A.

Luminol, (5-amino-2,3-dihydro-1,4-phthalazinedione) Sigma
Chemical Co., St. Louis, MO. U.S.A.

Lysozyme, Grade I, from chicken egg white, Sigma Chemical
Co., St. Louis, MO. U.S.A.

Micrococcus lysodeikticus, Sigma Chemical Co., St. Louis,
MO. U.S.A.

Superoxide dismutase, from bovine erythrocytes, Sigma
Chemical Co., St. Louis, MO. U.S.A.

Triton X-100, (OctylPhenoxy Polyethoxyethanol), Sigma
Chemical Co., St. Louis, MO. U.S.A.

Uric acid, (2,6,8-trioxypurine), Sigma Chemical Co., St.
Louis, MO. U.S.A.

REAGENTS AND SOLVENTS

Acetone, BDH.

Ethanol

Calcium acetate, Sigma.

Hydrochloric acid, BDH.

Orthophosphorous acid, 80% J.T. Baker.

Sodium chloride, BDH.

Sodium hydroxide, Fisher.

BUFFER SOLUTIONS

Hanks buffer (NaCl, 137 mM; KCl, 5.4 mM; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.33 mM; KH_2PO_4 , 0.44 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.41 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.3 mM; Glucose, 5.6 mM; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mM; NaHCO_3 , 4.2 mM), pH 7.4

Citrate buffer, pH 5.5, 0.1M

Potassium phosphate buffer pH 6.2, 0.065M

INSTRUMENTS

Bio-orbit 1250 Luminometer interfaced with PC; data collection software program obtained from manufacturer, Fisher Scientific Co. and equipped with Fisher Scientific Isotemp Dry Bath 147.

Dupont Differential Scanning Calorimeter Series 910 with Series 99 Thermal analyzer.

Eppendorf centrifuge 5412, Brinkmann Instruments.

Fisher Dyna-Mix

Fisher Scientific Accumet pH meter with Orion pH probe Model 91-02.

Haake circulating water bath

Hewlett Packard Vectra spectrophotometer interfaced with PC
and equipped with data collection software.

Hitachi S-57 scanning electron microscope and Hummer sputter
gold coater.

Magnetic stirrer and hotplate.

Mettler balances models AJ100 and AE163.

Nikon differential interference microscope Model R with
Nikon objective and ocular micrometers.

Oven, Johns Scientific

Perkin Elmer Differential Scanning Calorimeter, Model 1B.

Rigaku Giegerflex X-Ray Diffractometer System.

VWR Vortexer 2, Scientific Industries, N.Y.

LABWARE

Polypropylene tubes, 50 mL, 10 mL, Nalgene

Polypropylene Eppendorf tubes, 1.5 mL, 0.5 mL, Brinkmann
Instruments

Polystyrene cuvettes, 1 mL, Fisher Scientific

METHODS

1. PREPARATION OF CRYSTALS

1.1. MONOSODIUM URATE MONOHYDRATE (MSUM)

A modification of the method of Burt et al. (1983) was used to prepare MSUM samples. To 800 mL of distilled water was added 4.0 g of uric acid with stirring and the suspension heated to 55°C. Approximately 25 mL of 1M sodium hydroxide was then added slowly and the uric acid dissolved to give a clear solution. The stirring was continued for 45 minutes at 55°C after which the pH was checked. The pH was adjusted to 7.5 with 1M hydrochloric acid. The solution was filtered hot through Whatman # 1 filter paper under vacuum to remove any suspended particles. The filtrate was allowed to stand undisturbed for 24 hours at room temperature. The resulting crystals were harvested by filtration through Whatman # 1 filter paper under vacuum. The crystals were washed with 4 x 250 mL portions of distilled water saturated with MSUM at room temperature and were then allowed to dry overnight at 60°C. The crystals were stored in tightly capped amber glass bottles.

The crystals were characterized by X-ray diffraction analysis and differential scanning calorimetry. Particle size analysis was also done by optical microscopy.

1.2. CALCIUM PYROPHOSPHATE DIHYDRATE (CPPD)

The preparation of CPPD was a two step process, the first step being the preparation of calcium acid pyrophosphate/calcium dihydrogen pyrophosphate.

1.2.1. Synthesis of calcium dihydrogen pyrophosphate (CDPP)

In a 600 mL glass beaker, 250 mL of 80% orthophosphoric acid was added and heated with stirring to 215°C. Addition of calcium tetrahydrogen diorthophosphate was begun at an initial rate of 1 g/min. This was continued until about 40 g of calcium tetrahydrogen diorthophosphate had been added after which the rate of addition was about 0.5 g/min. This was continued till a further 35-40 g had been added. At this stage the mixture formed a white slurry and stirring became difficult. This slurry was then filtered through a sintered glass filter heated to approximately 170°C by passing orthophosphoric acid at that temperature through the filter. The crystals were allowed to cool to room temperature in the filter. They were then washed with three 100 mL aliquots of acetone to remove any adsorbed acid and allowed to air dry in the filter.

The crystals were characterized by X-ray diffraction to confirm that they were CDPP prior to use in the next step.

1.2.2. Synthesis of calcium pyrophosphate dihydrate (triclinic):

A 250 mL beaker containing 103 mL distilled water was heated in a water bath to $60 \pm 2^{\circ}\text{C}$ and stirred constantly with a Teflon coated stir bar. The stirring was slowed and 0.71 mL of concentrated hydrochloric acid and 0.32 mL of glacial acetic acid were added, followed by 0.6 g of calcium acetate. A 150 mL beaker containing 20 mL of distilled water was heated to 60°C in a water bath and 0.6 g of calcium acetate added. The rate of stir was increased in the 250 mL beaker, and 2 g of CDPP added rapidly. When the CDPP was nearly all dissolved, the rate of stirring was reduced for 5 minutes, then over a period of 15 seconds, the contents of the small beaker were poured into the large beaker with vigorous stirring. This led to the formation of a white gel. Stirring was discontinued and the gel allowed to stand undisturbed overnight. The gel collapsed with the formation of CPPD crystals. The crystals were washed in distilled water three times, washed in ethanol, then acetone and allowed to air dry.

The crystals were characterized by X-ray diffraction analysis and differential scanning calorimetry.

2. CHARACTERISATION OF CRYSTALS

2.1. X-RAY DIFFRACTION ANALYSIS:

The X-ray diffraction analyses were done using a Rigaku Giegerflex X-Ray Diffractometer System with a biplanar goniometer, a D/max-B controller interface between the goniometer and an IBM compatible 286 PC and equipped with a scintillation counter. The X radiation used was copper K wavelength = 1.56 Angstrom units, which was obtained by combining a copper source with a nickel filter. Data analyses were done with a software program provided by the manufacturer which allowed intensity and relative intensity determinations. The X-ray tube was operated at a potential of 40 KV and at a current of 20 mA. The sample was scanned over a range of 5 to 55 degrees 2θ at a rate of 5 degrees/minute. The instrument collected data every 0.05 degrees 2θ /sample. The unground samples were packed in an aluminum sample holder for MSUM and sprinkled onto a double sided Scotch tape attached to the holder for CPPD samples.

2.2. DIFFERENTIAL SCANNING CALORIMETRY

Weighed samples of MSUM prepared by both the method of Burt *et al.* (1983) and the modified method and CPPD were analyzed by DSC. The MSUM data were collected on a Dupont DSC under a nitrogen atmosphere at 20 psi, from an initial temperature of 25°C to a final temperature of 350°C, with a heating rate of 10°C/min in open crimped aluminum pans. The data for

CPPD crystals were also collected on the same instrument in open crimped aluminum pans in a nitrogen atmosphere and heated at 10°C/min from 150° to 350°C. CPPD samples were held isothermally at 350°C for 30 minutes.

Vaporization of water of hydration from the pans containing MSUM and CPPD samples was estimated quantitatively by weighing the pans after the appearance of the endothermic peak. Percent water losses were calculated for MSUM and CPPD samples.

2.3. PARTICLE SIZE ANALYSIS

The particle size distribution of the crystals was determined by optical microscopy using a 0.01 mm Nikon objective micrometer and eyepiece micrometer with a Nikon differential interference microscope. The samples were suspended in liquid paraffin and 100 crystals were measured for each of CPPD, MSUM (method of Burt *et al.*, 1983) and MSUM (modified method) samples.

2.4. SCANNING ELECTRON MICROSCOPY

Samples of MSUM and CPPD crystals were attached to the surface of a metal stub with graphite paint and the samples were gold coated on a Hummer sputter gold coater in an Argon atmosphere. The samples were then examined on a Hitachi S-57 scanning electron microscope.

3. PREPARATION OF NEUTROPHIL SUSPENSION:

Neutrophils were separated from freshly collected human whole blood obtained from the Red Cross, Vancouver, B.C. or from healthy volunteers. Red Cross blood was from a single donor and not pooled.

To 450 mL of citrate treated whole human blood in polypropylene stopper bottles were added 100 mL of 3% dextran solution in Hanks buffer and the blood/dextran suspension allowed to stand undisturbed. The erythrocytes sedimented in the presence of dextran and the neutrophil rich supernatant was removed as soon as it appeared, to reduce loss due to settling of the neutrophils. Aliquots of the neutrophil rich supernatant (5 mL) were layered over 4 mL of Ficoll-Paque in polypropylene tubes. The tubes were centrifuged at 400 x g at 20°C for 15 minutes. The neutrophil pellet thus obtained was suspended in 1.5 mL of distilled water at 4°C with gentle vortexing to lyse contaminating erythrocytes. Tonicity was restored after 10 seconds by the addition of 0.5 mL of 0.6 M sodium chloride. The tubes were centrifuged at 200 x g at 4°C for 5 minutes. The supernatant was discarded and the lysis treatment was repeated.

The pellet obtained after the second lysis step was resuspended in 6 mL Hanks buffer and kept on ice until used.

3.1. ESTIMATION OF NEUTROPHIL COUNT:

Samples of neutrophil suspensions obtained by the method described above were sent periodically to the Dept. of Laboratory Medicine, University Hospital (UBC Site) for differential cell counts.

On a routine basis, the total cell count (and therefore an estimate of the neutrophil count) in the cell suspension was determined from the lactate dehydrogenase (LDH) content in an aliquot of lysed cells using the Sigma LDH assay kit. A 10% solution of Triton X-100 was added to 0.4 mL of the cell suspension, vortexed for 5 minutes and centrifuged at 200 x g for 5 minutes. To 2.2 mL of lactate reagent at 30°C was added 110 μ L of supernatant in a UV/visible cuvette and the absorbance measured at 340 nm using the HP Vectra spectrophotometer in the kinetics mode for a period of 240 seconds. The absorbance was read every 30 seconds and the difference between the absorbances at 180 and 60 seconds used to calculate the change in absorbance per minute. The cell count was then estimated from a standard curve.

3.2. ESTIMATION OF NEUTROPHIL VIABILITY:

3.2.1. CL determination

The time elapsed between blood collection and preparation of the neutrophil suspension was between 24-48 hours for Red Cross blood and 4-6 hours for blood from UBC volunteers. As

a rapid test to determine whether Red Cross blood neutrophils were still viable following completion of the separation procedure, the MSUM stimulated chemiluminescent response of Red Cross blood neutrophils was compared to that of UBC volunteer blood neutrophils.

To a 1.5 mL polypropylene Eppendorf tube was added 5 mg uncoated MSUM, 10 μ l of 1×10^{-3} M luminol solution in DMSO (final concentration 10^{-5} M) and sufficient amount of the cell suspension to give a final neutrophil concentration of 2×10^6 cells/mL. The CL produced by the neutrophils was monitored on a Bio-Orbit 1250 Luminometer. The instrument was interfaced with an IBM compatible PC and the data collection was done through a software package for the same obtained from the manufacturer. The tube was placed in the chemiluminometer and maintained at 37°C by means of a jacketed tube holder connected to a circulating water bath. Every 3 minutes the tube was removed from the tube holder and the contents gently agitated. The agitation was required to overcome the problem of settling of the crystals within the tube leading to a reduced interaction of cells with the crystals. The response was studied for 30 minutes with 1000 sampling points in this interval i.e. readings were taken every 1.8 seconds.

3.2.2. Staining with Trypan blue

An aliquot of the neutrophil suspension was mixed with an equal volume of 0.5% trypan blue in 0.9% saline, incubated at room temperature for 5 minutes and the neutrophils examined under the microscope for dye uptake.

4. PROTEIN COATING OF CRYSTALS

Samples of 5 mg MSUM or 50 mg CPPD were weighed into 1.5 mL polypropylene Eppendorf tubes. Protein solutions were prepared in Hanks buffer for coating CPPD crystals or Hanks buffer presaturated with MSUM at 37° (Hanks saturated with MSUM) for coating MSUM. One mL of the following protein solutions was added to the crystals and the tubes tumbled at 37°C for 30 minutes: 6 mg/mL Ig G (3 mg/mL for CPPD), 16.67 mg/mL BSA and plasma diluted 50:50 with Hanks / Hanks saturated with MSUM. The tubes were centrifuged in an Eppendorf centrifuge at 16,000 x g for 5 minutes. The crystal pellet was washed by resuspending in 1 mL of Hanks buffer or Hanks saturated with MSUM, centrifugation at 16,000 x g and discarding the supernatant.

5. MEASUREMENT OF NEUTROPHIL RESPONSE:

5.1. SUPEROXIDE ANION RELEASE

5.1.1. Superoxide release from neutrophils on stimulation by uncoated MSUM

To a 1.5 mL Eppendorf tube was added 0.2 mL of a 25 mg/mL MSUM suspension in Hanks saturated with MSUM, 0.1 mL of a 10 mg/mL ferricytochrome c solution in Hanks saturated with MSUM, 0.2 mL of Hanks saturated with MSUM and 0.5 mL of a 1×10^7 cells/mL neutrophil suspension. The tubes contained 5 mg MSUM and 5×10^6 neutrophils. The tubes were rotated end-over-end at 15 rpm at 37°C for 180 minutes. At intervals, tubes were removed and centrifuged in an Eppendorf centrifuge at 16,000 x g for 30 seconds and stored on ice. A 0.75 mL aliquot of the supernatant was withdrawn and the absorbance of the sample at 550 nm measured on a diode array spectrophotometer in a polystyrene 1 mL cuvette. Controls were treated similarly to the samples with the substitution of 0.2 mL of Hanks saturated with MSUM for the 0.2 mL of crystal suspension. Blanks were 0.1 mL of ferricytochrome c solution diluted with buffer to 1 mL and treated similarly to the samples.

5.1.2. Superoxide release from neutrophils on stimulation by uncoated CPPD:

Tubes containing 50 mg CPPD and 5×10^6 neutrophils in Hanks buffer were prepared as described above and rotated end-over-end at 15 rpm and 37°C for 180 minutes. At intervals tubes were withdrawn and centrifuged in an Eppendorf centrifuge at $16,000 \times g$ for 30 seconds and stored on ice. A 0.75 mL aliquot of the supernatant was withdrawn and the absorbance of the sample at 550 nm measured on the HP Vectra spectrophotometer in a polystyrene 1 mL cuvette. Controls were treated similarly to the samples with the substitution of 0.2 mL of Hanks buffer for the 0.2 mL of crystal suspension. Blanks were 0.1 mL of ferricytochrome c solution diluted with buffer to 1 mL and treated similarly to the samples.

The rate of superoxide production was determined from the linear change in absorbance at 550 nm that occurs after an initial phase of no absorbance change followed by an increasing rate of change. Rates were reported in nmoles $\text{O}_2^-/\text{min}/10^6$ PMNL. The rate was calculated using the formula shown below:

$$\text{nmoles } \text{O}_2^-/\text{min}/10^6 \text{ PMNL} = \frac{A_{550}}{(0.0211)(\text{cell conc})(10^{-6})}$$

where 0.0211 is the extinction coefficient of ferricytochrome c.

5.1.3. Effect of SOD on superoxide release

To show that ferricytochrome c was reduced by superoxide anion and not other species, the levels of reduced ferricytochrome c were monitored in the presence of SOD, an enzyme which dismutates superoxide. These experiments were performed as described above with the following modifications: 0.1 mL of 3000 U/mL SOD solution in Hanks buffer (300 U/tube) was added to the tubes instead of 0.1 mL of buffer and tubes were withdrawn only at 10 minutes in the case of MSUM and at 60 minutes in the case of CPPD. Control tubes were as described previously.

5.1.4. Adsorption of SOD by MSUM crystals

In the following studies MSUM crystals were incubated with solutions containing SOD, centrifuged and the supernatant added to tubes containing CPPD and neutrophils. The rationale for this approach was that if MSUM crystals adsorb significant amounts of SOD from solution, then the reduced concentration of SOD in the supernatant added to the CPPD/neutrophil suspensions should result in a decreased inhibition of CPPD induced superoxide release.

To each of three 1.5 mL Eppendorf tubes was added 0.2 mL of a 20 mg/mL ferricytochrome c solution (4 mg/tube), 0.2 mL of Hanks saturated with MSUM, 0.1 mL of a 3000 U/mL SOD solution (300 U/tube) and 10 mg MSUM crystals. Another set of three tubes without the crystals was run as controls.

The tubes were tumbled end-over-end at 15 rpm at 37°C for 30 minutes. The tubes were then centrifuged in an Eppendorf centrifuge at 16,000 x g for 30 seconds and 0.5 mL of the supernatant added to another set of tubes containing 0.2 mL of a 250 mg/mL CPPD crystal suspension (50 mg/tube). Five hundred microliters of a 1×10^7 cells/mL cell suspension were added to the tubes which were then incubated at 37°C for 60 minutes. Another set of tubes with CPPD crystals but not containing any SOD was also run as a positive control. The absorbance was measured at 550 nm as described earlier.

5.1.5. Superoxide release from neutrophils on stimulation by protein coated MSUM and CPPD

To tubes containing 5 mg protein coated MSUM or 50 mg protein coated CPPD were added 0.1 mL of a 10 mg/mL ferricytochrome c solution (1 mg/tube), 0.3 mL of Hanks saturated with MSUM or Hanks buffer and 0.5 mL of a 1×10^7 cells/mL neutrophil suspension. Tubes containing uncoated crystals, ferricytochrome c and neutrophils were also prepared as described above. Control tubes (crystals absent) were prepared as above. The tubes were tumbled end over end at 15 rpm at 37°C for 10 minutes for MSUM containing tubes and 60 minutes for CPPD containing tubes. All tubes were centrifuged at 16,000 x g for 30 seconds, 0.75 mL of the supernatant withdrawn and the absorbance at 550 nm measured.

5.2. CHEMILUMINESCENCE

5.2.1 Chemiluminescent response of neutrophils on stimulation by uncoated MSUM and CPPD crystals

To a 1.5 mL plastic Eppendorf tube was added, 2, 5, 10 mg of uncoated MSUM or 10, 20, 30, 40, 50 mg of uncoated CPPD crystals, 10 μ l of a 1×10^{-3} M luminol solution in DMSO (final concentration 10^{-5} M) and 0.5 mL of a 1×10^7 cells/mL neutrophil suspension. The tubes were maintained at 37°C in the Fisher Isotemp Dry Bath and read in the tube holder of the chemiluminometer at the same temperature. Every 1.5-3 minutes the tube was removed from the tube holder and the contents gently agitated. The response was studied for 60 minutes with 2000 sampling points in this interval i.e. readings were taken every 1.8 seconds or till the response abated. The response was recorded as a graph of mV versus time.

5.2.2. Chemiluminescent response of neutrophils on stimulation by protein coated MSUM and CPPD crystals

Tubes containing 5 mg of uncoated or protein coated MSUM and 30 mg of uncoated or protein coated CPPD were prepared. To each tube was added 0.5 mL of a 1×10^7 cells/mL neutrophil suspension and the tubes maintained at 37°C in the Fisher Isotemp Dry Bath. The response was recorded for 15 seconds for each tube in succession, i.e. uncoated crystal tube followed by tubes containing BSA-coated, Ig G-coated and

plasma protein coated crystals in that order for a period of 30 minutes. The tubes were mixed prior to measurement.

The rationale for this particular sequence of measurements was because it was necessary to minimize any differences in response due to changing cell viability.

5.3. DEGRANULATION INDICATOR: RELEASE OF MPO AND LYZ

Degranulation and release of MPO and LYZ were studied in neutrophils pretreated with cytochalasin B to prevent phagocytosis of crystals and subsequent neutrophil cytolysis.

A 1 mg/mL cytochalasin B solution was added to a 2.5×10^7 cells/mL neutrophil suspension to give a final concentration of 10 μ g/mL cytochalasin B and the suspension incubated at 37°C for 30 minutes.

5.3.1. MPO and LYZ release from neutrophils on stimulation by uncoated MSUM and CPPD crystals

To a 0.5 mL Eppendorf tube containing 0.4 mL of cytochalasin B pretreated neutrophil suspension at 2.5×10^7 cells/mL was added 0.1 mL of either a 25 mg/mL MSUM suspension in Hanks saturated with MSUM or a 250 mg/mL CPPD suspension in Hanks buffer. Control tubes (crystals absent) were also prepared as described above. The tubes were tumbled end over end at 15 rpm at 37°C and withdrawn at 5, 10, 20, 30, 45, 60, 90, 120, 150 and 180 minutes. The tubes were

centrifuged in a Beckmann Centrifuge at 200 x g for 2 minutes at 0°C and the supernatant removed for analysis.

5.3.2. Measurement of LYZ

LYZ was determined using the method of Ginsberg et al. (1977). A standard curve for the LYZ assay was prepared over a concentration range of 0-200 units/mL. LYZ from chicken egg white, obtained from Sigma Chemical Co. was used as the standard. A series of solutions containing 25, 50, 75, 100, 125, 150, 175 and 200 units/mL were prepared and the change in absorbance at 450 nm per minute, produced when 100 microliters of each LYZ solution were added to a suspension of Micrococcus lysodeikticus at 25°C was monitored. The change in absorbance per minute was plotted against the concentration of LYZ in units/mL.

To a quartz cuvette containing 2.5 mL of Micrococcus lysodeikticus suspension ($A_{450\text{nm}} = 0.6-0.7$) was added 100 microliters of supernatant obtained from cytochalasin B pretreated neutrophil/crystal incubations and the decrease in turbidity monitored over a period of 120 seconds. The change in absorbance per minute was calculated and the concentration of LYZ calculated using the equation for the standard curve.

5.3.3. Measurement of MPO

A 0.05 mL aliquot of the supernatant obtained from cytochalasin B pretreated neutrophil/crystal incubations was added to a 1 mL cuvette containing 0.89 mL 3.2 mM dianisidine solution in pH 5.5 citrate buffer, 0.05 mL of 1% Triton X-100, and 0.01 mL of 10 mM H₂O₂ and the change in absorbance at 450 nm measured for 120 seconds at 25°C.

The rate of oxidation of dianisidine which is directly proportional to MPO concentration was calculated from the following equation:

$$\text{Rate (nmol/min)} = 50 \times A \text{ (units)}$$

where A is the change in absorbance

5.3.4. MPO and LYZ release from neutrophils on stimulation by protein coated MSUM and CPPD crystals

Tubes containing 2.5 mg protein coated MSUM or 25 mg protein coated CPPD were prepared and incubated as described above except that incubation times were 45 minutes for MSUM containing tubes and 60 minutes for CPPD containing tubes.

6. STATISTICAL TESTS

Statistical evaluations were performed using either a two sample t test or one way ANOVA to compare mean values between groups. A significance level of $p < 0.05$ two tailed was used. All tests were done using the Statview program on

a Macintosh computer. The values shown in the graphs represent the mean \pm standard error of the mean.

RESULTS AND DISCUSSION

1. PREPARATION OF CRYSTALS

1.1. MONOSODIUM URATE MONOHYDRATE (MSUM)

MSUM had earlier been prepared in this laboratory by the method described by Burt et al. (1983). The previous procedure involved dissolution of uric acid in distilled water to which sodium hydroxide had been added, followed by addition of sodium hydroxide to adjust the pH of the reaction mixture to 8.9. The particle size distribution of the resulting crystals showed a high proportion with large particle sizes (over 50% of crystals were 75 μm or greater) Figure 4. This necessitated an additional step of sizing of the crystals in which centrifugation was used to physically separate the smaller size crystals for use in experiments (Burt and Jackson, 1989).

Theoretically, an increase in the degree of supersaturation of the sodium urate solution would increase the driving force for crystallization leading to the production of smaller size crystals.

The degree of supersaturation of monosodium urate in the solution can be increased by either adding more solute or by altering the conditions such that the solubility is reduced. The solubility of sodium urate at pH 8.9 is greater than at pH 7.5. Hence, it was felt that a decrease in the pH of the

PARTICLE SIZE OF MSUM CRYSTALS
(Method of Burt and Jackson, 1983)

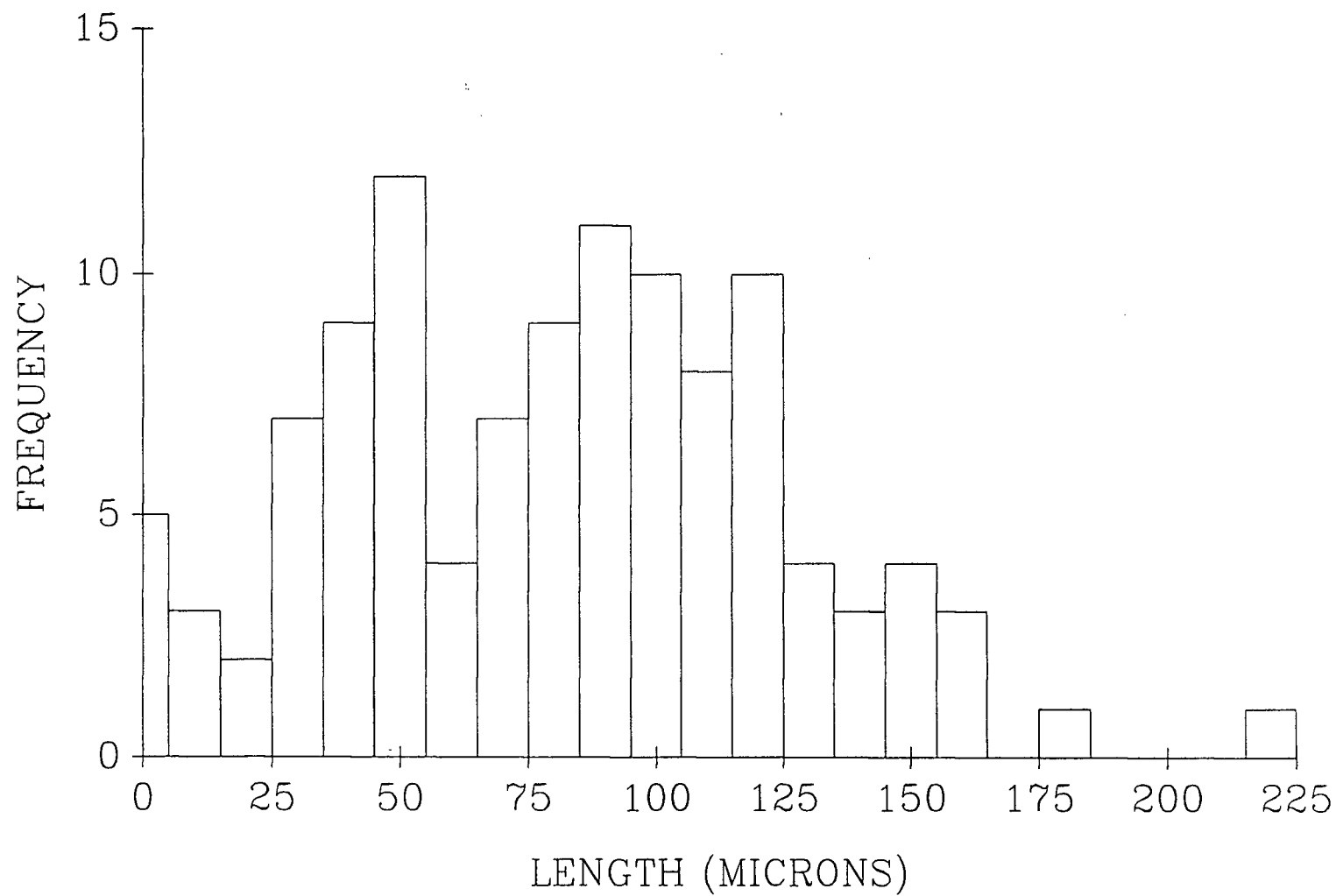


FIGURE 4

supersaturated solution from 8.9 to 7.5 would cause a significant increase in the degree of supersaturation and result in the production of smaller crystals of MSUM.

The particle size distribution of MSUM crystals obtained by the modified method is shown in Figure 5. The modification, which involved alteration of pH of the solution prior to crystallization, resulted in smaller particle sizes. The range of particle size was between 0.1-50 μm with a mean particle size of 21 μm and a smaller standard deviation about the mean.

1.2. CALCIUM PYROPHOSPHATE DIHYDRATE (CPPD)

1.2.1. Synthesis of calcium dihydrogen pyrophosphate (CDPP)

The synthesis of CDPP was carried out using the previously reported method (Burt and Jackson, 1987). The CDPP crystals obtained were characterized by X-ray powder diffraction prior to use in the synthesis of CPPD (Figure 6, Table 1). In Tables 1-4, the term 'd space' refers to the distance between the crystal planes while 'I (rel)' refers to the relative intensity of the peak compared to the intensity of the strongest peak. CDPP previously synthesized in the lab was used for comparison purposes. The diffraction peaks at 2 values of 23.8, 25.25, 26.7, 28.0, 33.25 and 40.55 were used for identification of CDPP.

PARTICLE SIZE OF MSUM CRYSTALS
(Modified method)

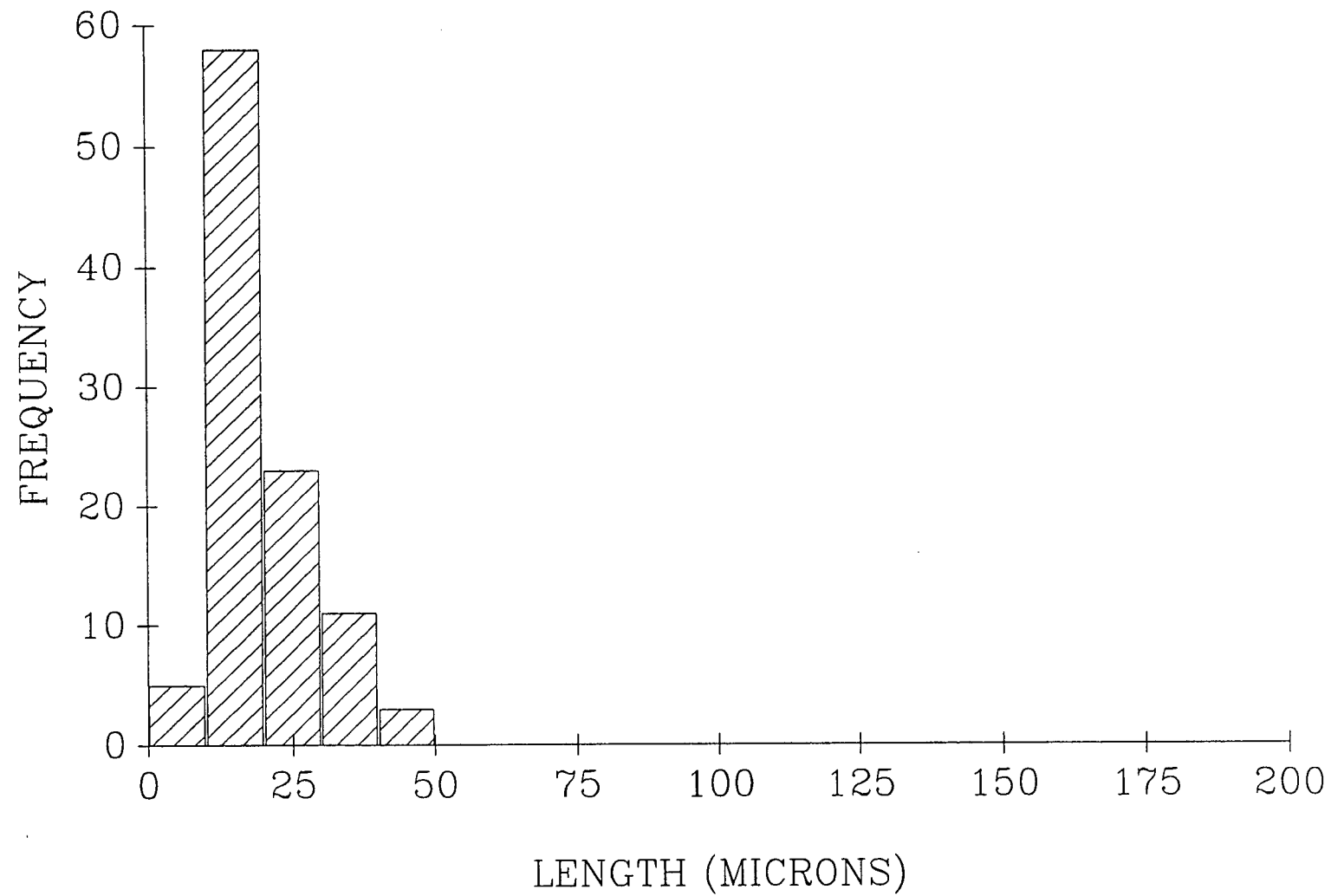


FIGURE 5

XRAY DIFFRACTION PATTERN OF
CALCIUM DIHYDROGEN PYROPHOSPHATE

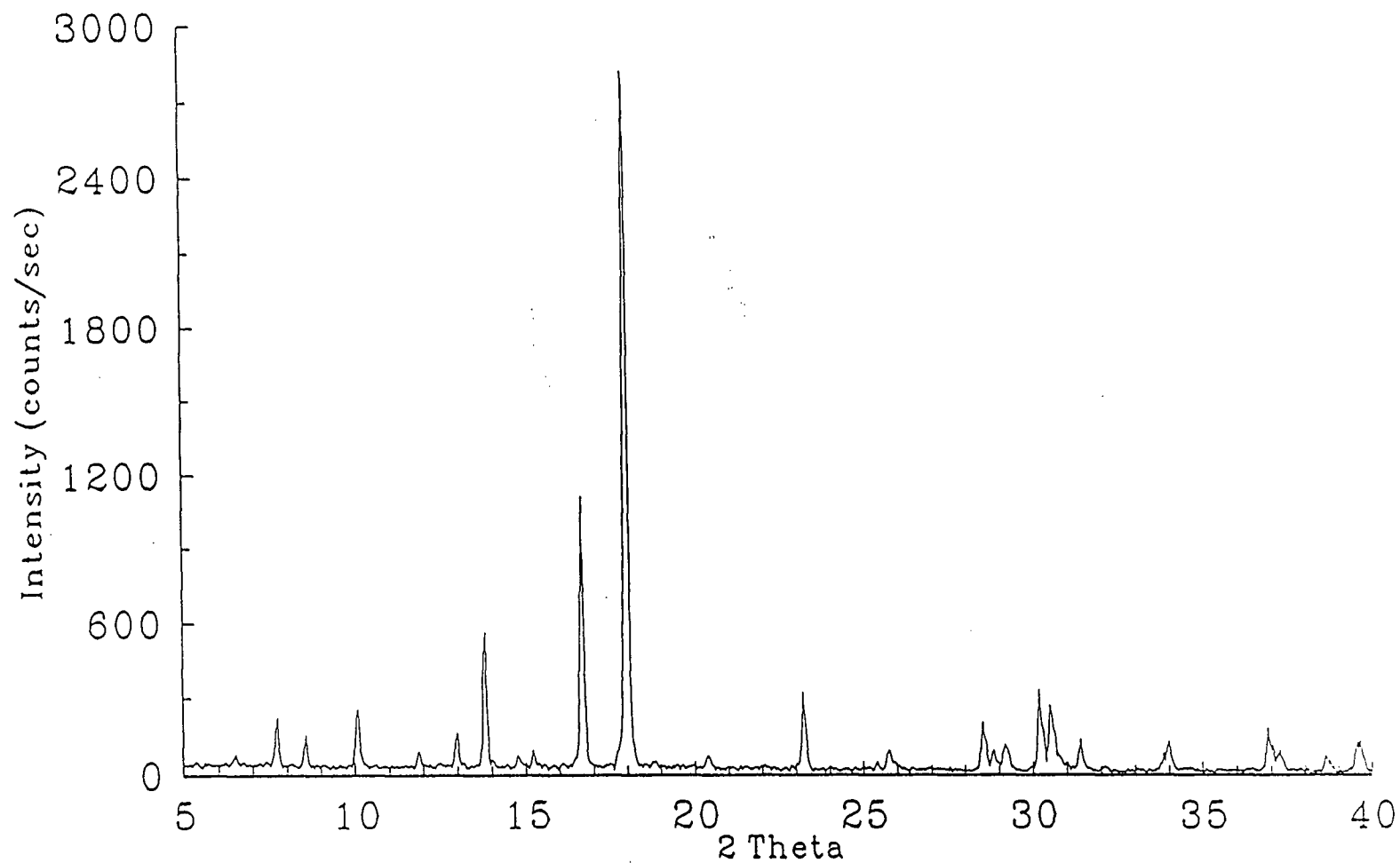


FIGURE 6

TABLE 1

X-RAY PEAKS OBTAINED FOR CDPP

Sample Identification: Calcium dihydrogen pyrophosphate
(CDPP)

Peak	2-Theta	d space	I (rel)
1	17.700	5.0069	7.94
2	20.050	4.4250	8.59
3	23.800	3.7356	20.24
4	26.650	3.3422	39.92
5	28.000	3.1841	100.00
6	33.200	2.6963	11.23
7	40.200	2.2415	11.43
8	40.550	2.2229	8.41

1.2.2. Synthesis of calcium pyrophosphate dihydrate (triclinic)

Triclinic crystals of CPPD were obtained within a period of 24 hr to 4 days following synthesis.

The particle size distribution of CPPD crystals is shown in Figure 7. The range of particle size was between 0.1-95 μm with a mean particle size of 29 μm .

2. CHARACTERIZATION OF CRYSTALS

2.1. X-RAY DIFFRACTION

MSUM and CPPD crystals were identified by the X-ray diffraction patterns obtained on a Rigaku Giegerflex X-ray diffractometer. The 2θ values, d spacings and relative intensities of the diffraction peaks of MSUM prepared by the previous method of Burt et al. (1983) and the modified method are given in Tables 2 and 3 respectively while the diffraction patterns are shown in Figures 8 and 9 respectively. The diffraction peaks at 2θ values of 3.16, 3.48, 4.69, 4.92, 7.58 and 9.40 were compared to confirm that the crystals prepared by the modified method were those of monosodium urate monohydrate.

The X-ray diffraction pattern of CPPD crystals is shown in Figure 10 and the data given in Table 4. The diffraction peaks at 2θ values of 3.12, 3.24, 7.07 and 8.1 were compared to the standard pattern for CPPD (Joint Committee on Powder

PARTICLE SIZE DISTRIBUTION OF CPPD CRYSTALS (n = 100)

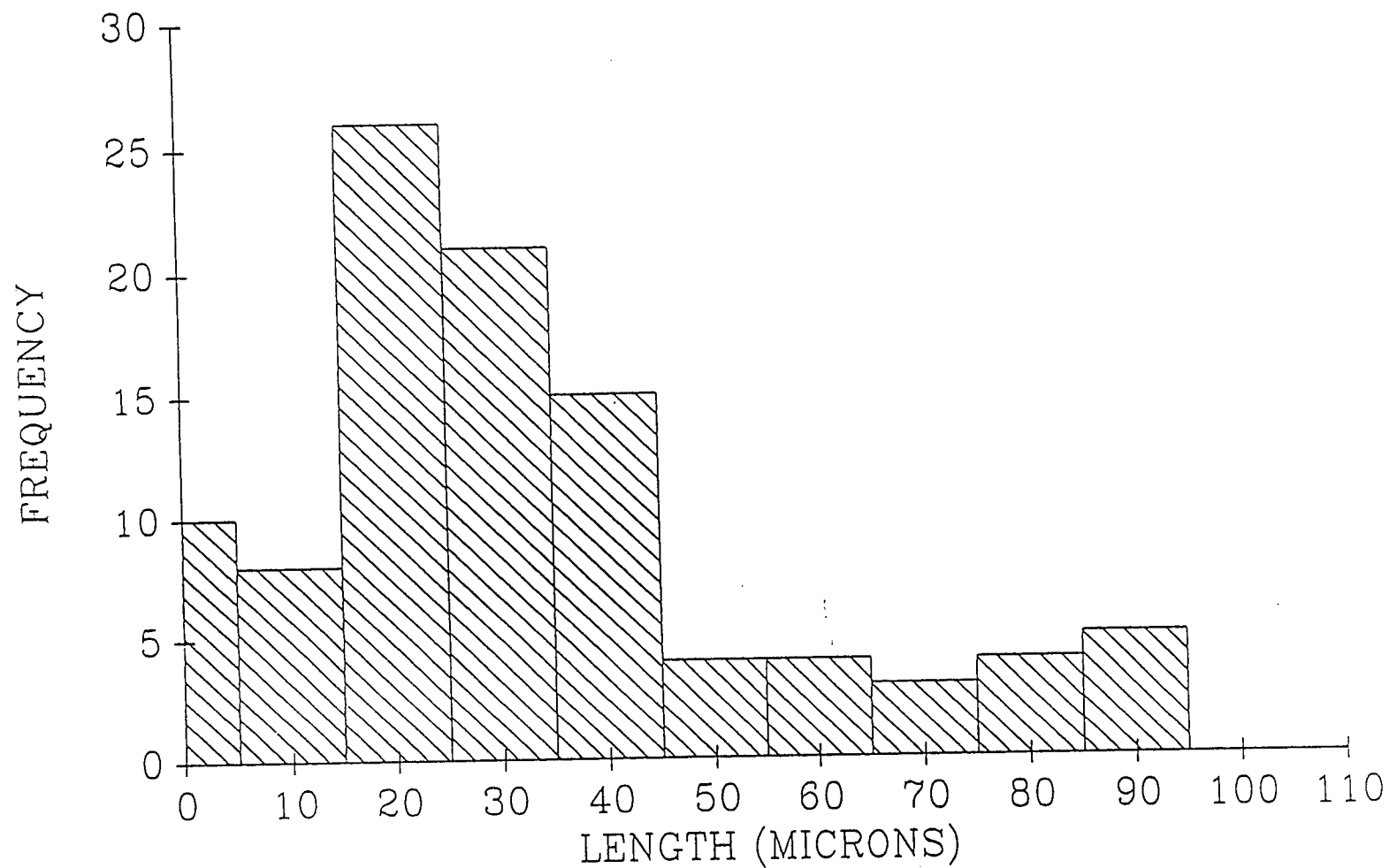


FIGURE 7

XRAY DIFFRACTION PATTERN OF
MONOSODIUM URATE MONOHYDRATE (Burt et al., 1983)

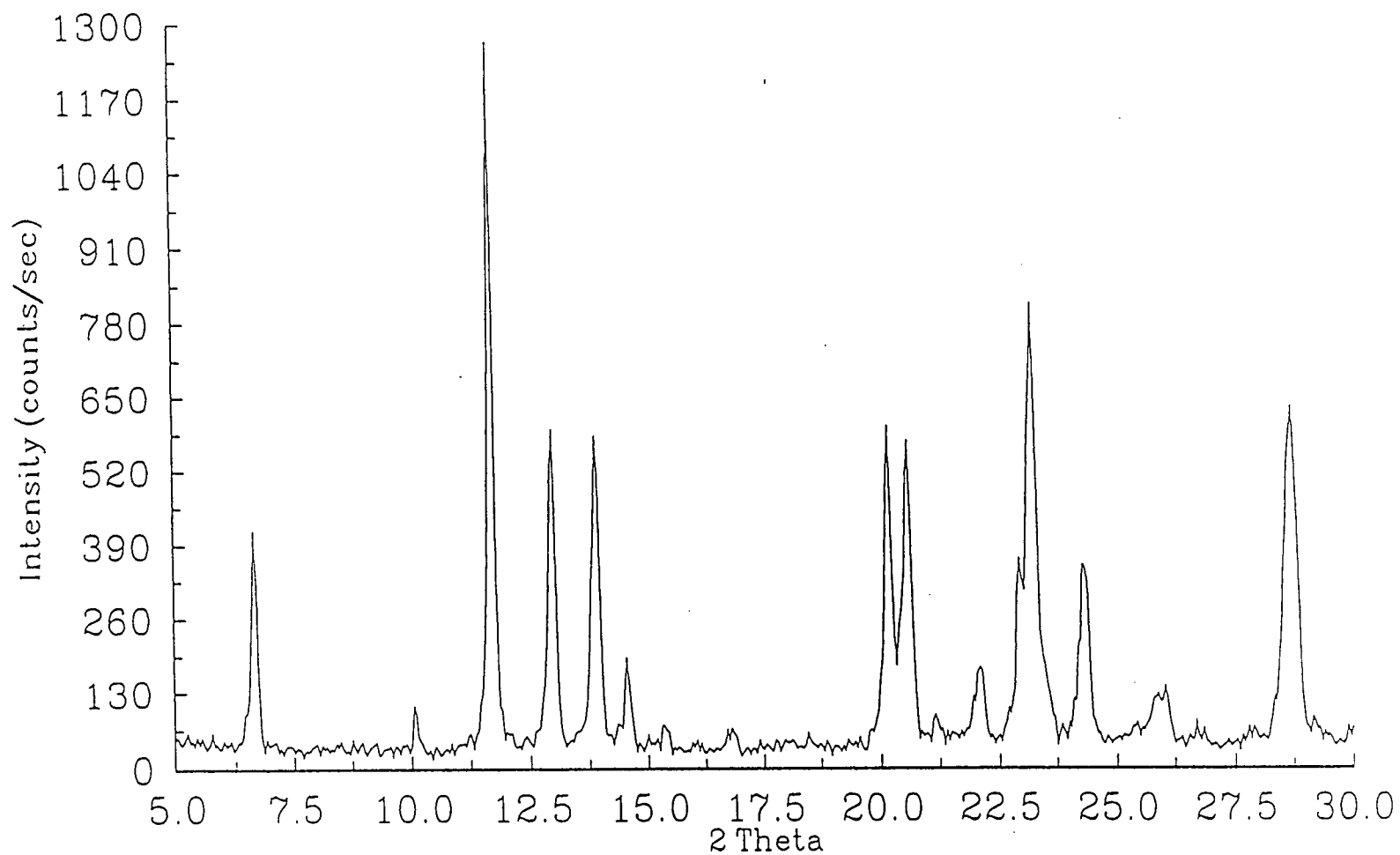


FIGURE 8

TABLE 2
X-RAY PEAKS OBTAINED FOR MSUM (Burt et al., 1983)

Peak	2-Theta	d space	I (rel)
1	11.650	7.5899	32.31
2	16.700	5.3044	100.00
3	17.950	4.9377	41.13
4	18.900	4.6916	45.14
5	19.550	4.5371	14.38
6	25.150	3.5381	40.43
7	25.600	3.4769	44.66
8	27.100	3.2877	12.64
9	27.900	3.1953	22.07
10	28.200	3.1620	53.19
11	29.300	3.0457	26.59
12	33.700	2.6574	44.99

XRAY DIFFRACTION PATTERN OF
MONOSODIUM URATE MONOHYDRATE (MODIFIED METHOD)

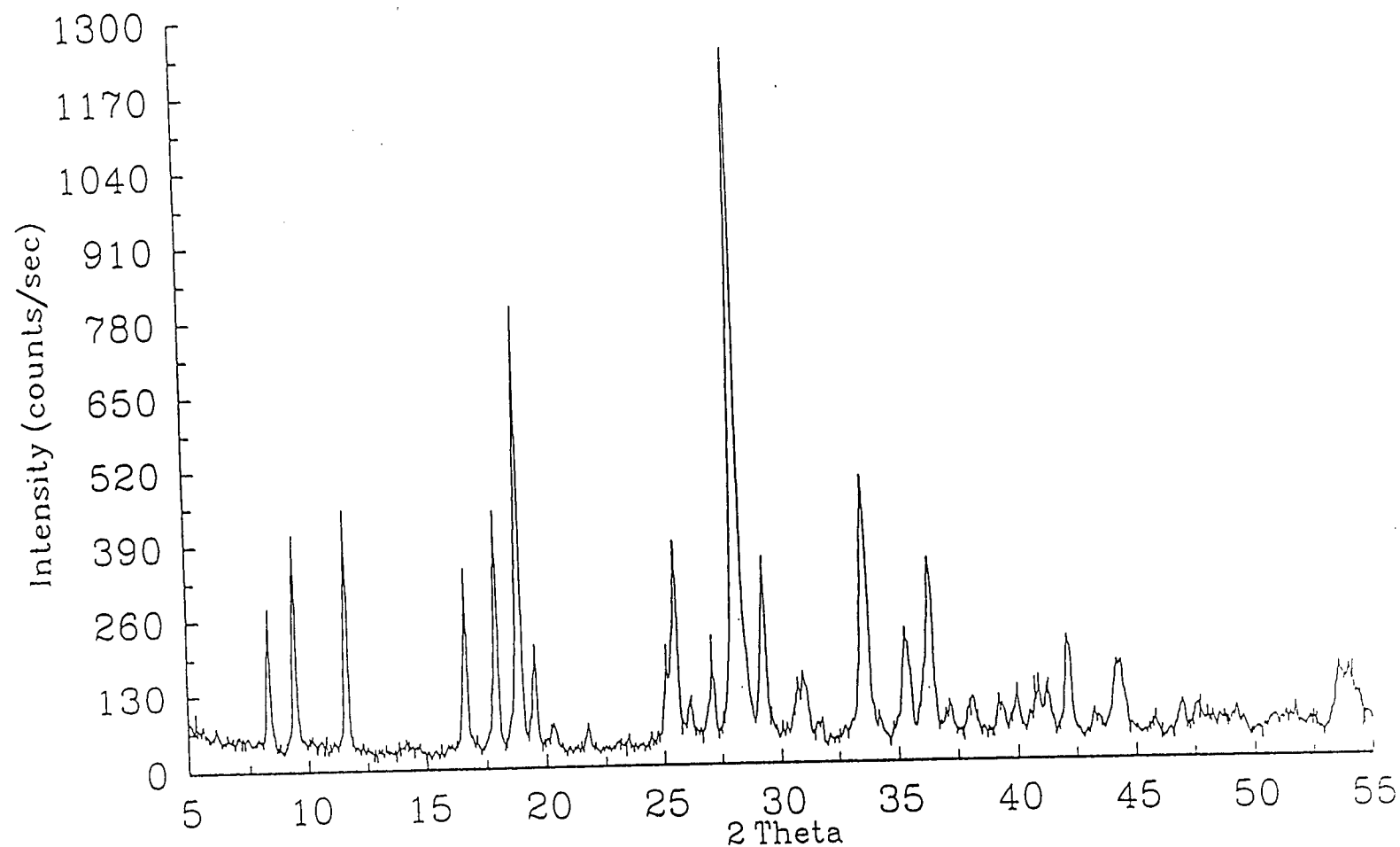


FIGURE 9

TABLE 3

X-RAY PEAKS OBTAINED FOR MSUM (modified method)

Peak	2-Theta	d space	I (rel)
1	8.300	10.6442	22.65
2	9.400	9.4009	31.86
3	11.650	7.5899	37.45
4	16.650	5.3202	23.82
5	17.950	4.9377	37.01
6	18.900	4.6916	60.54
7	19.550	4.5371	17.57
8	25.150	3.5381	16.26
9	25.550	3.4836	31.14
10	28.200	3.1620	100.00
11	29.300	3.0457	27.09
12	33.650	2.6613	39.96
13	36.400	2.4663	26.56

XRAY DIFFRACTION PATTERN OF
CALCIUM PYROPHOSPHATE DIHYDRATE

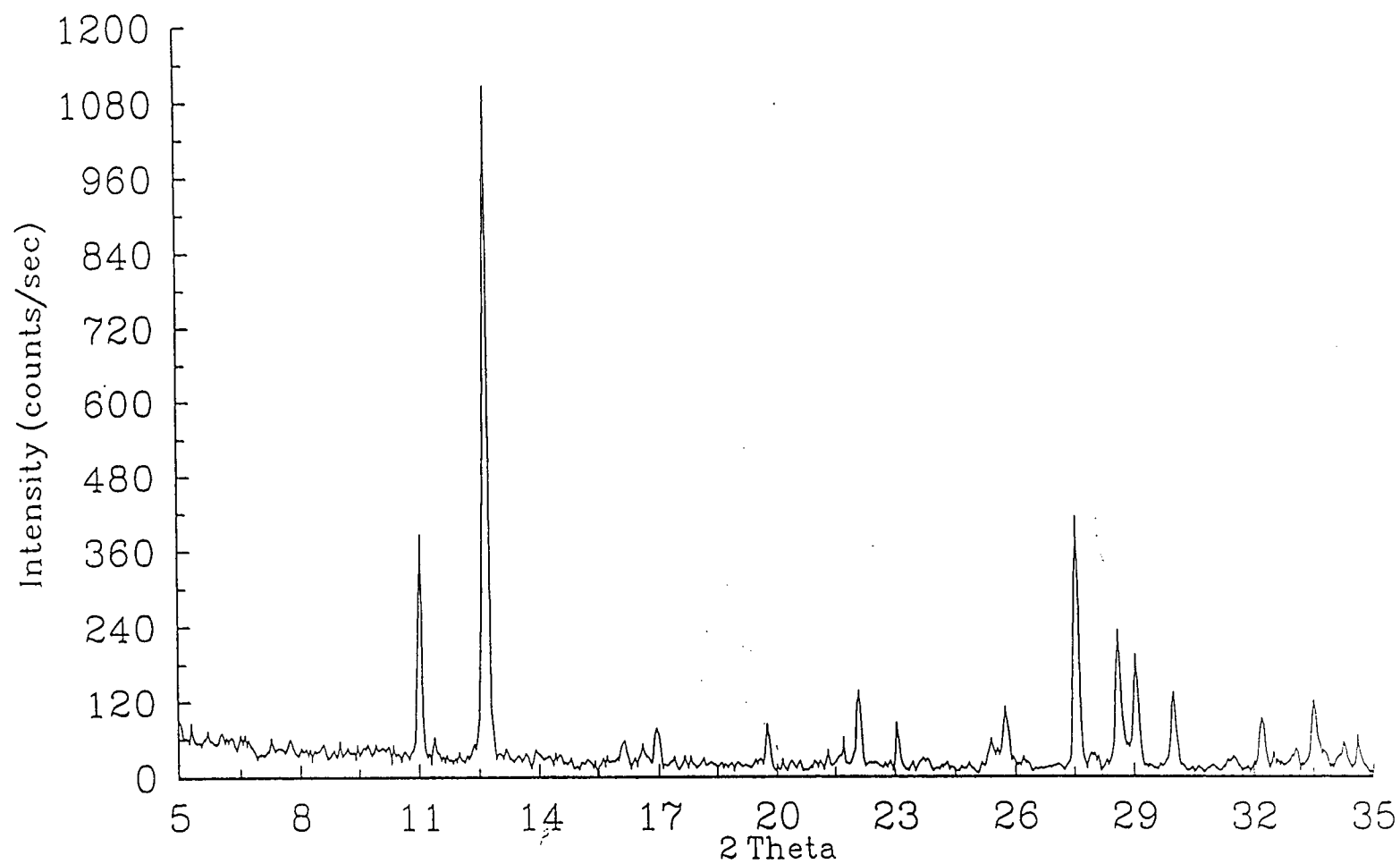


FIGURE 10

TABLE 4

X-RAY PEAKS OBTAINED FOR CPPD

Peak	2-Theta	d space	I (rel)
1	11.000	11.9443	32.84
2	12.650	10.3915	100
3	16.950	7.7679	6.79
4	19.750	6.6753	7.60
5	22.100	5.9730	13.49
6	23.050	5.7299	7.99
7	25.750	5.1377	10.72
8	27.550	4.8079	43.03
9	28.600	4.6349	23.66
10	29.050	4.5646	19.57
11	30.000	4.4232	13.13
12	32.200	4.1282	8.75
13	33.500	3.9723	12.03

Diffraction Standards, JCPDS pattern) (Burt and Jackson, 1987) to confirm that the prepared crystals were those of calcium pyrophosphate dihydrate (triclinic).

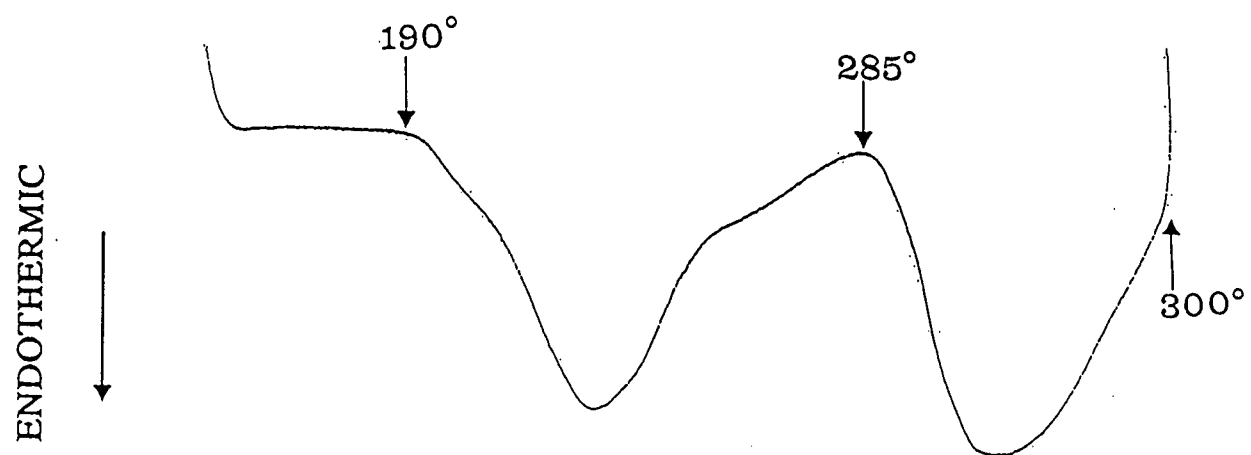
2.2. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

The DSC curve obtained for calcium pyrophosphate dihydrate samples is shown in Figure 11. The presence of two endothermic peaks, the first between 190 to 285°C and the second between 285 to 300°C corresponded to the loss of two moles of water from the crystals (average weight loss 12.3% at 300°C; molecular weight of CPPD 290) confirming the presence of the calcium pyrophosphate as a dihydrate.

Crystals of monosodium urate prepared by the Burt et al. (1983) method as well as the modified method were analyzed by DSC and the curves obtained are shown in Figures 12 and 13 respectively. The broad endothermic peak between 230 to 300°C corresponded to the loss of one mole of water of hydration (average weight loss 10.2%; molecular weight of MSUM 209) confirming the monohydrate form of these crystals.

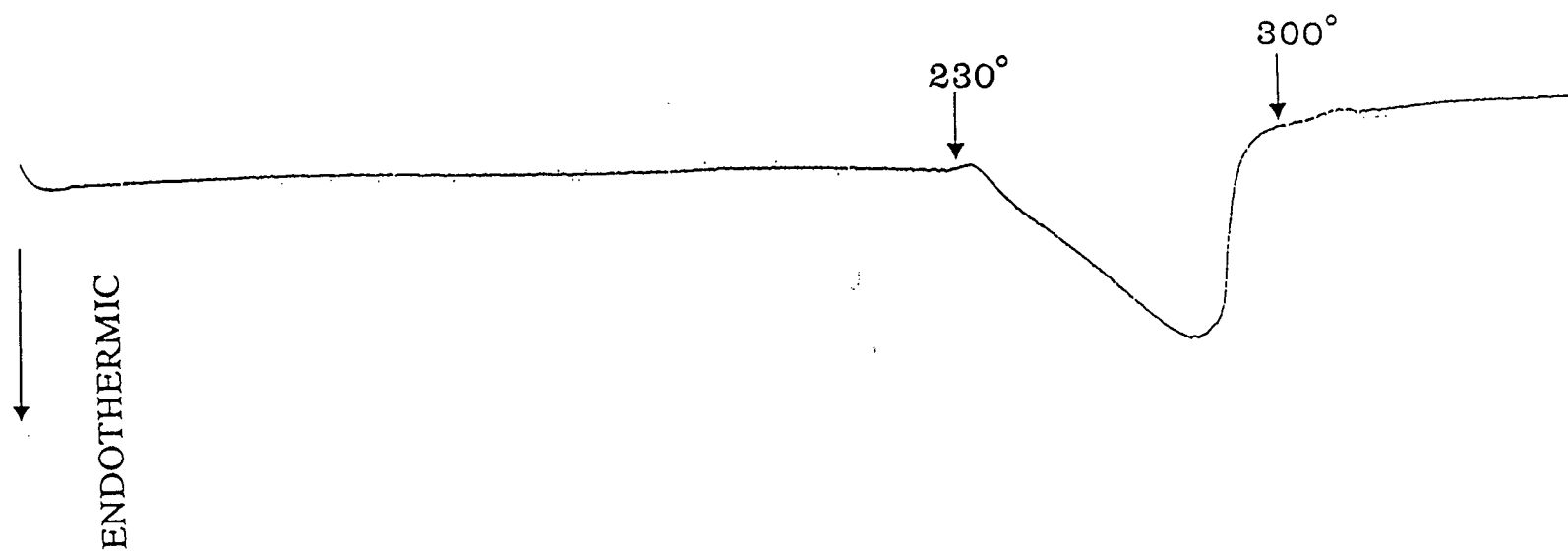
2.3. SCANNING ELECTRON MICROSCOPY (SEM)

Figures 14-16 show scanning electron micrographs of CPPD crystals and MSUM crystals prepared by the method of Burt et al. (1983) and the modified method. The micrometer length printed in the lower right hand corner of the micrographs refers to the length of the largest crystal in the



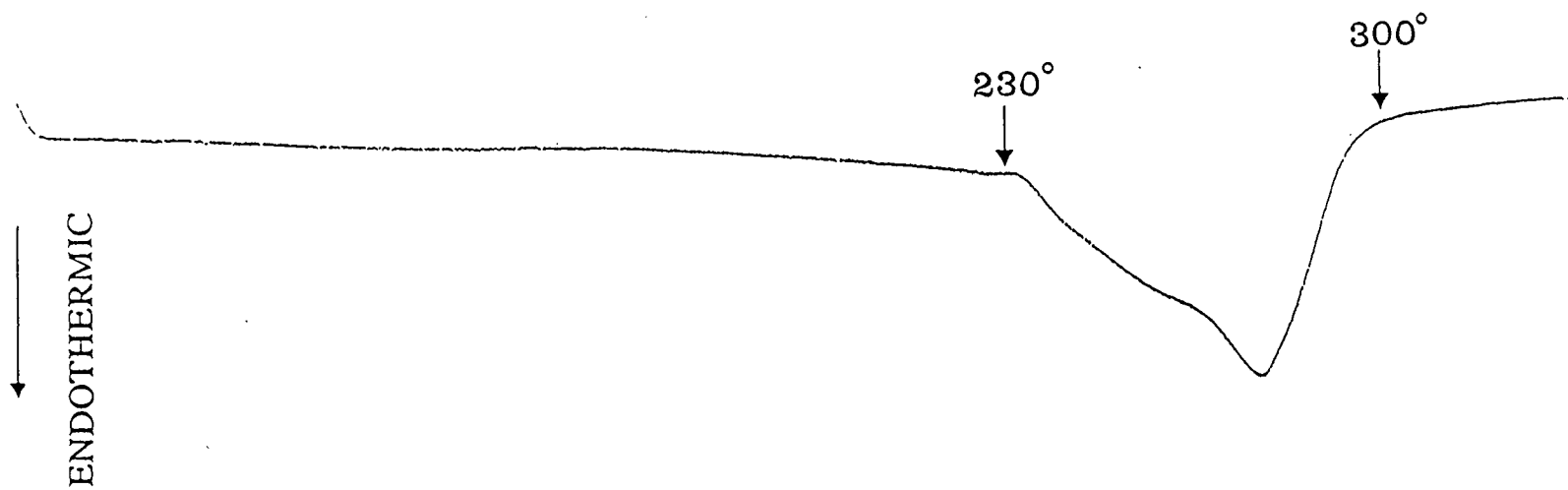
DSC THERMOGRAM OF CPPD CRYSTALS

FIGURE 11



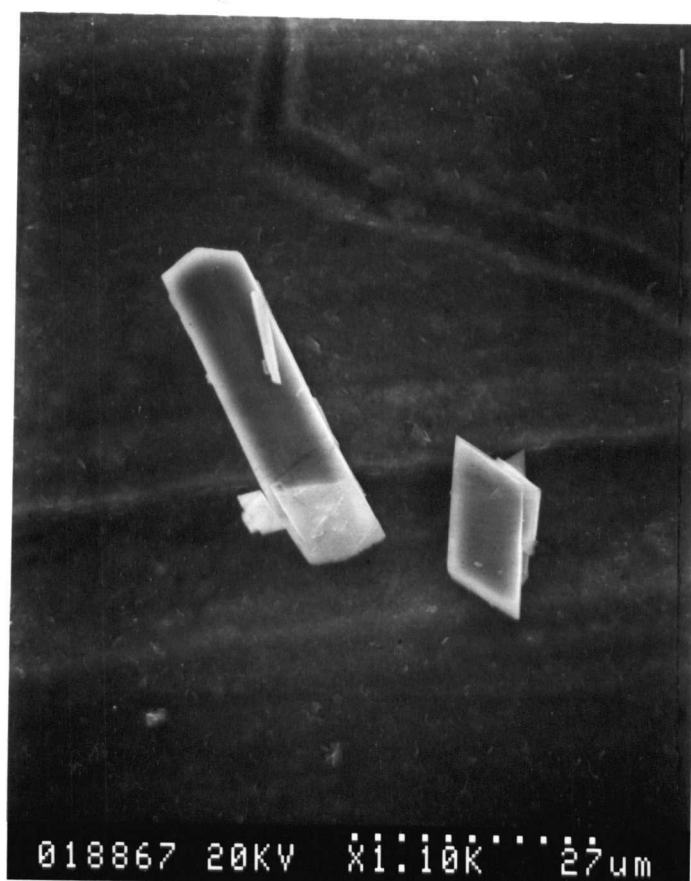
DSC THERMOGRAM OF MSUM CRYSTALS (BURT et al., 1983)

FIGURE 12



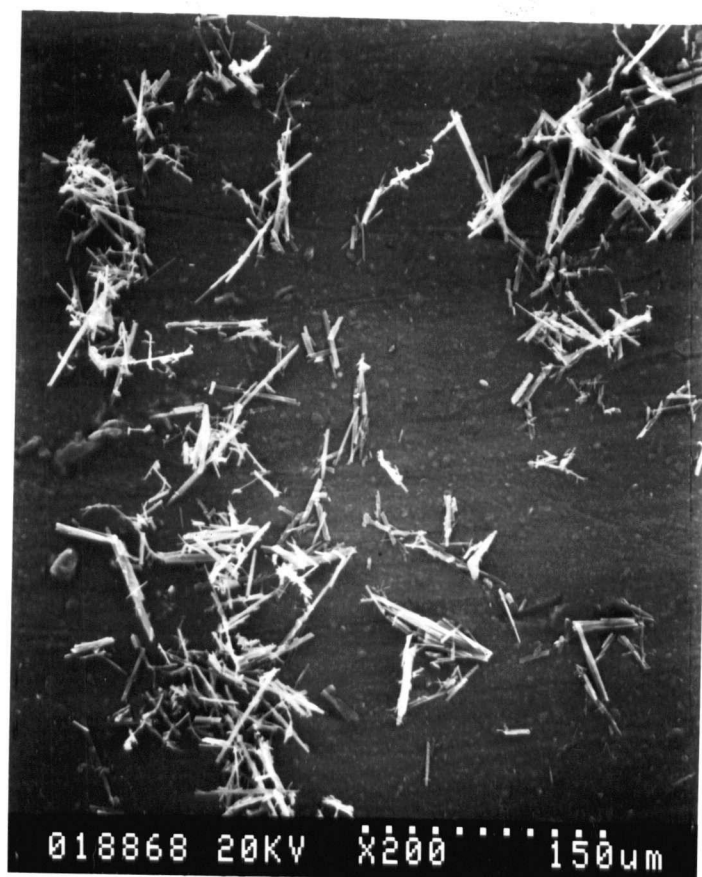
DSC THERMOGRAM OF MSUM CRYSTALS (Modified method)

FIGURE 13



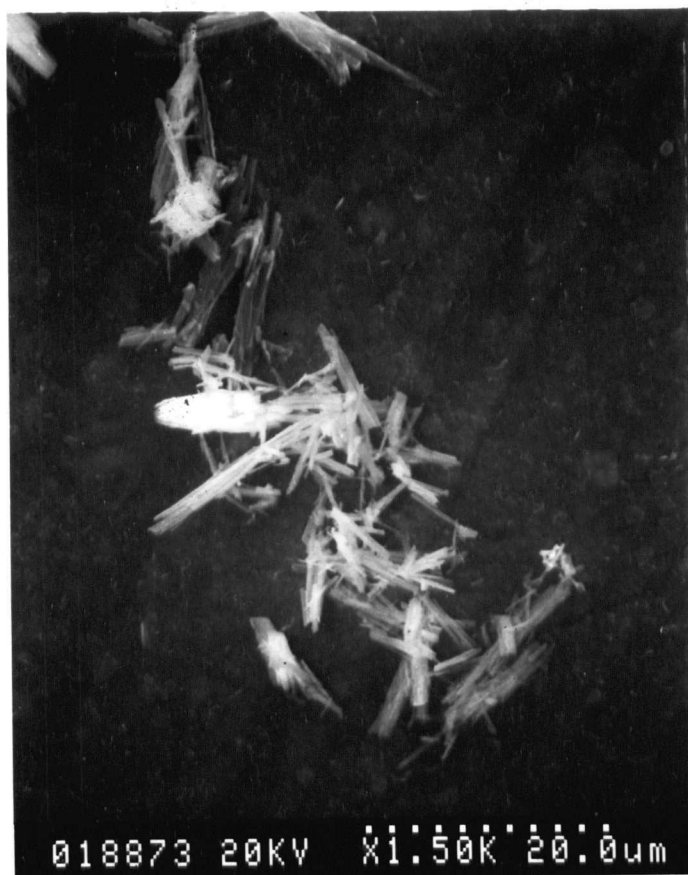
SCANNING ELECTRON MICROGRAPH OF CPPD CRYSTALS

FIGURE 14



SCANNING ELECTRON MICROGRAPH OF MSUM CRYSTALS (Burt
et al., 1983)

FIGURE 15



SCANNING ELECTRON MICROGRAPH OF MSUM CRYSTALS
(Modified method)

FIGURE 16

micrograph . The MSUM crystals prepared by either method were found to have the typical long needle shaped crystal habit and the CPPD crystals the typical elongated prismatic habit.

3. ESTIMATION OF NEUTROPHIL COUNT AND VIABILITY

Differential counts of the prepared neutrophil suspension were used to determine the % purity of the preparation as well as to determine the cell count. The neutrophil count thus obtained was similar to the count estimated by the release of LDH from neutrophils lysed with Triton X-100. The prepared neutrophil suspensions were found to be more than 95% pure i.e. >95% of the cells were polymorphonuclear leukocytes.

The ability of the viable neutrophil to exclude the dye trypan blue from the cell has previously been used as a measure of the viability of isolated human neutrophils (Babior and Cohen, 1981). The prepared neutrophil suspension when examined by this method had >95% cell viability. Thus the method of isolation of neutrophils was found to yield cell preparations of >95% purity and >95% viability.

The ability of neutrophils to produce CL depends on the viability of the cells. Thus the chemiluminescent response of neutrophils on stimulation with particulate or soluble stimuli could serve as an indicator of the viability of the

cell preparation. Hence, the chemiluminescent response of neutrophils separated from Red Cross blood (processing time 24-48 hr) and stimulated with uncoated MSUM crystals in the presence of luminol was used as a rapid test of the viability of the cell preparation. All observed responses were compared to the response obtained when the neutrophil cell preparation isolated from blood collected from human volunteers (processing time, 4-5 hours) was stimulated with MSUM in the presence of luminol. The viability of a cell preparation was considered acceptable if the CL response was greater than or equal to 150 mV with a time to maximum response of between 5-10 minutes.

4. MEASUREMENT OF NEUTROPHIL RESPONSES

The weights chosen represented the weights corresponding to the maximal response. In our studies, surface areas of 5 mg MSUM and 50 mg CPPD were similar (Burt et al., 1989).

4.1. CRYSTAL STIMULATED SUPEROXIDE RELEASE FROM NEUTROPHILS

Figure 17 shows the typical time course of superoxide anion generation by neutrophils stimulated with uncoated MSUM and CPPD crystals. There was a rapid increase in superoxide levels up to about 20 minutes for MSUM to reach a peak value of about 15 nmoles O_2^- generated per $10E6$ neutrophils/mL for MSUM. The response of the neutrophils to stimulation with CPPD crystals was slower than the response to stimulation with MSUM crystals though the magnitude of the responses

SUPEROXIDE GENERATION BY NEUTROPHILS STIMULATED BY UNCOATED MSUM AND CPPD CRYSTALS

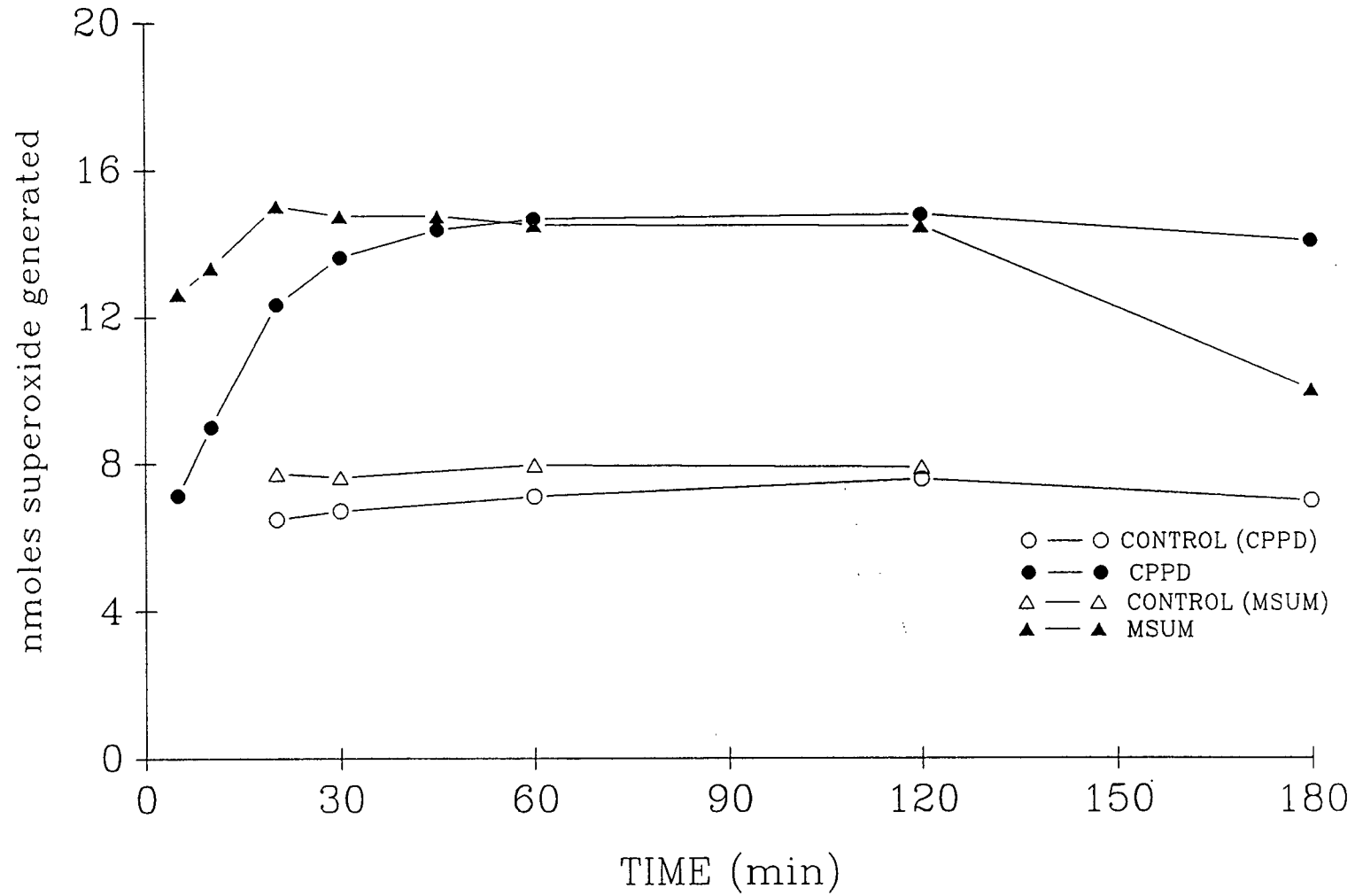


FIGURE 17

were not different. The maximum superoxide anion levels were attained in about 60 minutes for CPPD stimulated neutrophils and were about 14 nmoles per 10E6 neutrophils/mL. Controls were about 50% of the maximum values for superoxide release. Figures 18 and 19 show the graphs obtained for superoxide release when data from 4 experiments each for MSUM and CPPD were pooled. Naccache et al., (1991) also found that MSUM crystals (3 mg/mL) produced a more rapid generation of superoxide than CPPD crystals (3 mg/mL). The amounts of superoxide produced were maximal within 10 min for both crystals at about 9 nmoles and 5 nmoles superoxide per 10E6 neutrophils for MSUM and CPPD crystals, respectively. Terkeltaub et al. (1984, 1988) expressed O_2^- generation in terms of nmoles ferricytochrome c reduced. At crystal concentrations of 5 mg/mL for both MSUM and CPPD, they reported values of between 3.2-5.4 nmoles ferricytochrome c reduced per 10E6 neutrophils for MSUM and 2.4 nmoles ferricytochrome c reduced per 10E6 neutrophils for CPPD. Other studies of crystal-induced superoxide release have included cytochalasin B in the incubation medium to prevent phagocytosis and internalization of the membrane bound NADPH dependent oxidase involved in superoxide generation (Nagase et al., 1987; Rosen et al., 1986; Abramson et al., 1982; Higson et al., 1984). We have studied the effect of adding 10 μ g/mL cytochalasin B to the incubation medium on superoxide release and the results are shown in Figure 20. There was a

SUPEROXIDE GENERATION INDUCED BY MSUM CRYSTALS

(n = 4)

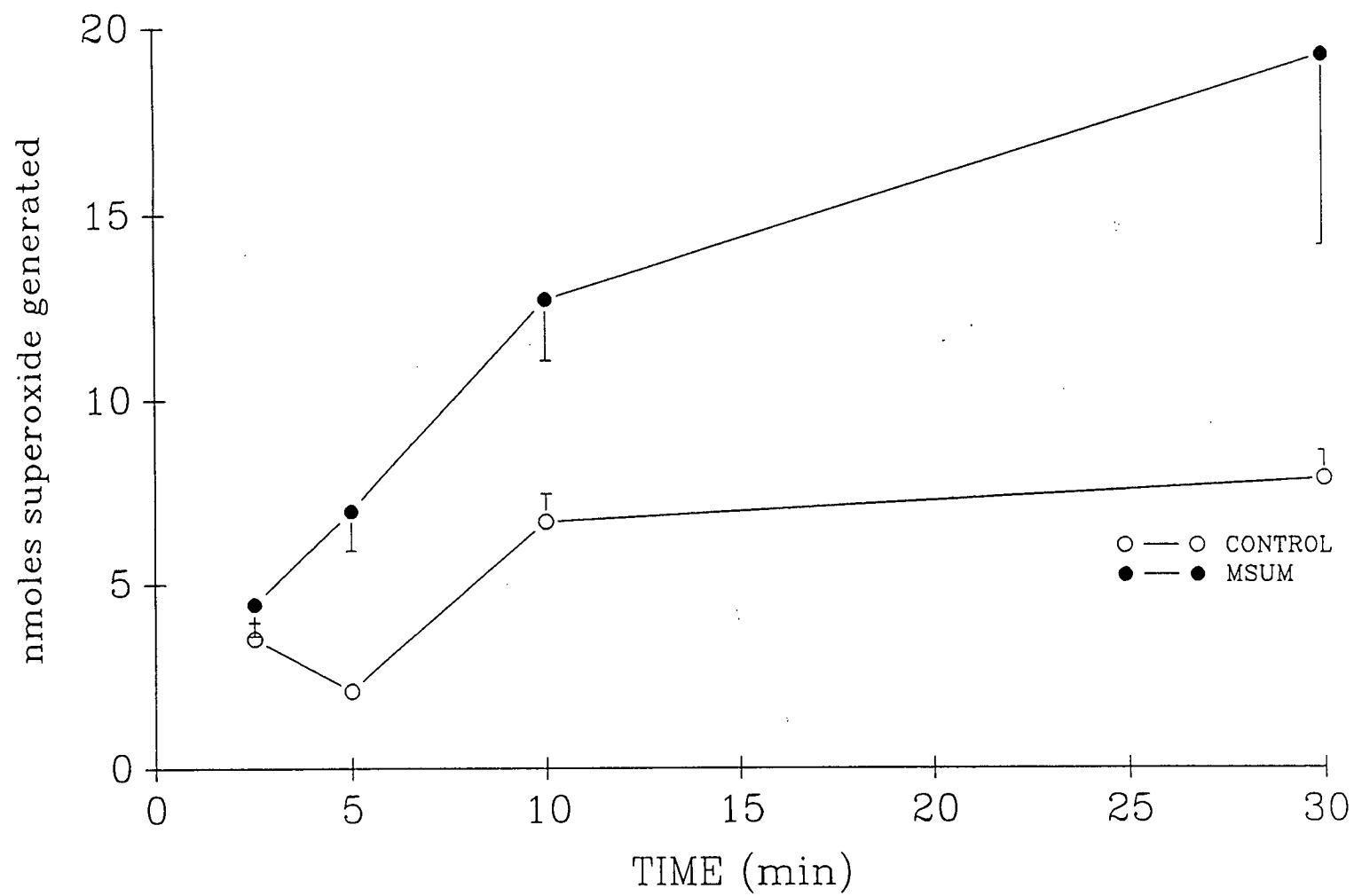


FIGURE 18

SUPEROXIDE GENERATION INDUCED BY CPPD CRYSTALS

(n = 4)

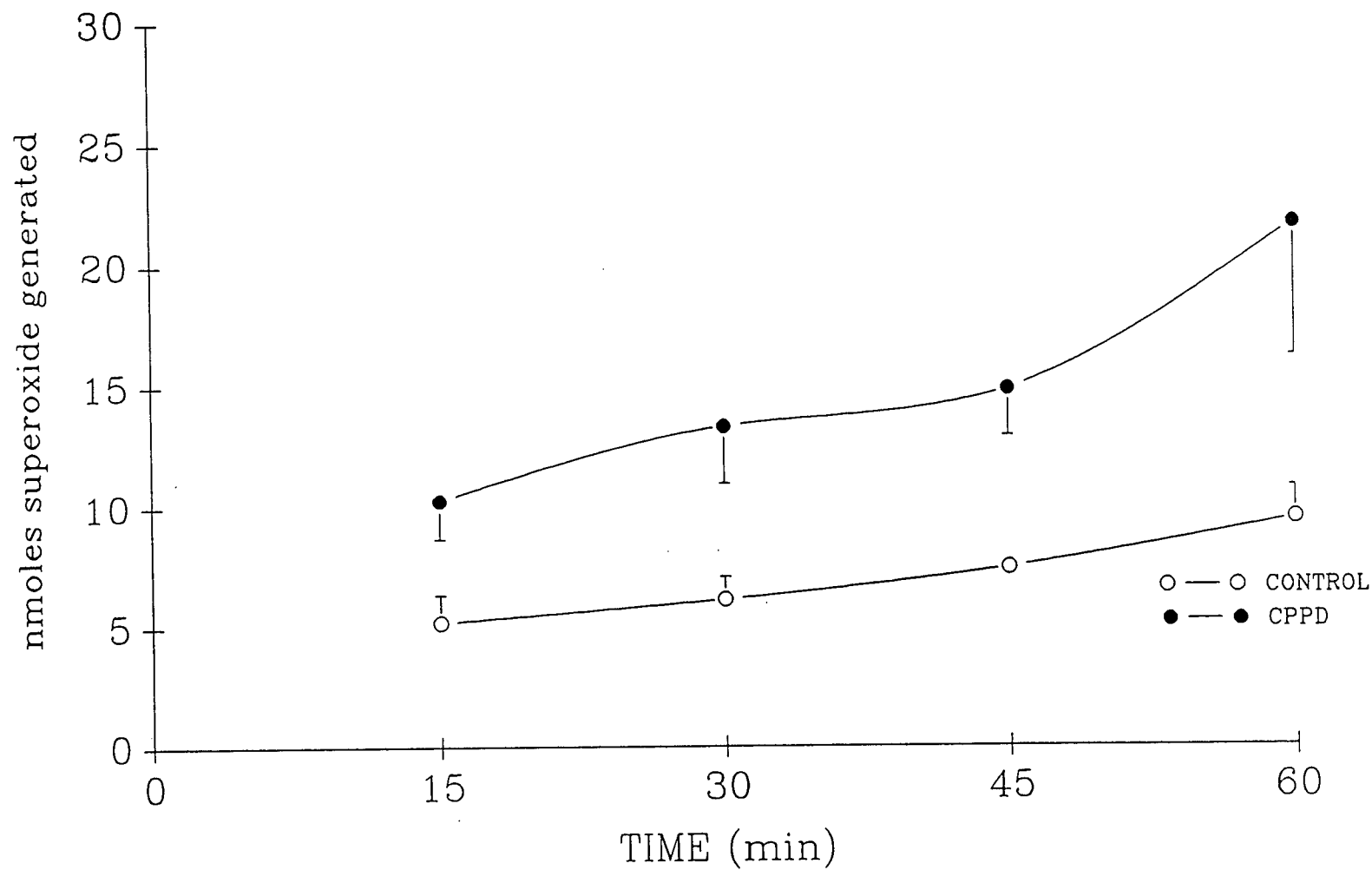


FIGURE 19

EFFECT OF CYTOCHALASIN B ON MSUM INDUCED SUPEROXIDE GENERATION

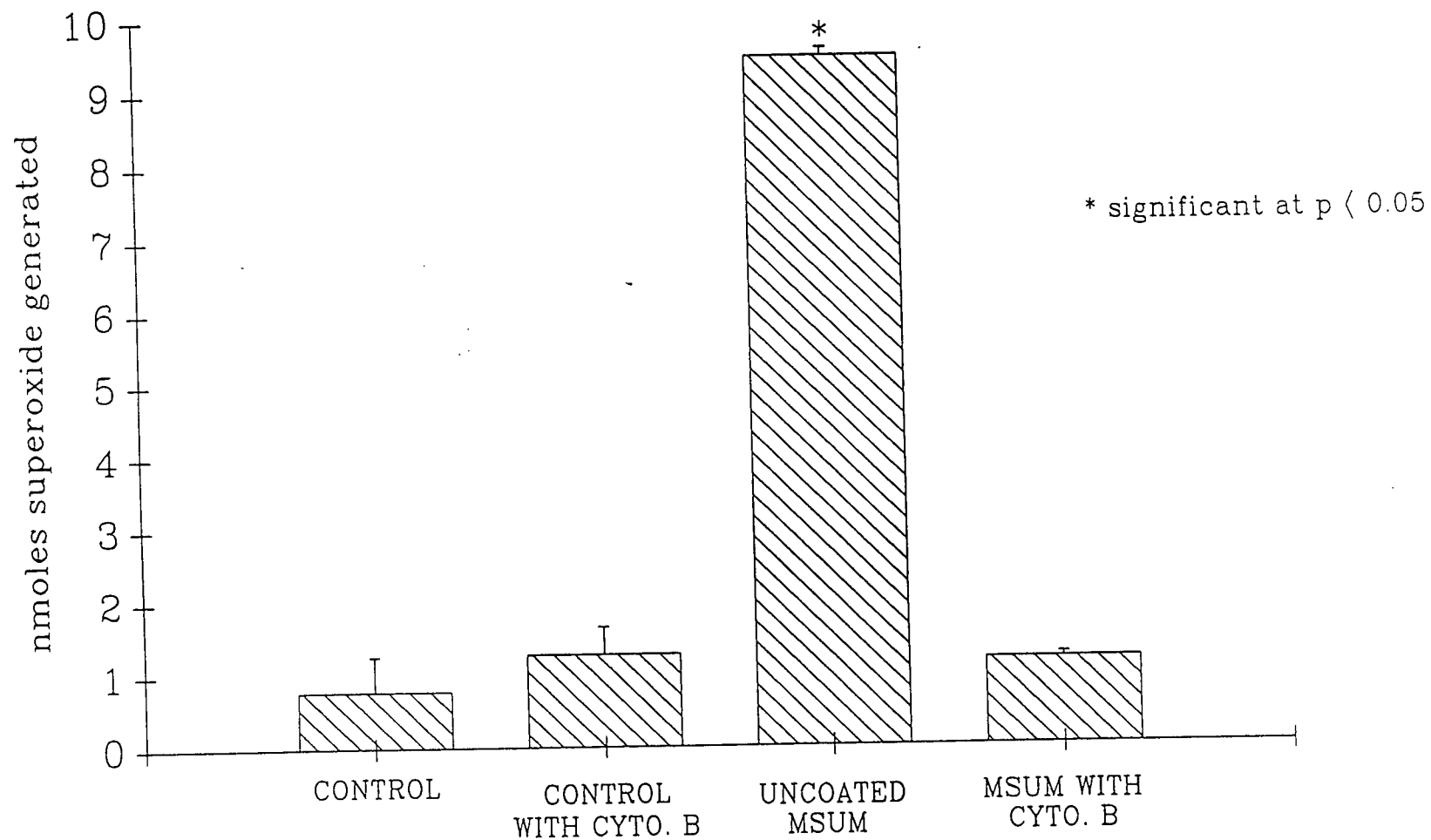


FIGURE 20

significant reduction in the superoxide generation induced by MSUM crystals ($p < 0.05$). Cytochalasin B is known to inhibit phagocytosis which is accompanied by the respiratory burst involving superoxide release and CL. Hence inhibition of phagocytosis may result in inhibition of the respiratory burst as well. Simchowitz et al. (1982) reported the inhibition of MSUM induced oxygen uptake by cytochalasin and suggested that the stimulation of the respiratory burst could be dependent on phagocytosis.

4.1.1. EFFECT OF SOD ON SUPEROXIDE RELEASE

The use of ferricytochrome c to monitor the release of superoxide is based on the reduction of ferricytochrome c to ferrocytochrome c. This reduction can be effected by several agents but only the reduction of ferri to ferro by superoxide can be inhibited by the enzyme SOD. To confirm that the reduction being measured in the assay was indeed due to the superoxide being generated, the reduction of ferricytochrome c was monitored in the presence of the enzyme SOD. Results are expressed in terms of nmoles superoxide generated which represents the nmoles of ferricytochrome c reduced. The results obtained when CPPD was incubated with neutrophils in the presence of SOD are shown in Figure 21. The reduction of ferricytochrome c by superoxide generated by CPPD stimulated neutrophils was suppressed in the presence of SOD indicating that the reduction of ferricytochrome c measured when CPPD crystals

EFFECT OF SOD ON CPPD INDUCED SUPEROXIDE RELEASE FROM NEUTROPHILS

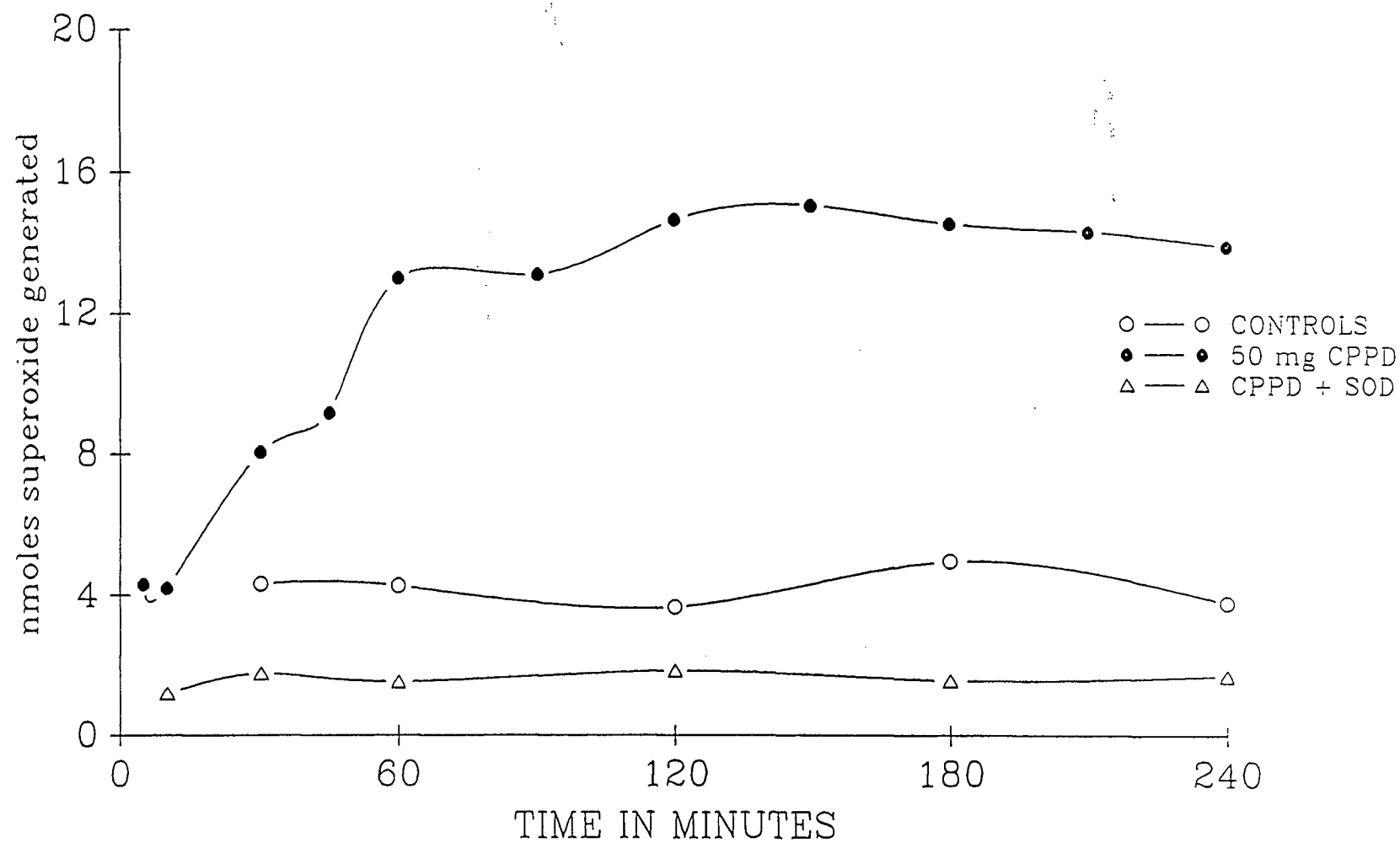


FIGURE 21

were incubated with neutrophils was due to the presence of superoxide.

The results obtained when MSUM was incubated with neutrophils in the presence of SOD are shown in Figure 22. The reduction of ferricytochrome c by superoxide produced by MSUM stimulated neutrophils was not significantly suppressed in the presence of SOD. This could be caused either by the reduction of ferricytochrome c being driven by another reducing species other than superoxide, or by the inactivation of SOD for example by adsorption of this protein onto the MSUM crystals. To determine whether SOD was being adsorbed to the MSUM crystal surface and thereby being inactivated, the supernatant from the incubation of SOD with MSUM crystals was added to CPPD/neutrophil incubations. The results of these incubations are shown in Figure 23. The levels of reduced ferricytochrome c for CPPD/neutrophil incubations to which the MSUM/SOD supernatants had been added were not decreased and in fact were greater than for CPPD/neutrophil incubations. It was likely the SOD had been inactivated, probably by adsorption onto the surface of uncoated MSUM crystals.

The SOD inhibitable reduction of ferricytochrome c assay has been widely used to monitor the generation of superoxide anion by neutrophils incubated with MSUM crystals (Terkeltaub et al., 1984; Abramson et al., 1982; Rosen et al., 1986; Naccache et al., 1991). However the inability of

SUPEROXIDE PRODUCTION BY MSUM STIMULATED NEUTROPHILS IN THE PRESENCE OF SUPEROXIDE DISMUTASE

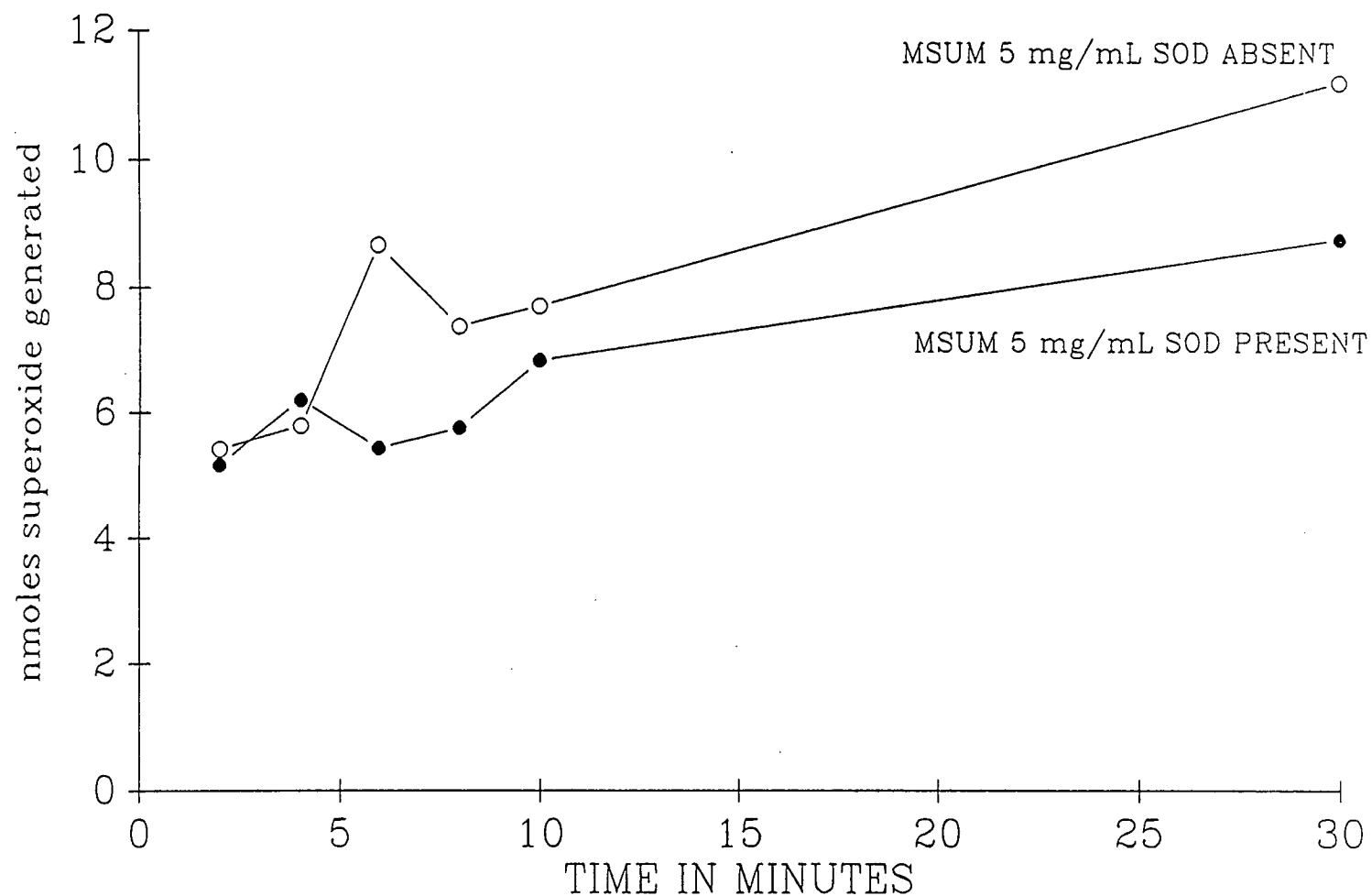
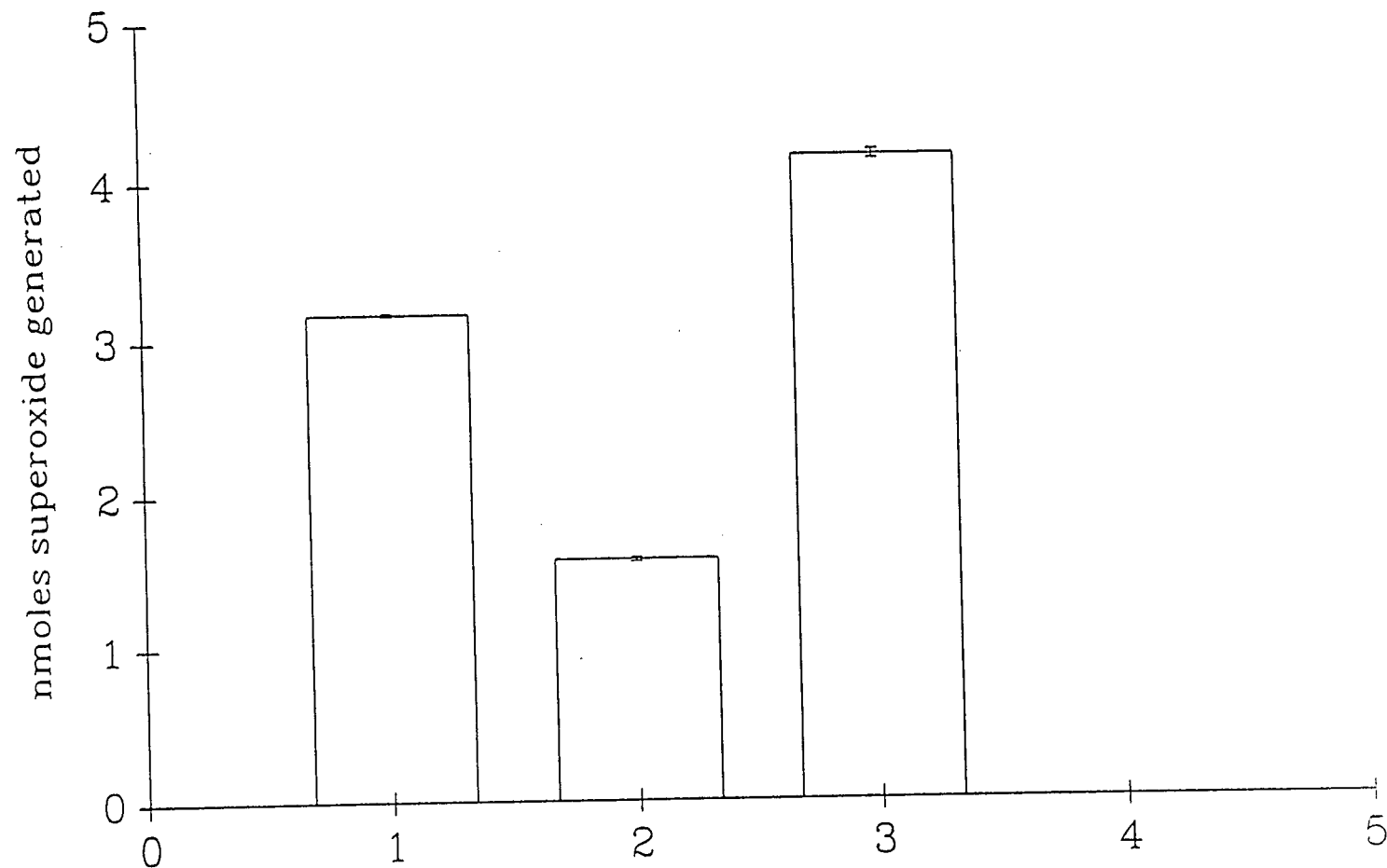


FIGURE 22

EFFECT OF MSUM TREATED SOD ON CPPD INDUCED SUPEROXIDE RELEASE FROM NEUTROPHILS



1: CPPD 50 mg/mL ; 2: CPPD 50 mg/mL + SOD 300 U/mL ; 3: CPPD 50 mg/mL + MSUM TREATED SOD

FIGURE 23

SOD to inhibit the reduction of ferricytochrome c by superoxide generated from MSUM stimulated neutrophils has not been previously reported. The presence of BSA in the buffers used in previous studies may have prevented any inactivation of SOD due to preferential adsorption of BSA onto the MSUM crystal surface thus inhibiting any subsequent adsorption of SOD onto the MSUM crystals.

4.1.2. EFFECT OF PROTEINS ON SUPEROXIDE RELEASE

The effect of protein coating on the MSUM crystal-induced superoxide generation by neutrophils is shown in Figure 24. There were no significant differences ($p < 0.05$) between the superoxide released by uncoated MSUM compared to Ig G, BSA or plasma protein coated MSUM. Other studies have shown that precoating of MSUM crystals with Ig G significantly enhanced MSUM induced superoxide generation (Abramson et al., 1982; Rosen et al., 1986; Nagase et al., 1989). However the superoxide release from neutrophils induced by uncoated MSUM in our work was approximately 3 fold greater than in the previous studies. It is possible that under the assay conditions used in our studies, the neutrophils were maximally stimulated by the uncoated MSUM crystals such that the coating of the crystals with Ig G could not enhance the generation of superoxide from the neutrophils.

Abramson et al. (1982) showed that precoating MSUM with plasma did not affect superoxide generation from

EFFECT OF PROTEIN COATING ON THE GENERATION OF SUPEROXIDE INDUCED BY MSUM

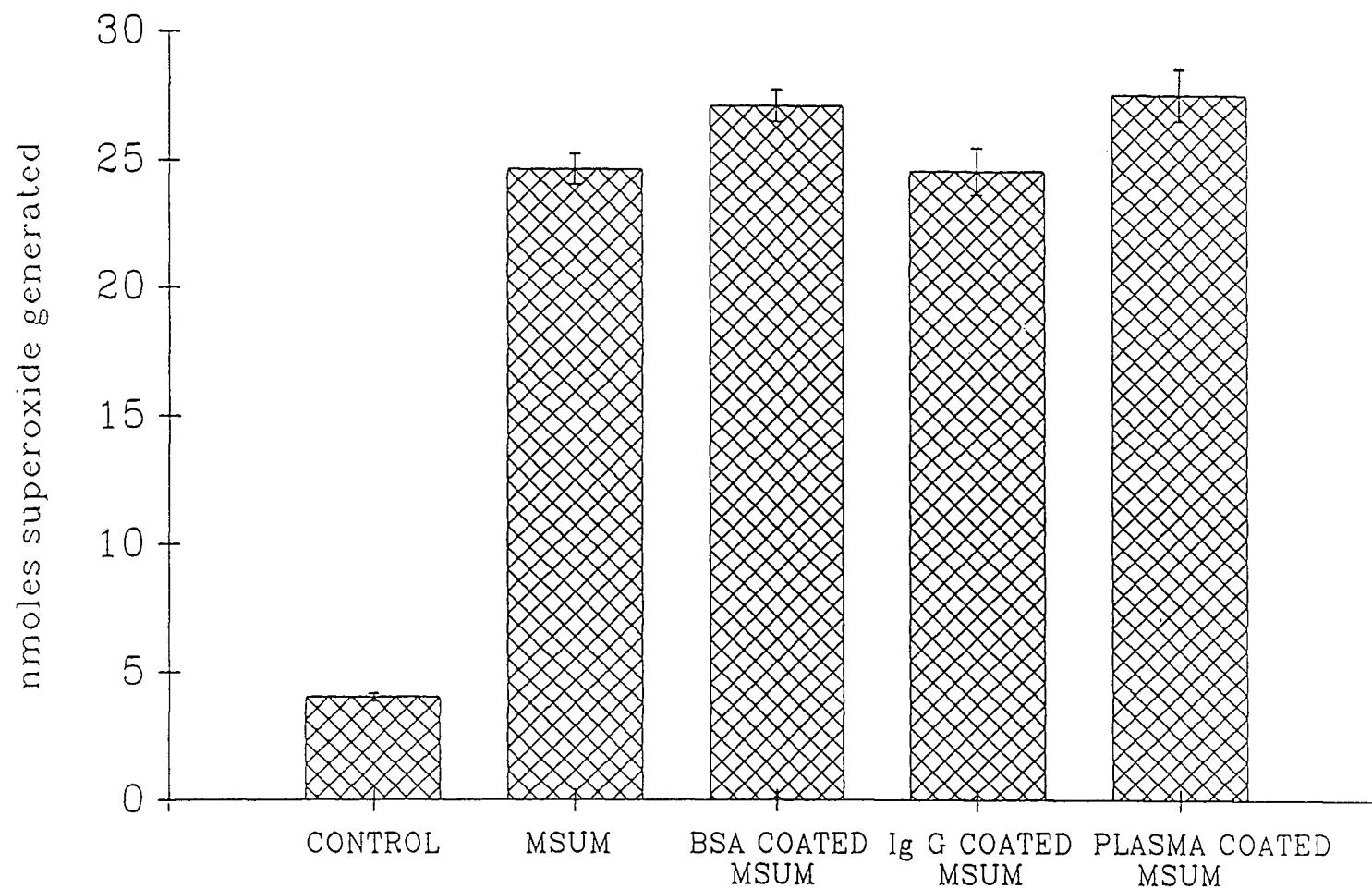


FIGURE 24

neutrophils, whereas Terkeltaub et al. (1984) found that precoating MSUM with plasma significantly inhibited MSUM induced superoxide generation.

Figure 25 shows the effect of protein coating on the generation of superoxide induced by incubation with CPPD crystals. There were no significant differences ($p < 0.05$) in the superoxide release induced by uncoated CPPD compared to Ig G or plasma protein coated CPPD. Again, it is possible that neutrophils were maximally stimulated by both uncoated CPPD and Ig G-coated CPPD. Nagase et al. (1989) found no significant enhancement of superoxide generation when CPPD crystals were coated with Ig G. To our knowledge, there are no other reports on the effect of protein coatings on CPPD crystal-induced superoxide generation.

Although no differences in superoxide release were observed for protein coated MSUM and CPPD versus uncoated crystals, all the studies were carried out at an incubation time of 30 min for MSUM and 60 min for CPPD. It is possible that there may be differences in the rates of superoxide generation for neutrophils stimulated by protein coated versus uncoated crystals.

4.2. CRYSTAL STIMULATED CHEMILUMINESCENT RESPONSE OF NEUTROPHILS

The chemiluminescent response of neutrophils to stimulation by 5 mg of uncoated MSUM crystals is shown in Figure 26.

EFFECT OF PROTEIN COATING ON SUPEROXIDE GENERATION INDUCED BY CPPD CRYSTALS

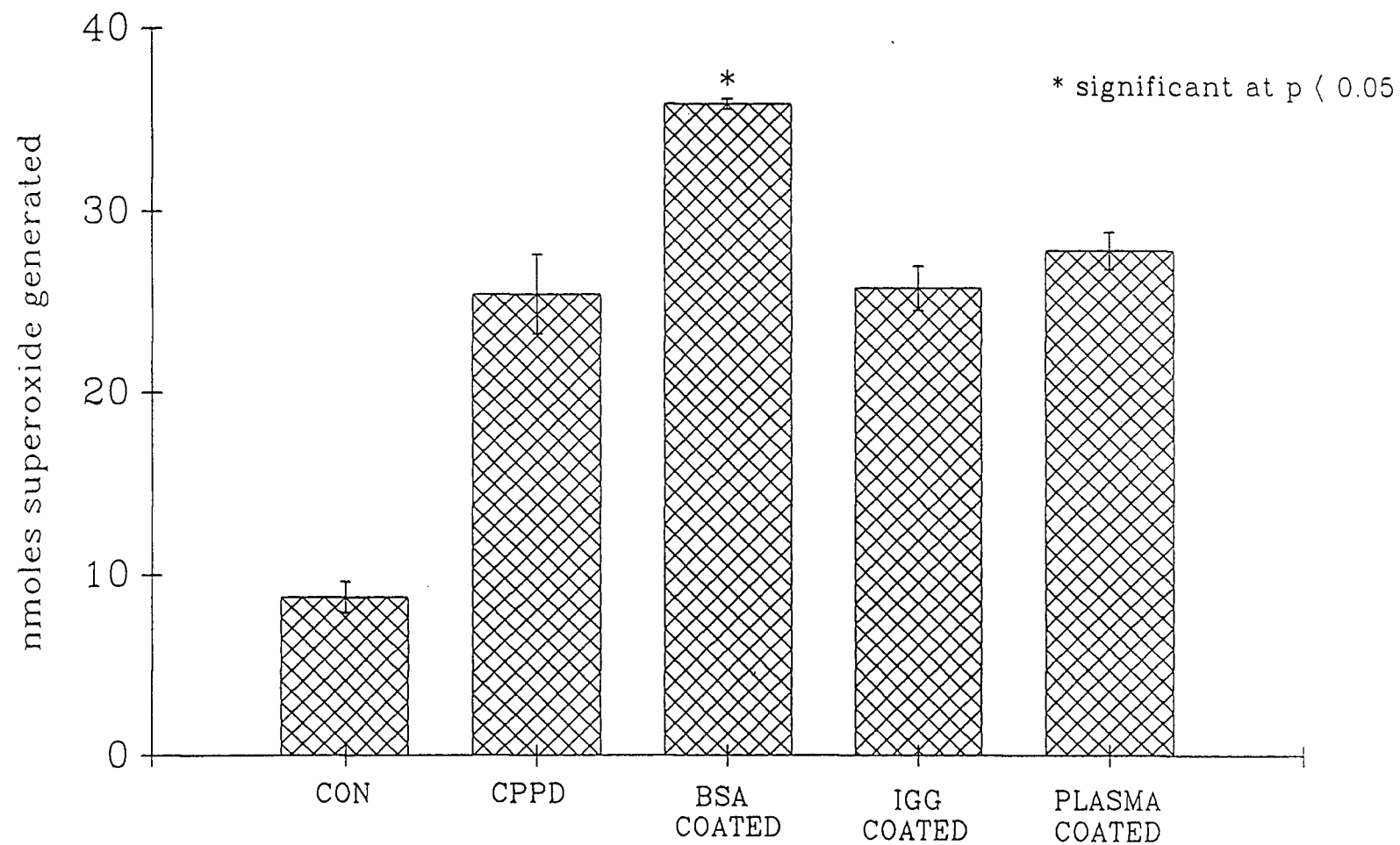


FIGURE 25

CHEMILUMINESCENCE GENERATED BY NEUTROPHILS STIMULATED BY 5 mg UNCOATED MSUM

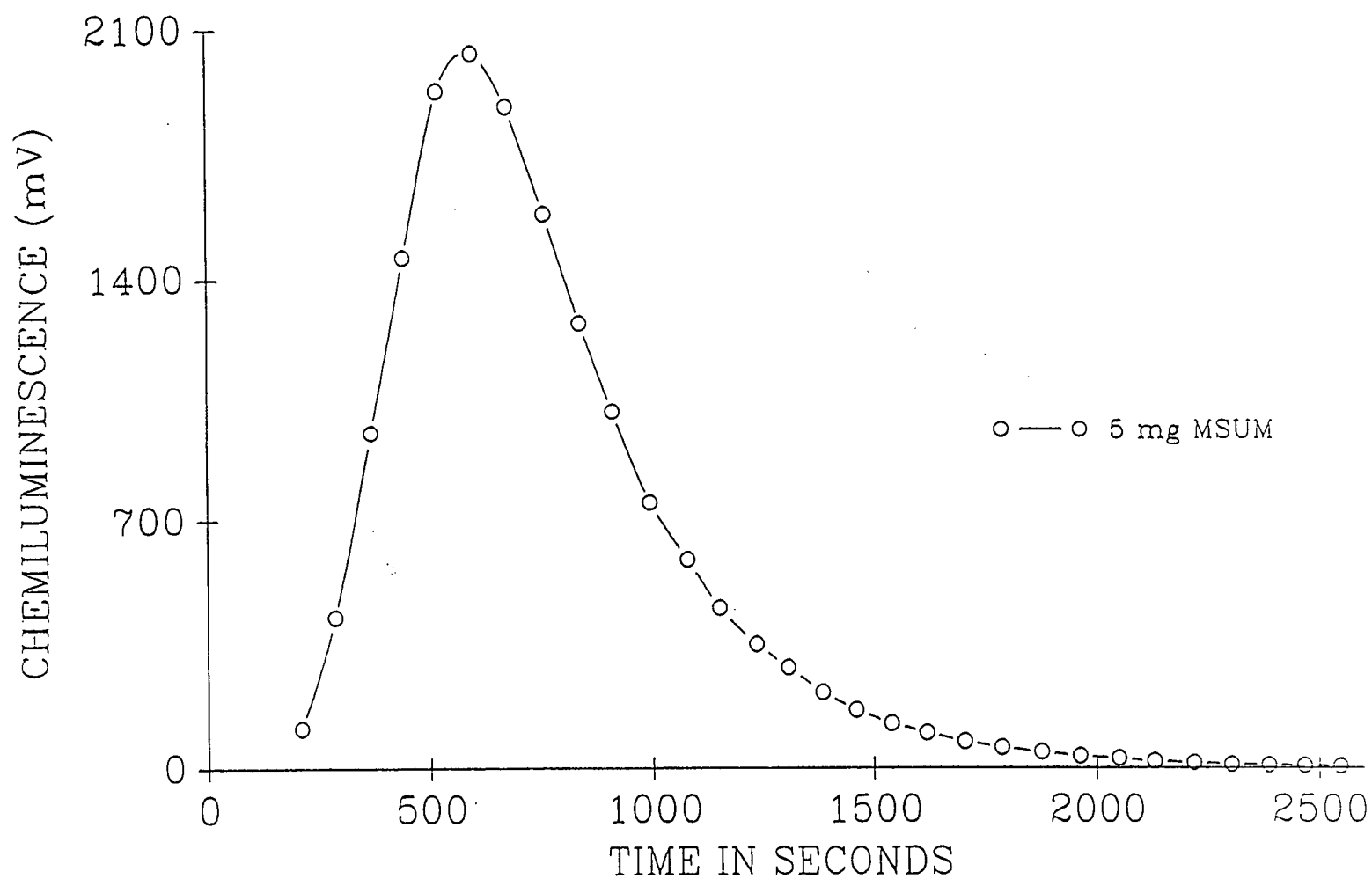


FIGURE 26

The bell shaped response curve was affected by changes in crystal concentration as seen in Figure 27. Table 5 shows the data expressed in terms of the area under the curve (AUC) of the plots of CL response in mV versus time at each crystal concentration. An increase in the crystal concentration from 2 mg to 5 mg/mL resulted in a higher maximum response and a decrease in the time required to elicit the maximal response. However further increases in MSUM crystal concentration to 10 mg/mL did not significantly enhance the response observed compared to 5 mg/mL MSUM. This could be due to interference in measurement due to light scattering by the crystals at the higher crystal concentrations or due to the limited metabolic capacity of the isolated neutrophil. The CL response of the neutrophils incubated with 5 mg/mL MSUM may be near the maximum possible thus allowing only a small increase when the crystal concentration is doubled to 10 mg/mL.

The chemiluminescent response of neutrophils to 50 mg/mL CPPD crystals is shown in Figure 28. The effect of CPPD concentration on neutrophil CL was studied over the concentration range of 10-50 mg/mL and the results obtained are shown in Figure 29 and Table 6. The CL generated was influenced by the CPPD crystal concentration. The CL produced increased as the CPPD concentration was increased from 10 mg/mL to 30 mg/mL but further increases in the CPPD concentration to 50 mg/mL resulted in a reduction of the

EFFECT OF MSUM CONCENTRATION ON CHEMILUMINESCENCE

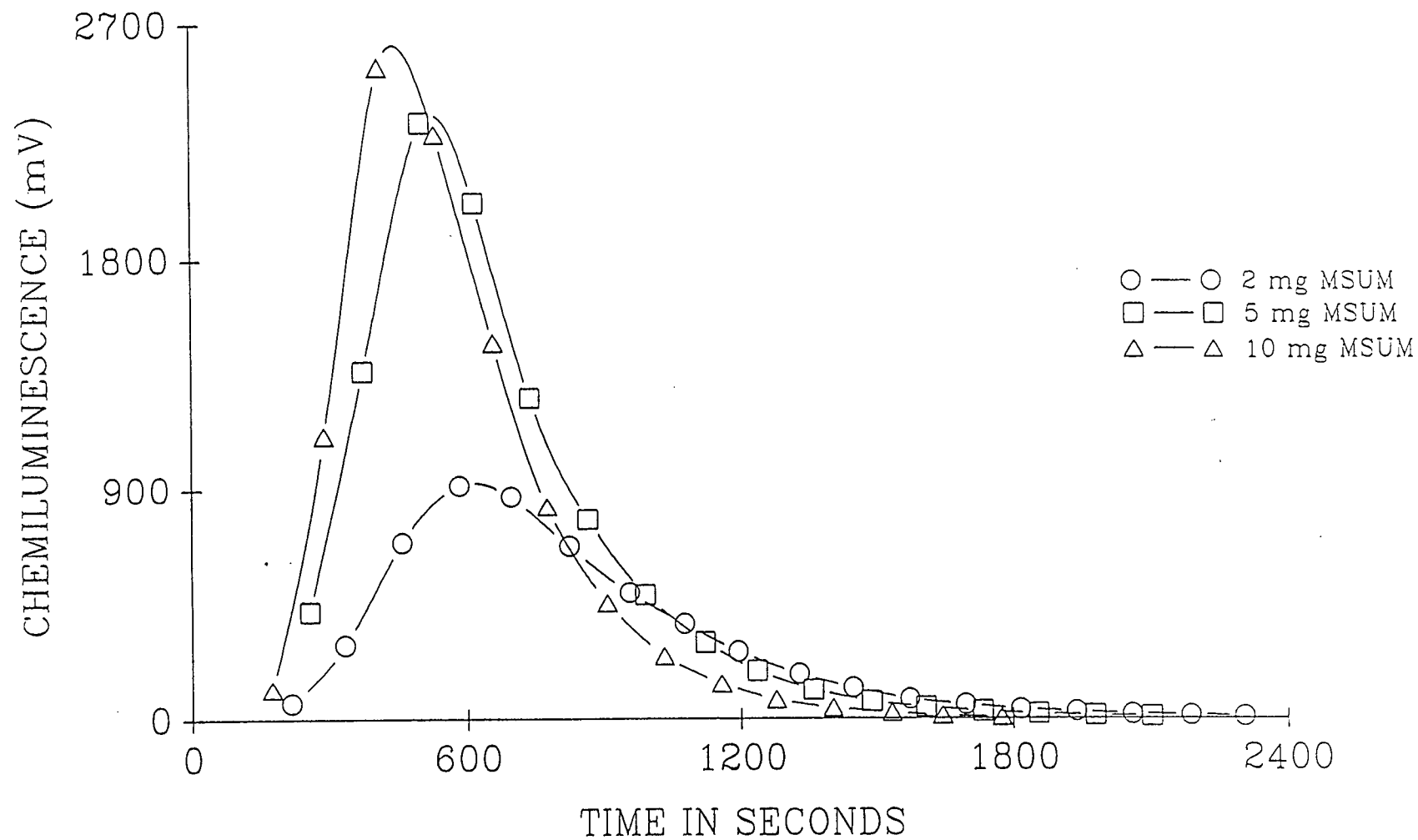


FIGURE 27

TABLE 5

EFFECT OF MSUM CRYSTAL CONCENTRATION ON CHEMILUMINESCENCE

CRYSTAL CONC. (mg/mL)	AUC ¹ (V.s)
2	656 (629 - 682)
5	1192 (1127 - 1256)
10	1140 (1139 - 1268)

Note : 1 - AUC is the area under the curve for plots of CL response in mV versus time. Values represent the mean of two determinations with the range in parentheses.

CHEMILUMINESCENCE GENERATED BY NEUTROPHILS STIMULATED BY
50 mg UNCOATED CPPD

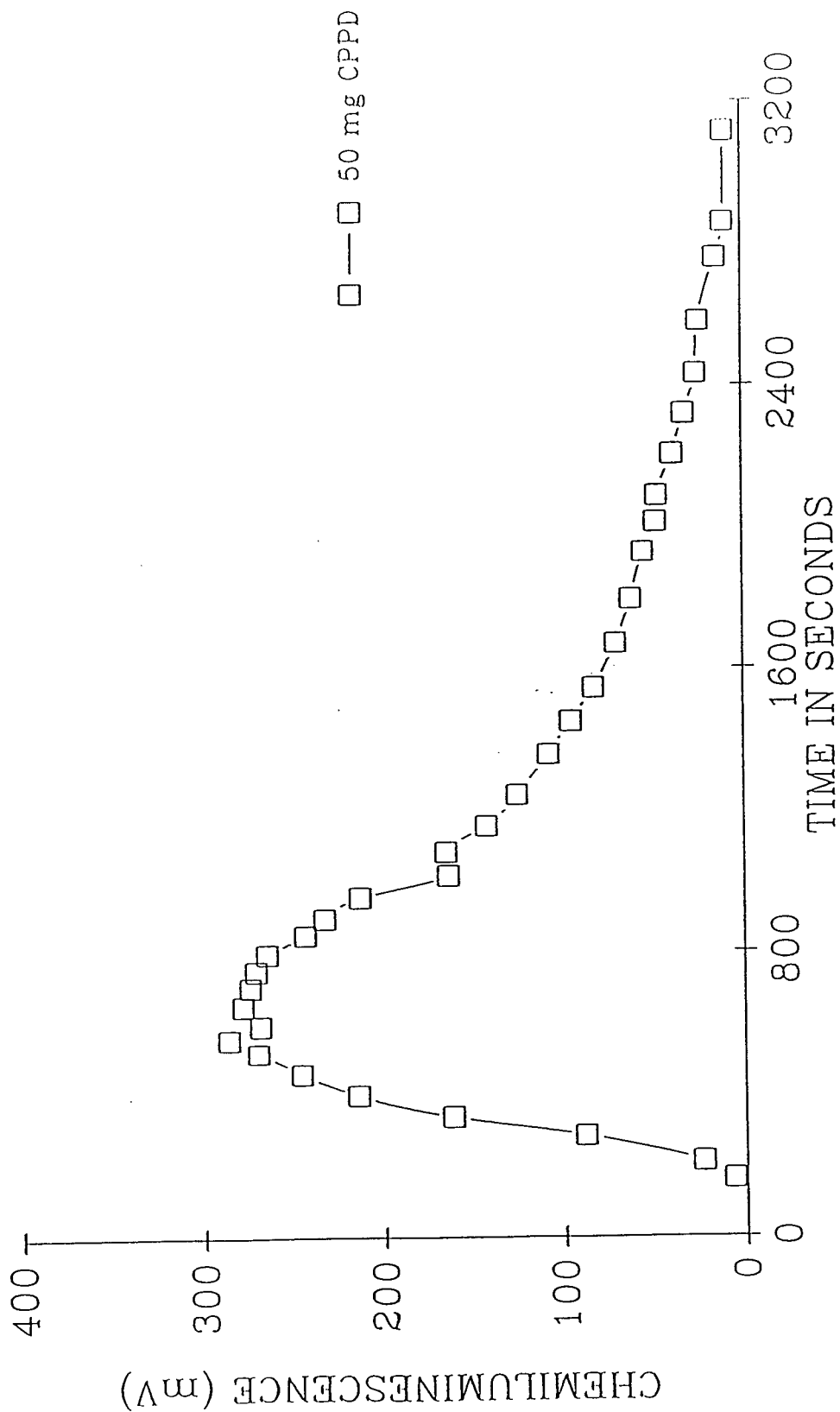


FIGURE 28

EFFECT OF CPPD CONCENTRATION ON NEUTROPHIL CHEMILUMINESCENCE

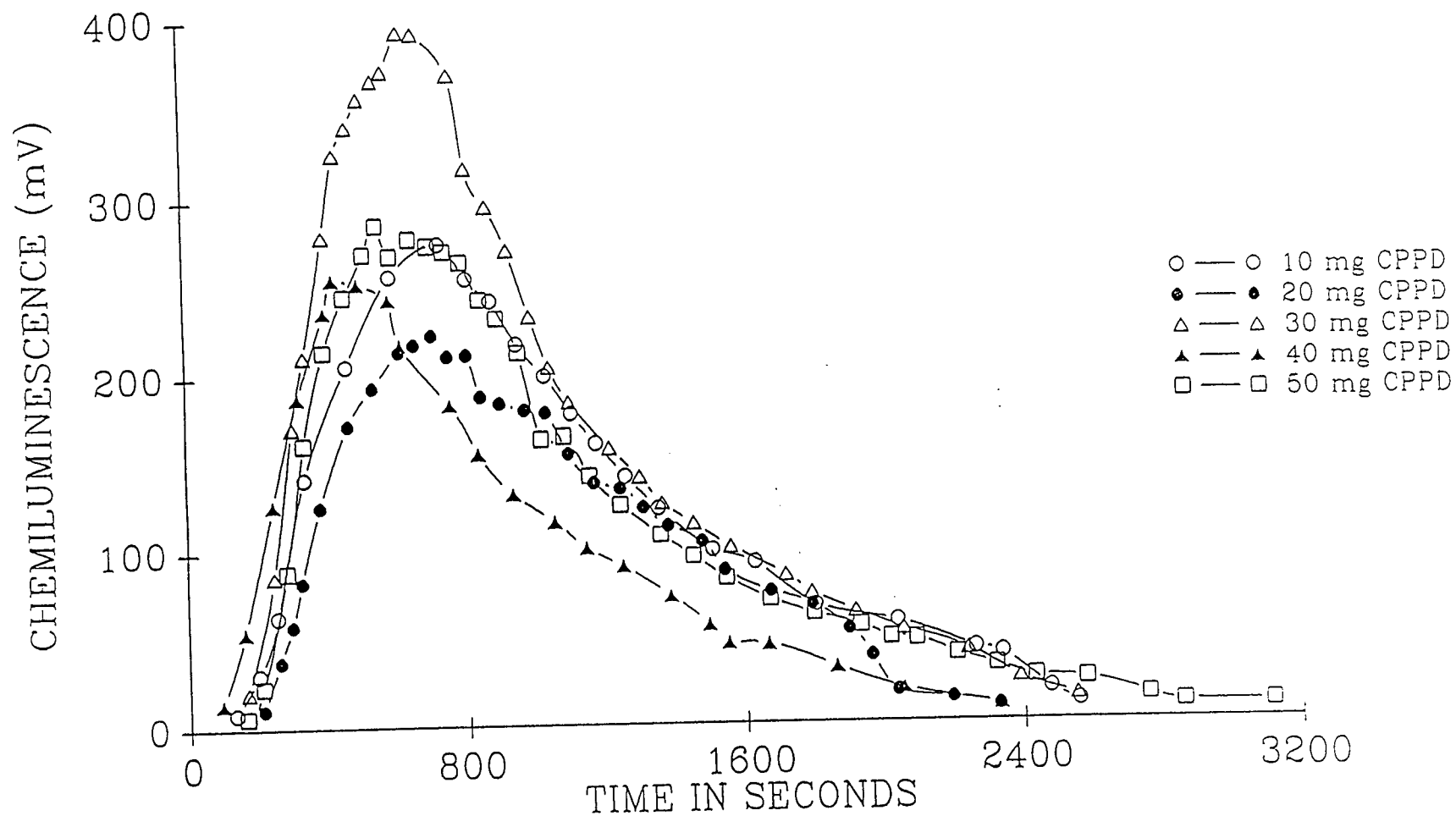


FIGURE 29

TABLE 6

EFFECT OF CPPD CRYSTAL CONCENTRATION ON CHEMILUMINESCENCE

CRYSTAL CONC. (mg/mL)	AUC ¹ (V.s)
10	62 (50 - 73)
20	57 (56 - 58)
30	79 (74 - 82)
40	63 (55 - 70)
50	55 (47 - 62)

Note : 1 - AUC is the area under the curve for plots of CL response in mV versus time. Values represent the mean of two determinations with the range in parentheses.

total amount of CL generated. A similar trend of increased CL with increasing CPPD concentrations upto 30 mg/mL was observed at 25°C. Though the number of crystal-neutrophil interactions would be expected to increase with crystal concentration, the accompanying increase in scatter could reduce the amount of light reaching the photomultiplier tube thus decreasing the CL detected. Another factor which may affect the CL response of neutrophils to CPPD crystals is the tendency of the crystals to settle rapidly even during the time of measurement (15 sec) at high crystal concentrations thus decreasing the number of possible crystal-neutrophil interactions. The neutrophil chemiluminescent response appears to be maximal at a CPPD concentration of 30 mg/mL. This CPPD concentration was used for all further CL experiments with CPPD.

To our knowledge there are no other reports of the effect of MSUM and CPPD crystal concentrations on the CL generated by neutrophils.

We have studied the effect of adding 10 μ g/mL cytochalasin B to the incubation medium on the CL produced. There was a significant ($p < 0.05$) reduction in the CL produced in the presence of cytochalasin B. The AUC for MSUM induced CL in the absence of cytochalasin B was 588 ± 108 V.s (mean of three determinations) while the AUC for MSUM induced CL in the presence of cytochalasin B was 5.46 ± 3 V.s (mean of three determinations). Hence cytochalasin B was found to

inhibit the generation of superoxide and CL production by neutrophils. This may be due to the inhibition of phagocytosis by cytochalasin B which may result in the inhibition of the accompanying respiratory burst (Simchowitz et al., 1982).

4.2.1. EFFECT OF TEMPERATURE ON THE CL RESPONSE

The CL generated by neutrophils in response to crystal-induced stimulation was dependent on the temperature of the suspending medium for neutrophils. Table 7 gives the AUC for the CL curves obtained when neutrophils were incubated with MSUM crystals at 25°C and 37°C. The increase in AUC reveals that the CL response was significantly enhanced when the neutrophils were maintained at 37°C presumably due to an enhanced oxidative metabolic activity of the neutrophils at 37° compared to that at 25°. Hence the neutrophils were maintained at 37°C for all further experiments. These results are in good agreement with those reported by Harber and Topley (1986).

4.2.2. EFFECT OF PROTEINS ON THE CHEMILUMINESCENT RESPONSE

Figures 30 and 31 show the typical CL curves obtained when neutrophils were exposed to protein coated MSUM and CPPD respectively. It was not possible to measure the CL generated by a control set of tubes since the CL values were extremely low and below the detection limits of the chemiluminometer. The AUCs of the plots of CL response in

TABLE 7
EFFECT OF SAMPLE TEMPERATURE ON MSUM INDUCED
CHEMILUMINESCENCE

MSUM (mg/mL)	AUC ¹	
	SAMPLE TEMPERATURE	
	25°C	37°C
2	443 (414 - 470)	656 (629 - 682)
5	787 (686 - 886)	1191 (1127-1257)
10	967 (877 - 1056)	1198 (1139-1268)

Note : 1 - AUC is the area under the curve for plots of CL response in mV versus time. Values represent the mean of two determinations with the range in parentheses.

EFFECT OF PROTEIN COATING ON CHEMILUMINESCENCE RESPONSE TO MSUM

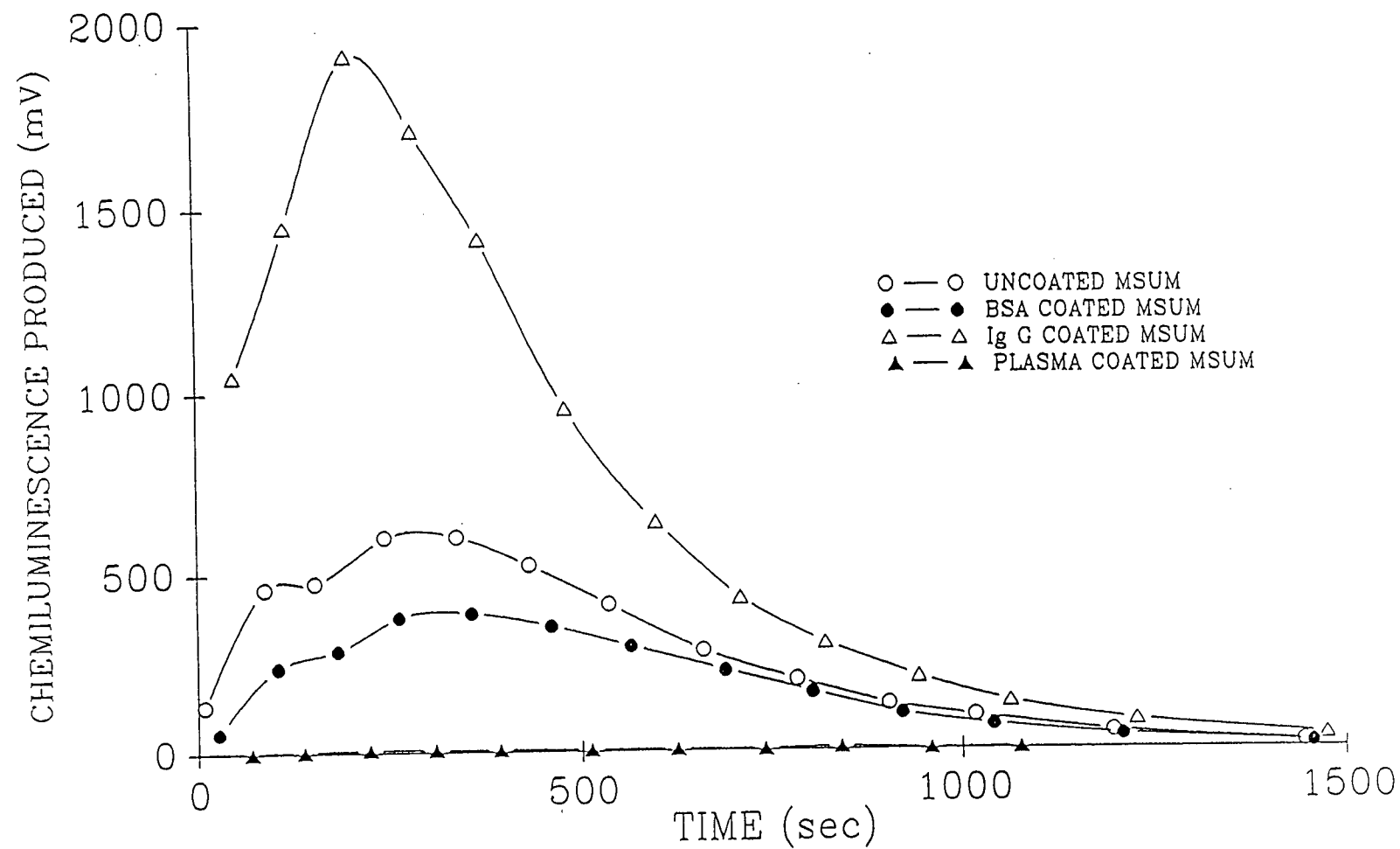


FIGURE 30

EFFECT OF PROTEIN COATING ON THE CHEMILUMINESCENCE RESPONSE TO CPPD

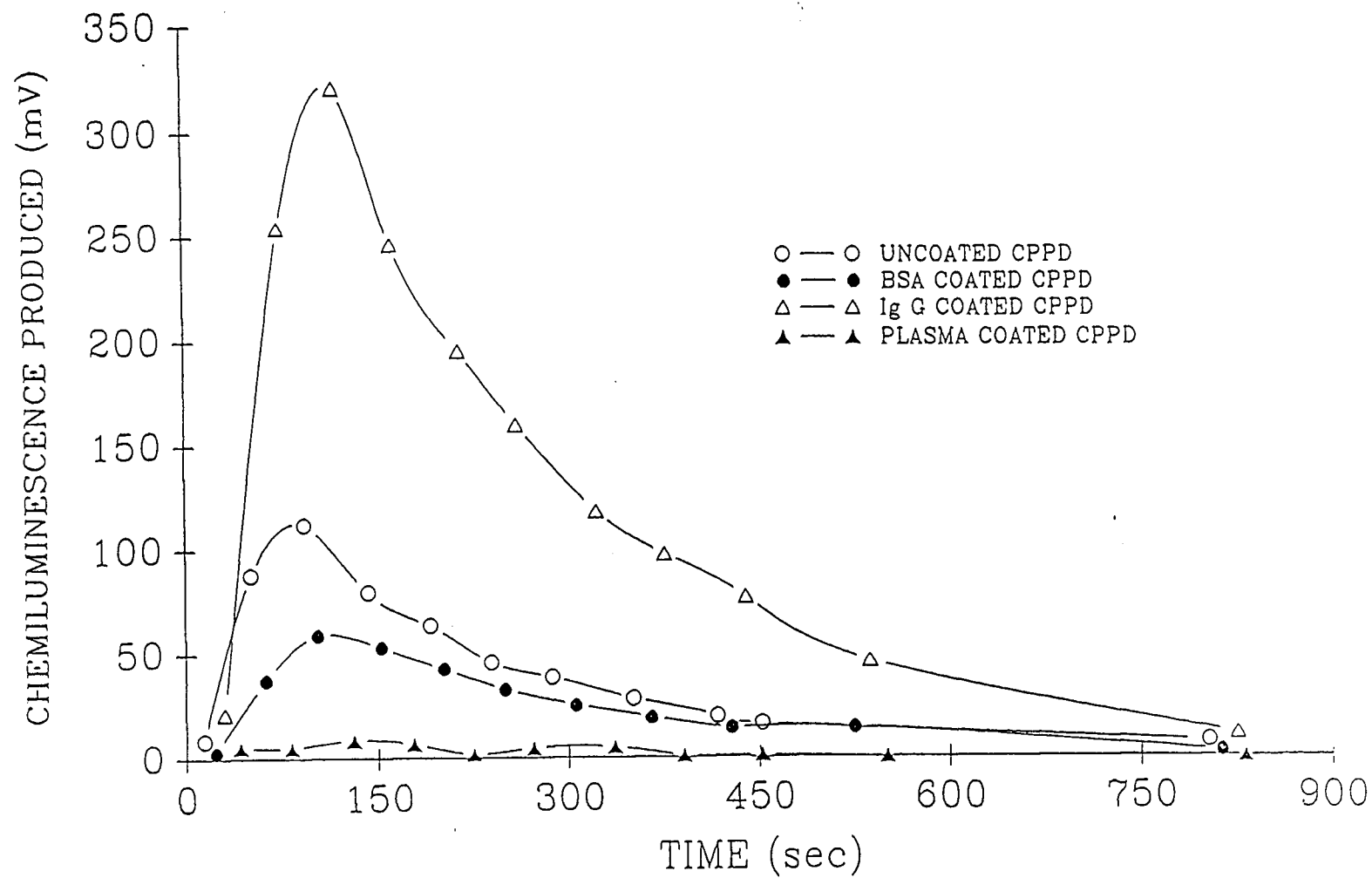


FIGURE 31

mV versus time for protein coated and uncoated MSUM are given in Table 8. There was significant enhancement of the CL response when the crystals were coated with immunoglobulin g. This may be due to the opsonizing effect of Ig G which facilitates an interaction between the F_C portion of Ig G on the crystal surface and the F_C receptors present on the neutrophil membrane. Statistical analysis of the data revealed that there was a significant suppression of the neutrophil CL response when the MSUM crystals were precoated with plasma proteins. Terkeltaub et al. (1984) reported that plasma precoating of MSUM crystals inhibited the CL generated by >50%. They suggested that the low density lipoprotein fraction bound to MSUM from plasma was responsible for a major portion of the plasma inhibition of neutrophil CL responses to MSUM. This is an interesting observation because during a gout attack, the synovium of the joint becomes more permeable and synovial fluid levels of large molecules such as lipoproteins increases (Terkeltaub et al., 1986).

The effect of protein coating on CL induced by CPPD crystals followed a similar pattern as for MSUM crystals. The AUCs of CL versus time plots for protein coated and uncoated CPPD are shown in Table 8. Precoating of CPPD crystals with plasma was found to suppress the CL response though this suppression was not found to be statistically significant ($p < 0.05$) for CPPD. The enhancement in the CL generated when

TABLE 8

EFFECT OF PROTEIN COATED AND UNCOATED MSUM AND CPPD ON
CHEMILUMINESCENCE

	AUC ¹ (V.s)	
	MSUM	CPPD
	5 mg/mL	30 mg/mL
Uncoated crystals	306 ± 85	38 ± 8
IgG coated	570 ± 97	103 ± 22 (*)
BSA coated	97 ± 34	32 ± 9
Plasma coated	20 ± 10 (*)	17 ± 6

Note : 1 - AUC is the area under the curve for plots of CL response in mV versus time. Values represent the mean of 9 determinations for MSUM ± standard error and 7 determinations for CPPD ± standard error.

* significant at $p < 0.05$

crystals were precoated with Ig G was statistically significant ($p < 0.05$). Again, it is likely that adsorbed Ig G enhanced the F_C receptor mediated crystal-neutrophil activation and CL generation.

The slight reduction in CL observed when crystals were coated with BSA was not statistically significant ($p < 0.05$) for either MSUM or CPPD crystals.

4.3. CRYSTAL STIMULATED DEGRANULATION RESPONSE OF NEUTROPHILS

Degranulation refers to the release of the contents of the primary and azurophilic granules of the neutrophil either into the phagosome formed after phagocytosis or directly into the extracellular medium during invagination of the plasma membrane before phagocytosis is complete. The primary and azurophilic granules of the neutrophil contain several enzymes, among them, MPO, LYZ, alpha mannosidase, collagenase, gelatinase and β glucuronidase (Baggiolini and Dewald, 1984). Some of these enzymes are involved in the generation of toxic species such as the hydroxyl radical, hypochlorous acid and chloramines while others are proteolytic in action. The release of these lytic contents into the extracellular fluid either as a consequence of cell death or during the process of phagocytosis can have deleterious effects on the surrounding tissues. In order to study the kinetics of degranulation following crystal

stimulation it is necessary to alter the neutrophil such that phagocytosis or internalization of the crystal is inhibited and complete degranulation occurs into the extracellular medium. The cytochalasins are a group of compounds that inhibit phagocytosis but do not inhibit degranulation (Weissmann et al., 1972). Hence neutrophils were treated with cytochalasin B prior to stimulation with the particulate stimuli to study degranulation. Cytochalasin B has been previously used in studies with human neutrophils (Nagase et al., 1987; Rosen et al., 1986; Abramson et al., 1982; Higson et al., 1984). However, it is important to note that this alteration of the ability of the neutrophil to phagocytose could result in alteration of other functions of the cell as well. Furthermore some studies have revealed that cytochalasin B has a tendency to stimulate degranulation (Aaku et al., 1990; Hoffstein et al., 1980).

4.3.1. CRYSTAL STIMULATED MPO RELEASE FROM NEUTROPHILS

The determination of MPO released from neutrophils was based on the MPO catalyzed oxidation of o-dianisidine by hydrogen peroxide. Increasing amounts of supernatant obtained from Triton X-100 lysed neutrophil suspensions were added to a dianisidine reaction mixture to determine the relationship between the volume of supernatant added and the change in absorbance produced. The results are shown in Figure 32. The relationship was found to be linear within the range of

EFFECT OF VARIOUS CONCENTRATIONS OF SUPERNATANT ON
THE MPO ASSAY (AT T = 60 sec)

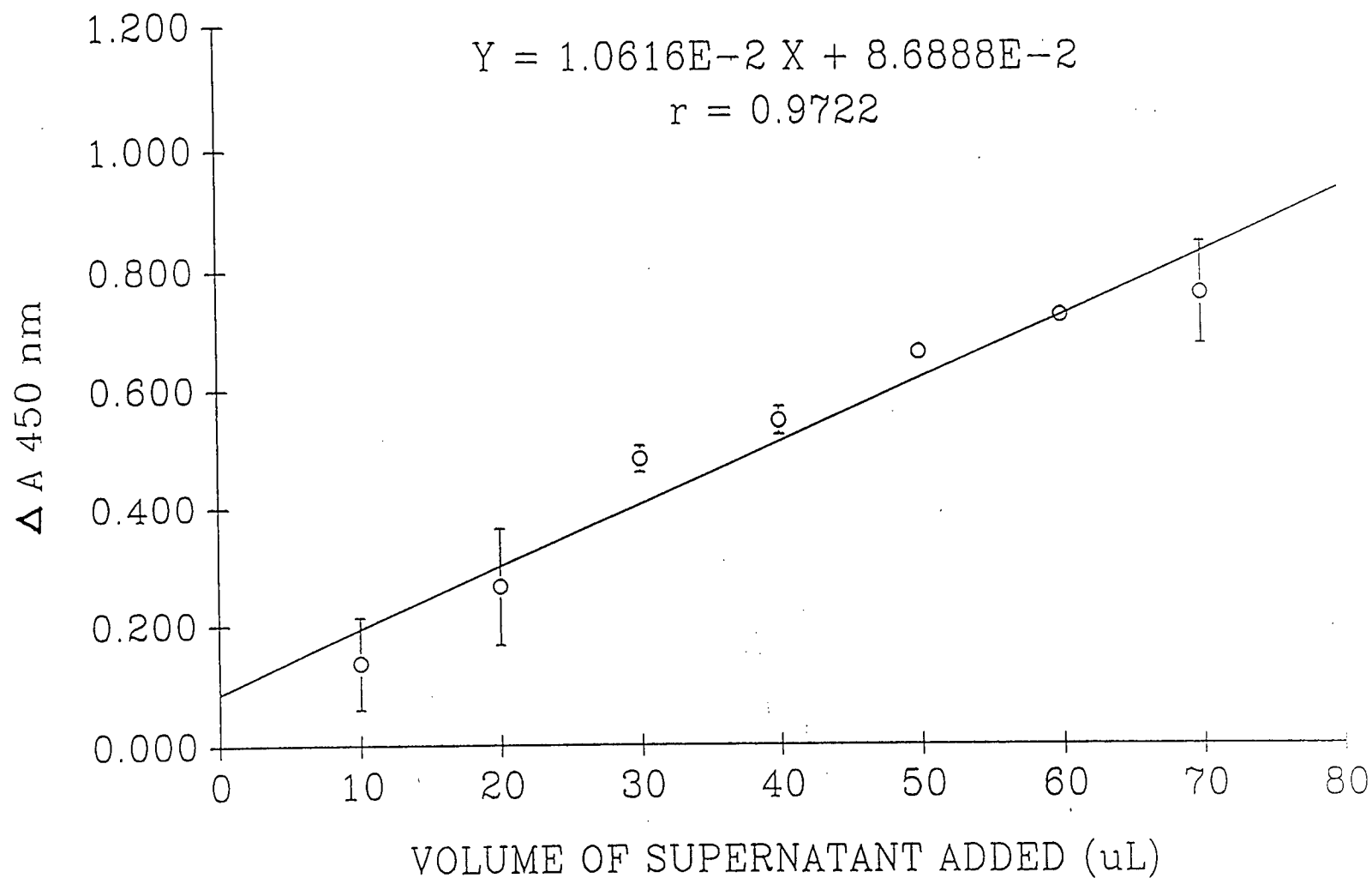


FIGURE 32

30 to 70 μ L of supernatant added. Hence 50 μ L of the supernatant were used in all further determinations. This method of analysis gives the rate of oxidation of o - dianisidine and this rate has been shown to be proportional to the concentration of the enzyme MPO (Babior and Cohen, 1981). The rate of appearance of MPO in the supernatant was calculated from the difference in the rate of oxidation at various time intervals.

The time course for the release of MPO obtained when cytochalasin B pretreated neutrophils were incubated with uncoated CPPD crystals is shown in Figure 33. The maximum amount of MPO present in the supernatant was between 10-45 min and the MPO levels observed on incubation with CPPD were significantly higher than control values. The time course for the release of MPO when cytochalasin B pretreated neutrophils were incubated with uncoated MSUM crystals is shown in Figure 34. The level of MPO in the supernatants resulting from the MSUM/neutrophil incubations was lower than that for the control set of experiments. This raised the possibility that the enzyme was being removed from the supernatant by adsorption onto the MSUM crystals. This was considered a likely possibility since MSUM crystals have been previously reported to adsorb many different proteins (Terkeltaub et al., 1983; Kozin and McCarty, 1976; Kozin et al., 1979). In order to test this hypothesis the supernatant from neutrophils lysed with Triton X-100 10%

MYELOPEROXIDASE RELEASE FROM CYTOCHALASIN B PRETREATED NEUTROPHILS
ON STIMULATION WITH UNCOATED CPPD CRYSTALS

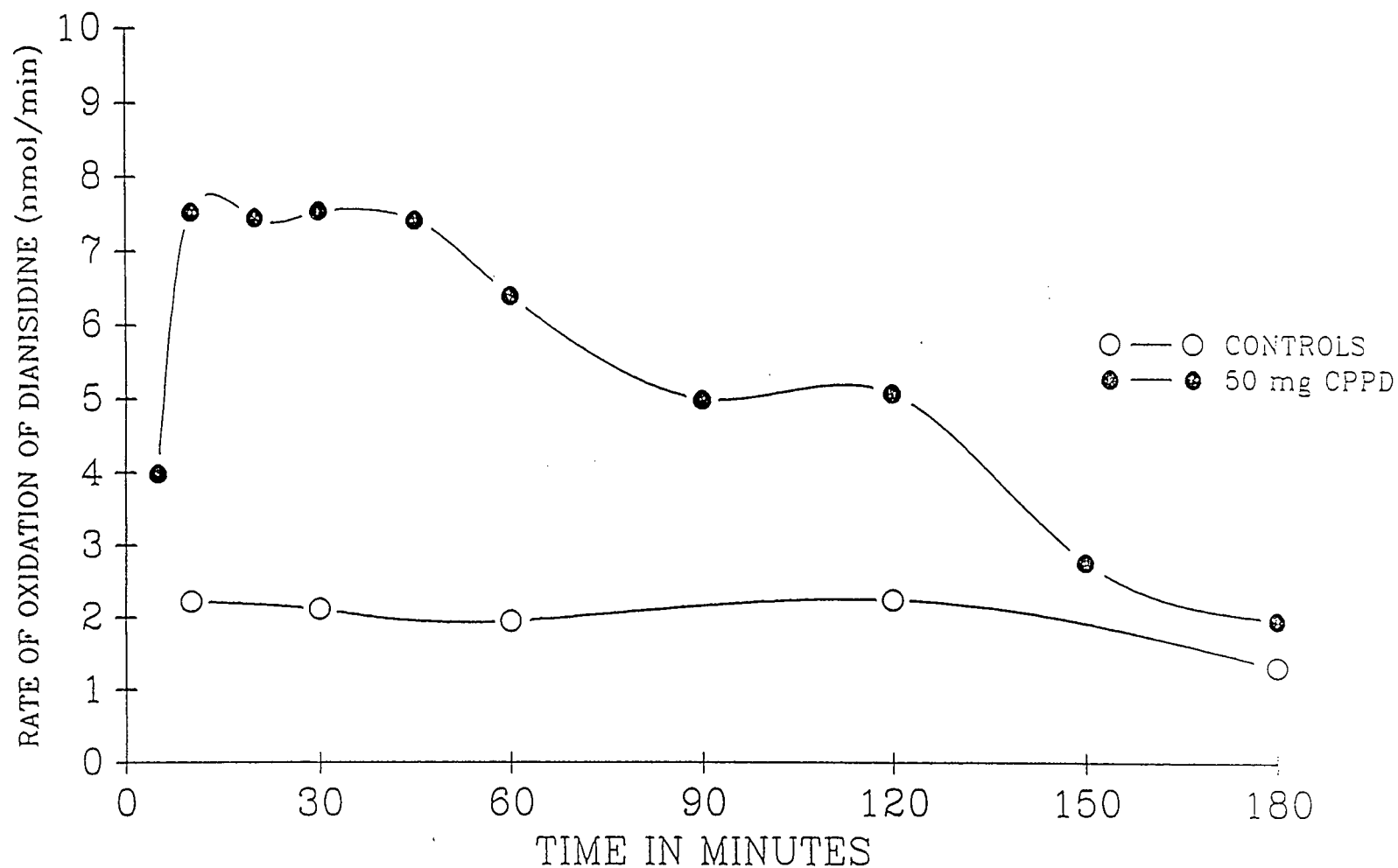


FIGURE 33

MYELOPEROXIDASE RELEASE FROM CYTOCHALASIN B PRETREATED NEUTROPHILS ON STIMULATION WITH UNCOATED MSUM CRYSTALS

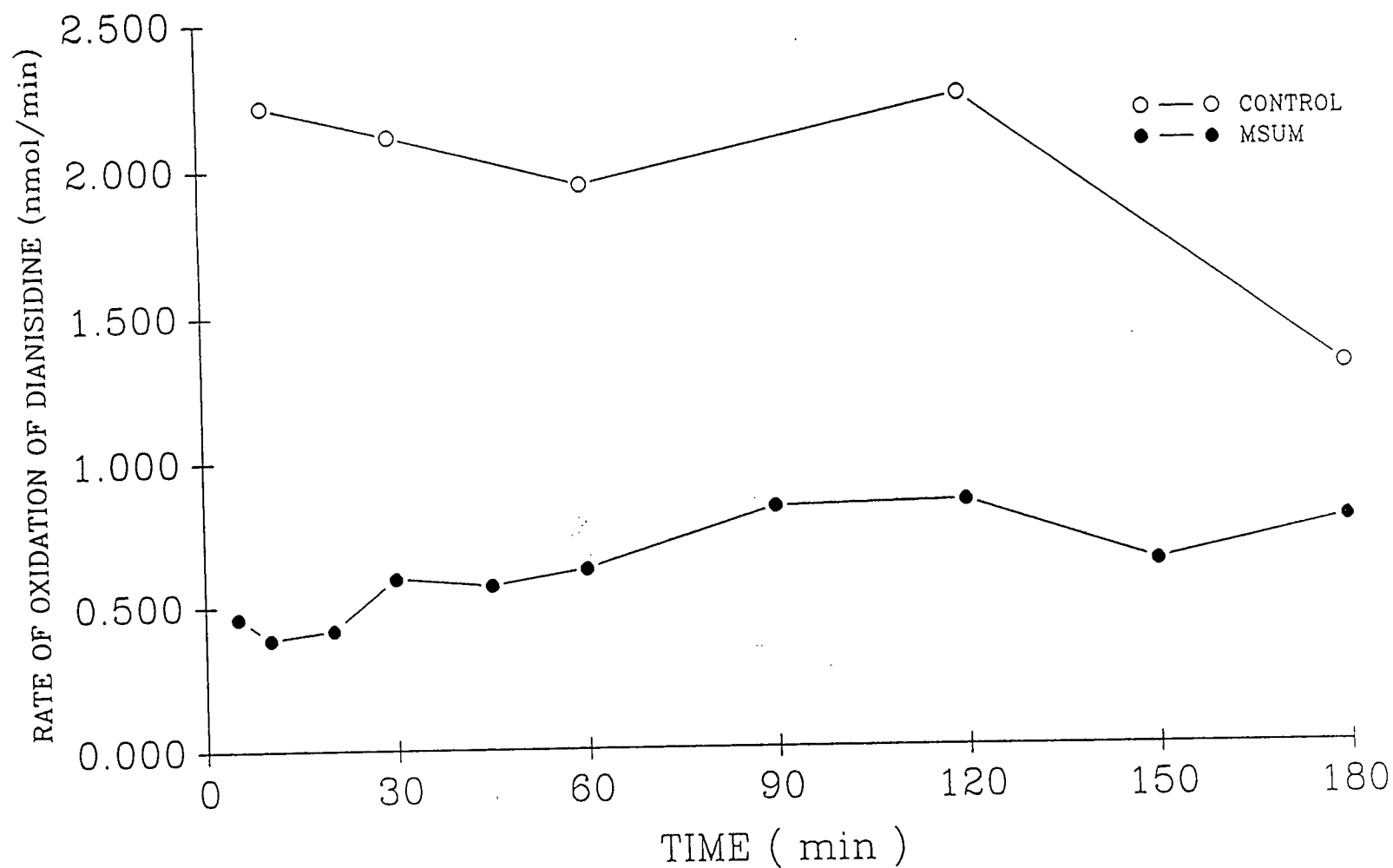


FIGURE 34

solution was incubated with increasing MSUM crystal concentrations from 0.1 mg/mL to 10 mg/mL. The effect of increasing the amount of MSUM added on the % reduction in MPO activity can be seen in Figure 35. Increasing amounts of MSUM resulted in significant reduction of MPO activity. Hence it is likely that MPO was being adsorbed onto the MSUM crystal surface from the supernatant. Adsorption of MPO appeared to take place even in the presence of many other proteins released by degranulation and cell death. Terkeltaub et al., (1991) studied the degranulation of neutrophils induced by MSUM but they studied the release of alpha mannosidase.

4.3.2. EFFECT OF PROTEINS ON MPO RELEASE

The effect of protein coating of the crystals on the release of MPO from cytochalasin B pretreated neutrophils stimulated with CPPD crystals is shown in Figure 36. Precoating of CPPD crystals with immunoglobulin g was found to significantly enhance the release of MPO, possibly through the interaction with the F_C receptors present on the neutrophil surface. Precoating with plasma and BSA did not have a significant effect on the release of MPO induced by CPPD crystals.

As seen in Figure 37, the MPO release induced by uncoated MSUM was less than control samples and precoating MSUM crystals with Ig G, BSA or plasma proteins produced

EFFECT OF ADDITION OF INCREASING AMOUNTS OF MSUM
ON MYELOPEROXIDASE ACTIVITY

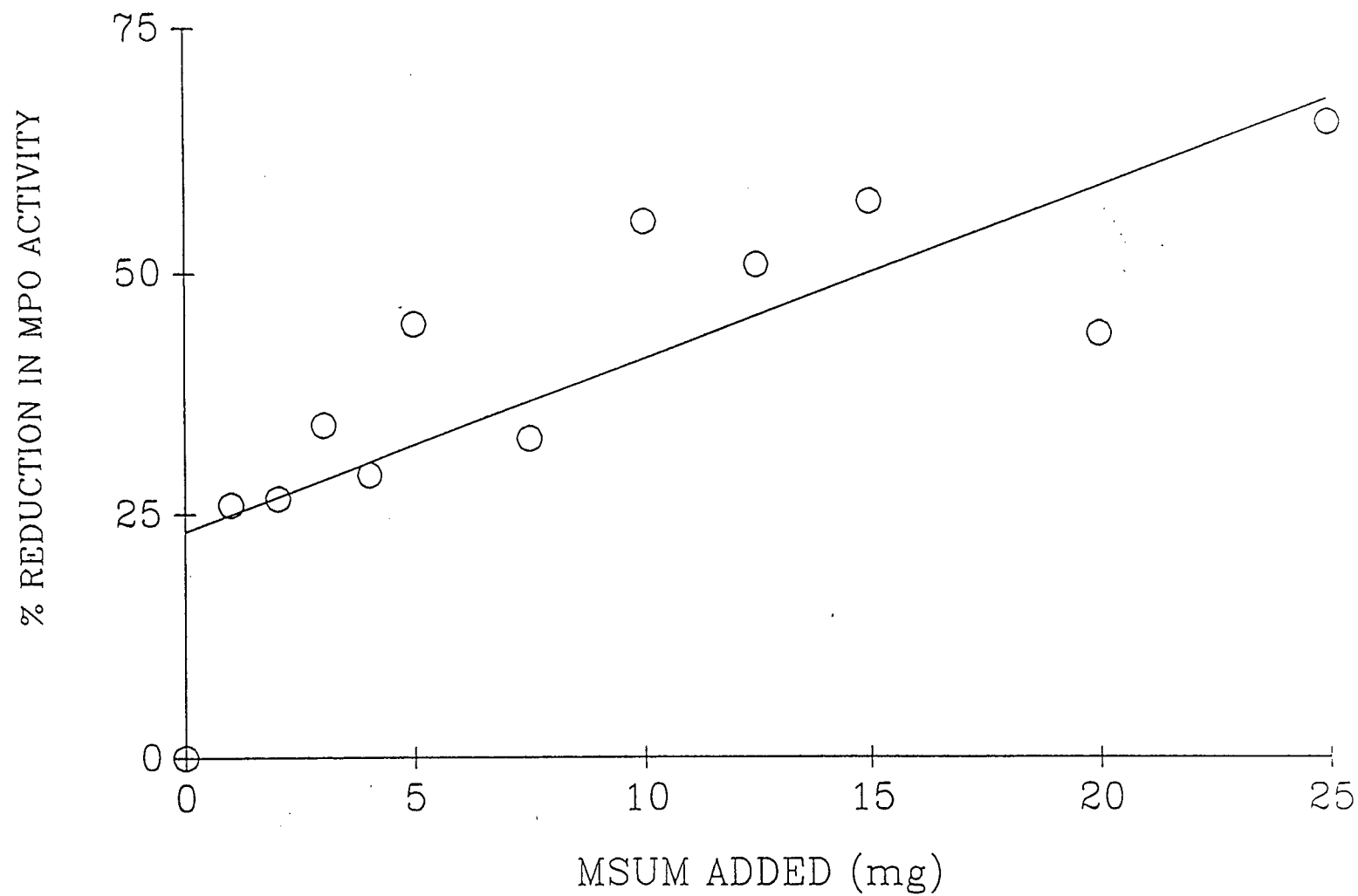


FIGURE 35

MYELOPEROXIDASE RELEASE FROM CYTOCHALASIN B PRETREATED NEUTROPHILS
ON STIMULATION WITH PROTEIN COATED CPPD (n=6)

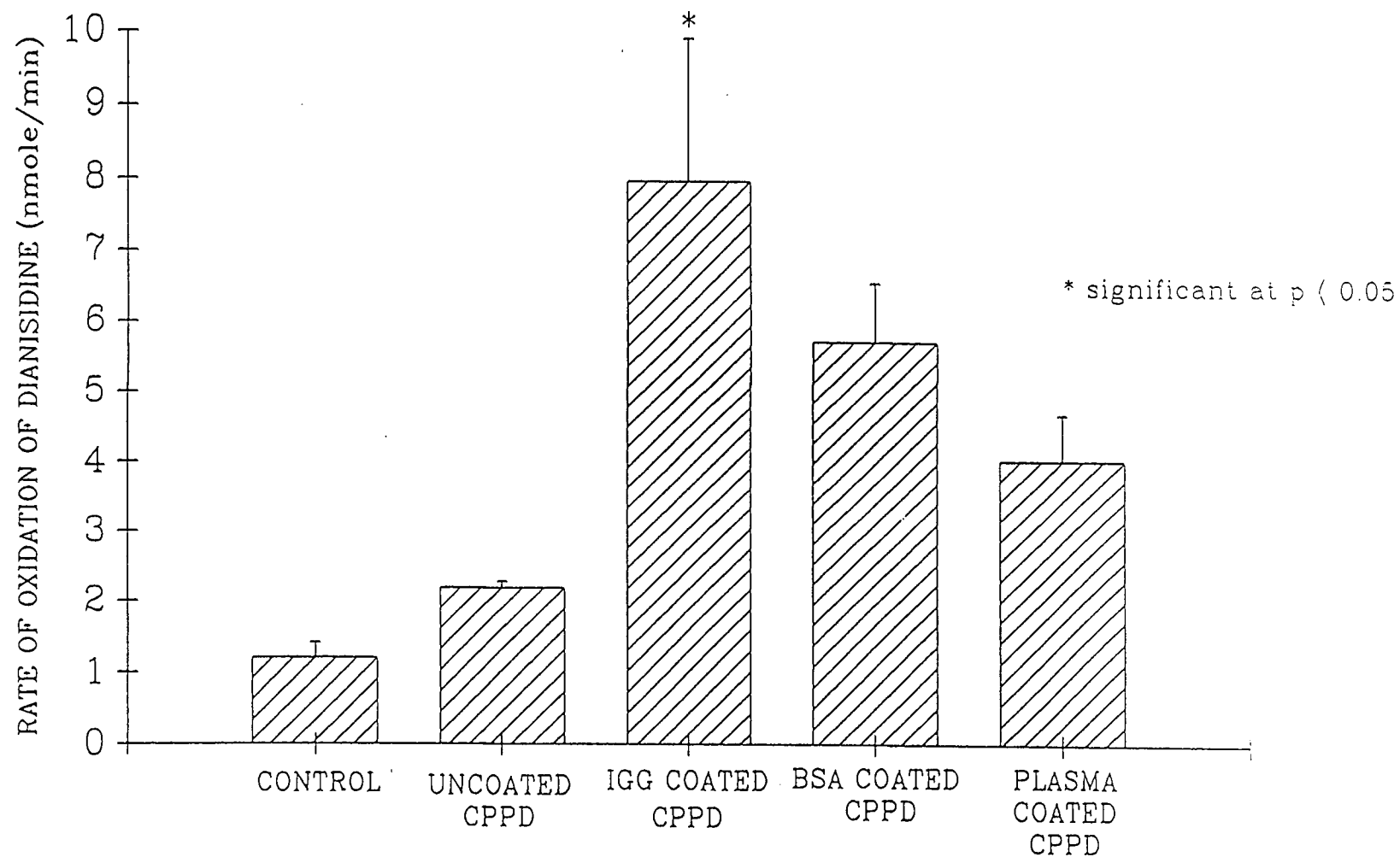


FIGURE 36

MYELOPEROXIDASE RELEASE FROM CYTOCHALASIN B PRETREATED NEUTROPHILS ON STIMULATION WITH PROTEIN COATED MSUM

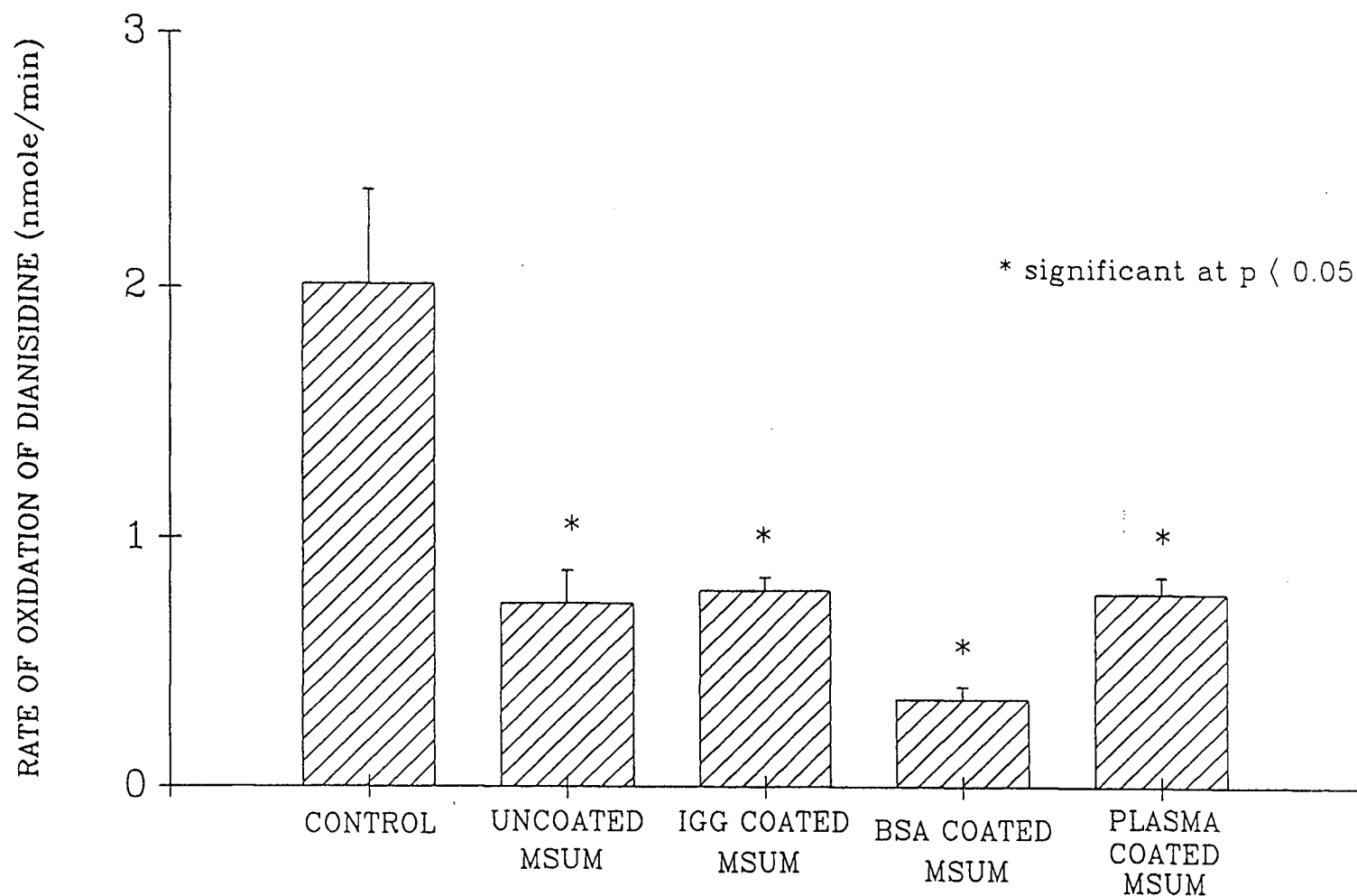


FIGURE 37

significantly lower MPO release than for controls ($p < 0.05$). Hence, it seems likely that even the presence of a protein coat on the crystal did not prevent further adsorption of MPO onto the crystal surface.

Due to the adsorption of MPO onto the crystal surface it was not possible to monitor the release of MPO as an indicator of degranulation occurring when cytochalasin B pretreated neutrophils were stimulated with MSUM crystals. Hence it was decided to study the release of another enzyme released during degranulation, LYZ.

4.3.3. CRYSTAL STIMULATED LYZ RELEASE FROM NEUTROPHILS

Figure 38 shows the standard curve obtained by measuring the change in absorbance of a *Micrococcus lysodeikticus* suspension at 450 nm when standard egg white LYZ solution was added to it in amounts ranging from 500 to 3500 U/mL. The relationship was found to be linear over the concentration range 0.1 to 200 LYZ units/mL.

The time course for the release of LYZ from cytochalasin B pretreated neutrophils stimulated with uncoated MSUM is shown in Figure 39. The maximal response was observed between 30-60 min and was in the range of 990 ± 290 U/mL. This represented about 40% of total enzyme activity released by lysis with Triton X-100, which is in good agreement with values reported by Simchowitz et al. (1982).

STANDARD CURVE FOR LYSOZYME ASSAY BY MICROCOCCUS METHOD

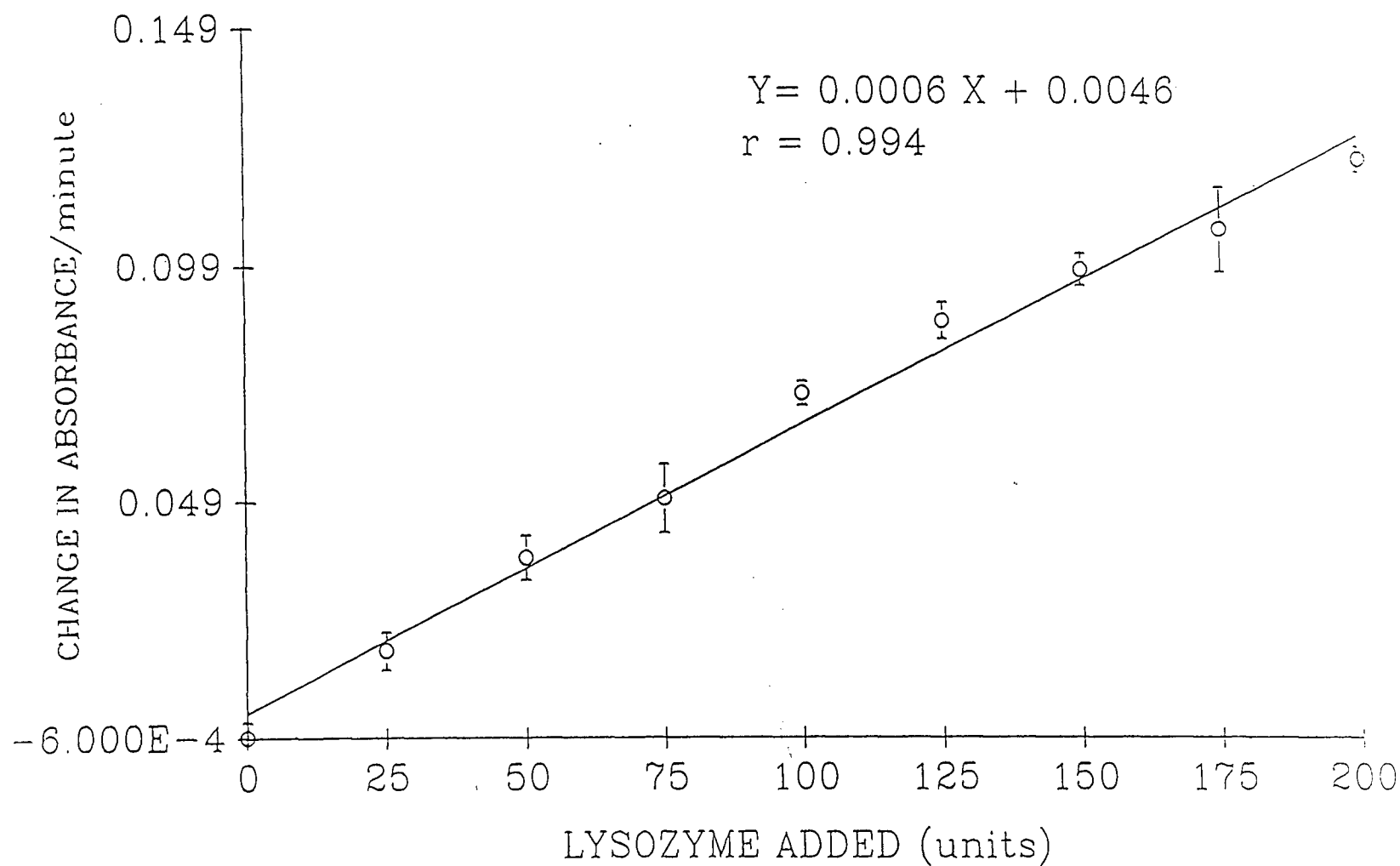


FIGURE 38

LYSOZYME RELEASE INDUCED BY MSUM

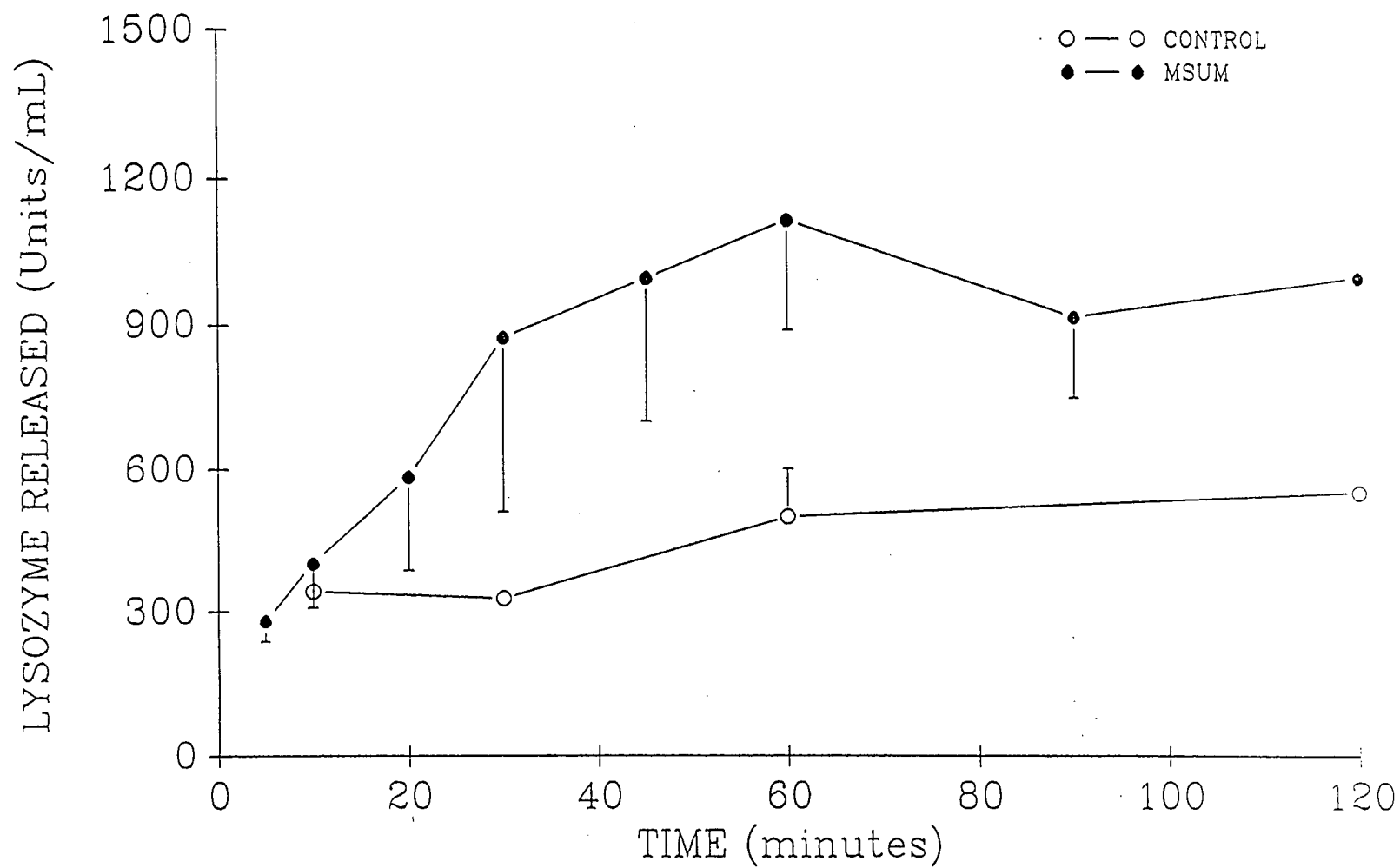


FIGURE 39

The time course for the release of LYZ induced by CPPD crystals is shown in Figure 40. The response reached approximately 1500 ± 240 U/mL in 45-60 min and reached a maximum of 2200 ± 120 at about 150 min. Figures 39 and 40 show that the control values remained in the same range over the time course for MSUM and CPPD crystals. LYZ release from neutrophils stimulated by MSUM crystals was lower and subject to greater variation compared to the LYZ release from CPPD stimulated neutrophils. This raised the possibility that analogous to MPO, LYZ was also being adsorbed onto the MSUM crystal surface.

Figure 41 shows the change in absorbance produced by solutions of egg white LYZ incubated with MSUM crystals. There was a slight reduction in activity of LYZ when incubated with MSUM crystals. Kozin and McCarty (1967) studied the adsorption of several proteins by MSUM crystals including LYZ, albumin and Ig G. They found that the adsorption of LYZ to MSUM was greater than the adsorption of BSA to MSUM. Kozin et al. (1979) also observed only small amounts of LYZ released from neutrophils stimulated by MSUM which was probably due to adsorption of LYZ by MSUM. Hence it is probable that the LYZ levels measured when neutrophils were incubated with uncoated MSUM crystals were being underestimated due to the adsorption of LYZ onto the MSUM crystals.

LYSOZYME RELEASE INDUCED BY CPPD

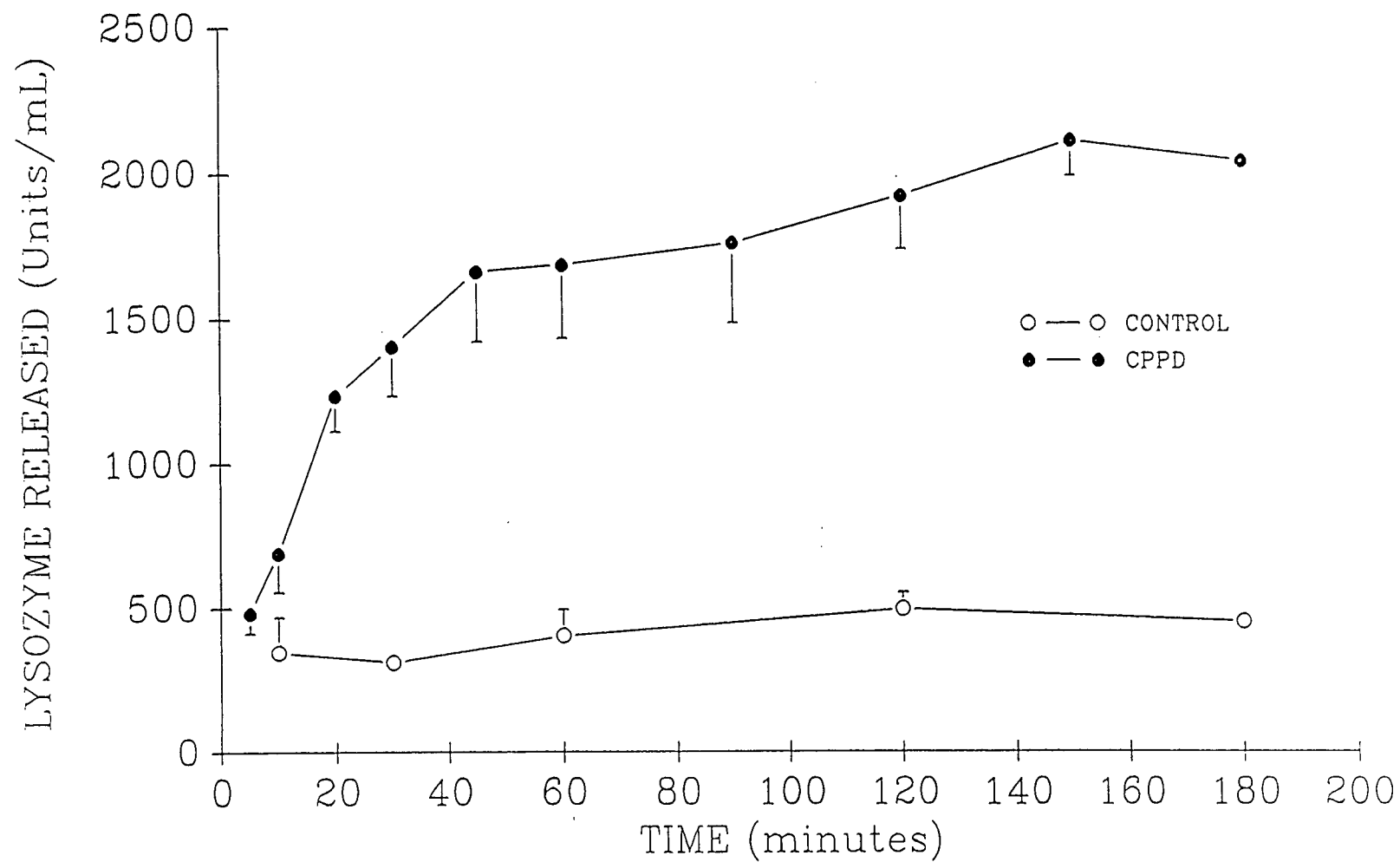


FIGURE 40

STUDY OF LYSOZYME ADSORPTION ONTO MSUM AND THE EFFECT ON THE LYSOZYME ASSAY

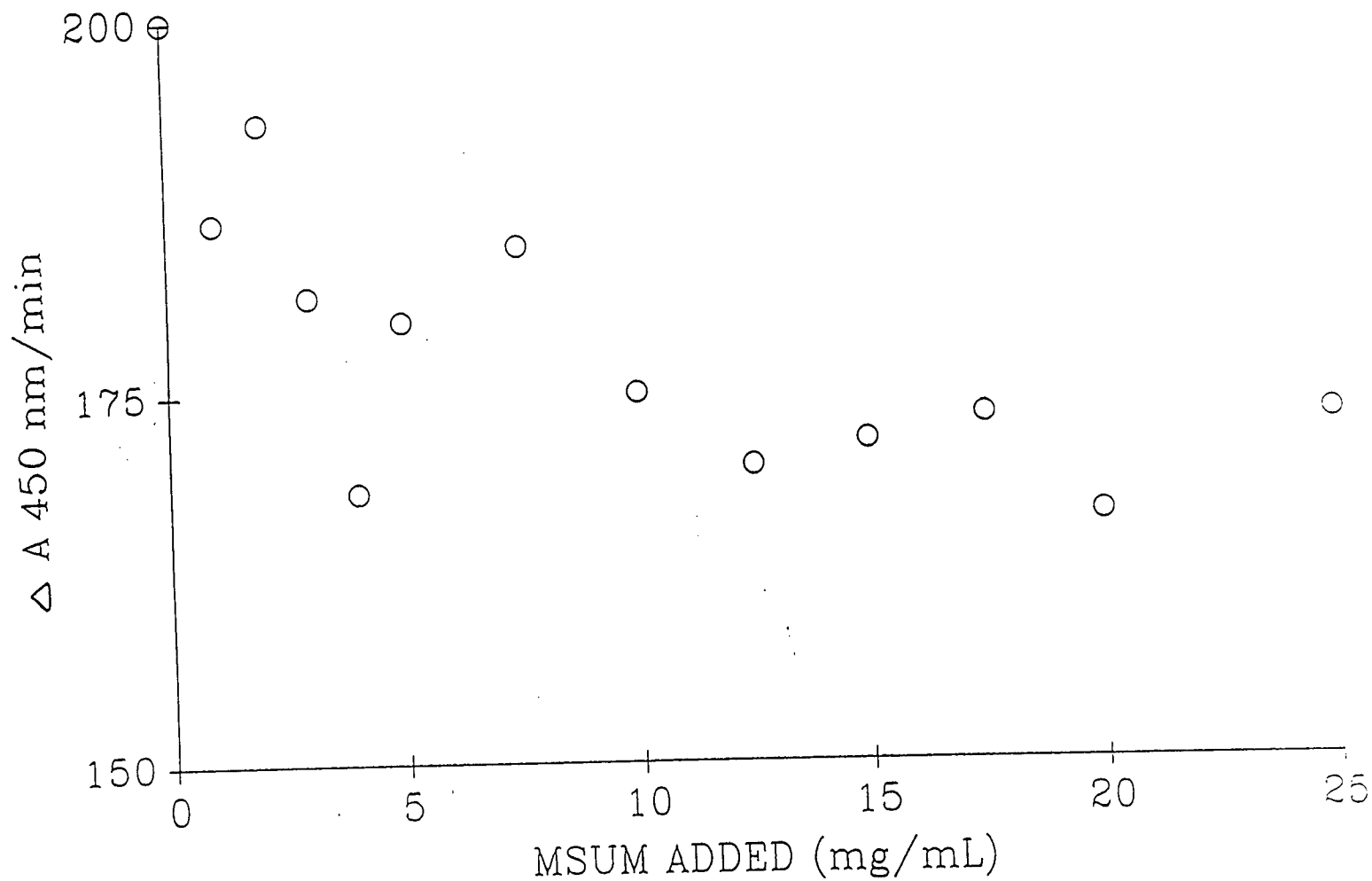


FIGURE 41

4.3.4. EFFECT OF PROTEINS ON LYZ RELEASE

The effect of protein coating of MSUM and CPPD crystals on degranulation from cytochalasin B pretreated neutrophils monitored by the release of LYZ is shown in Figures 42 and 43 respectively. The LYZ release from cytochalasin B pretreated neutrophils induced by CPPD crystals was not affected by the nature of the protein coating on the crystal as seen in Figure 43. Precoating of MSUM crystals with proteins had no significant effect on the LYZ release induced by MSUM.

5. FUTURE WORK

We have studied the modulation of neutrophil responses to MSUM and CPPD crystals by the presence of adsorbed proteins, such as immunoglobulin G, bovine serum albumin and plasma proteins, at specific time intervals. It would be interesting to study the time course of the neutrophil responses to protein coated MSUM and CPPD crystals, particularly since differences may exist in the rate of response to crystals coated with different proteins. The effect of other proteins such as low density lipoproteins and high density lipoproteins on the neutrophil responses such as superoxide generation and chemiluminescence production could be studied. The effect of drugs used in the treatment of crystal deposition diseases on the

LYSOZYME RELEASE INDUCED BY PROTEIN COATED MSUM

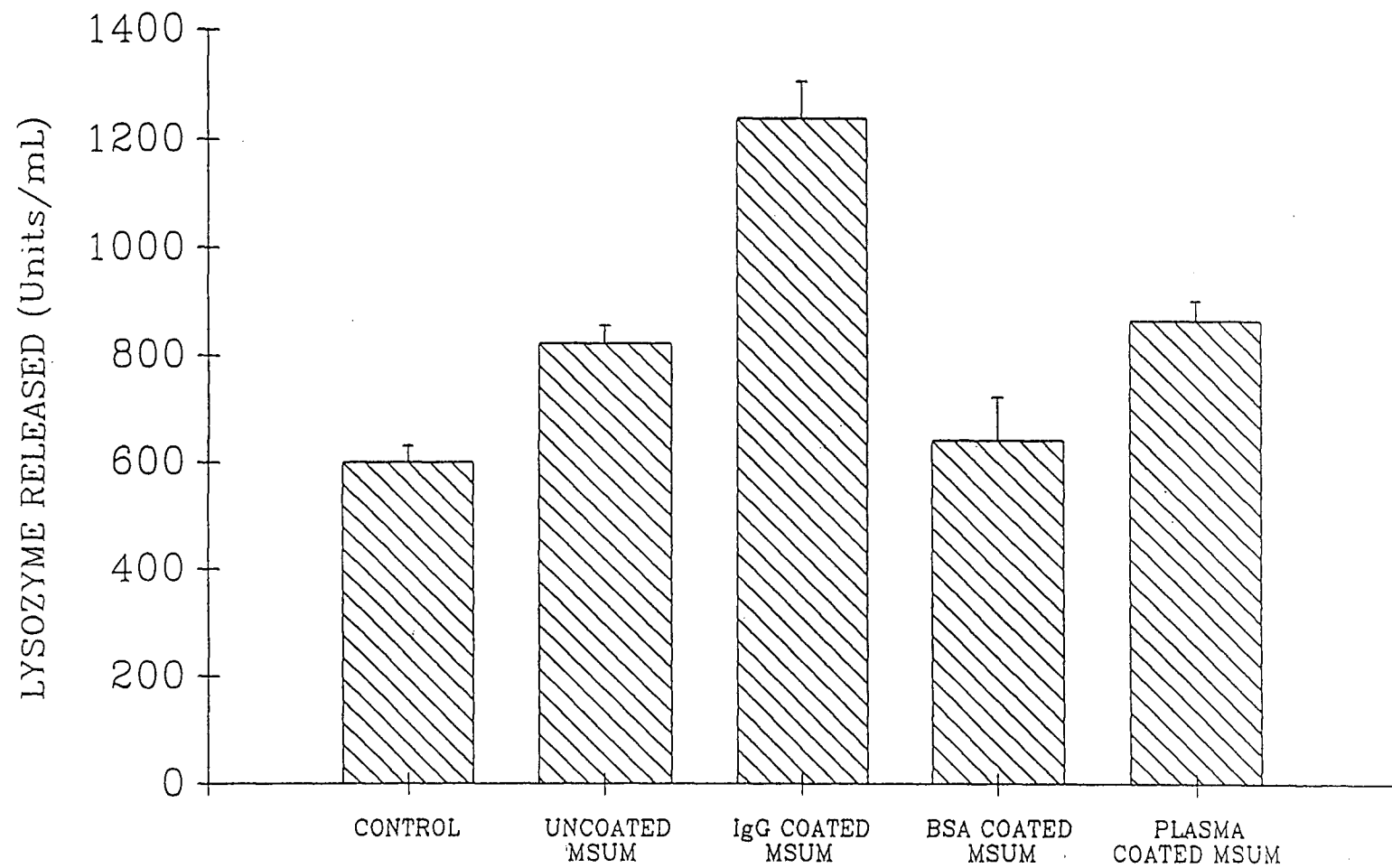


FIGURE 42

LYSOZYME RELEASE INDUCED BY PROTEIN COATED CPPD

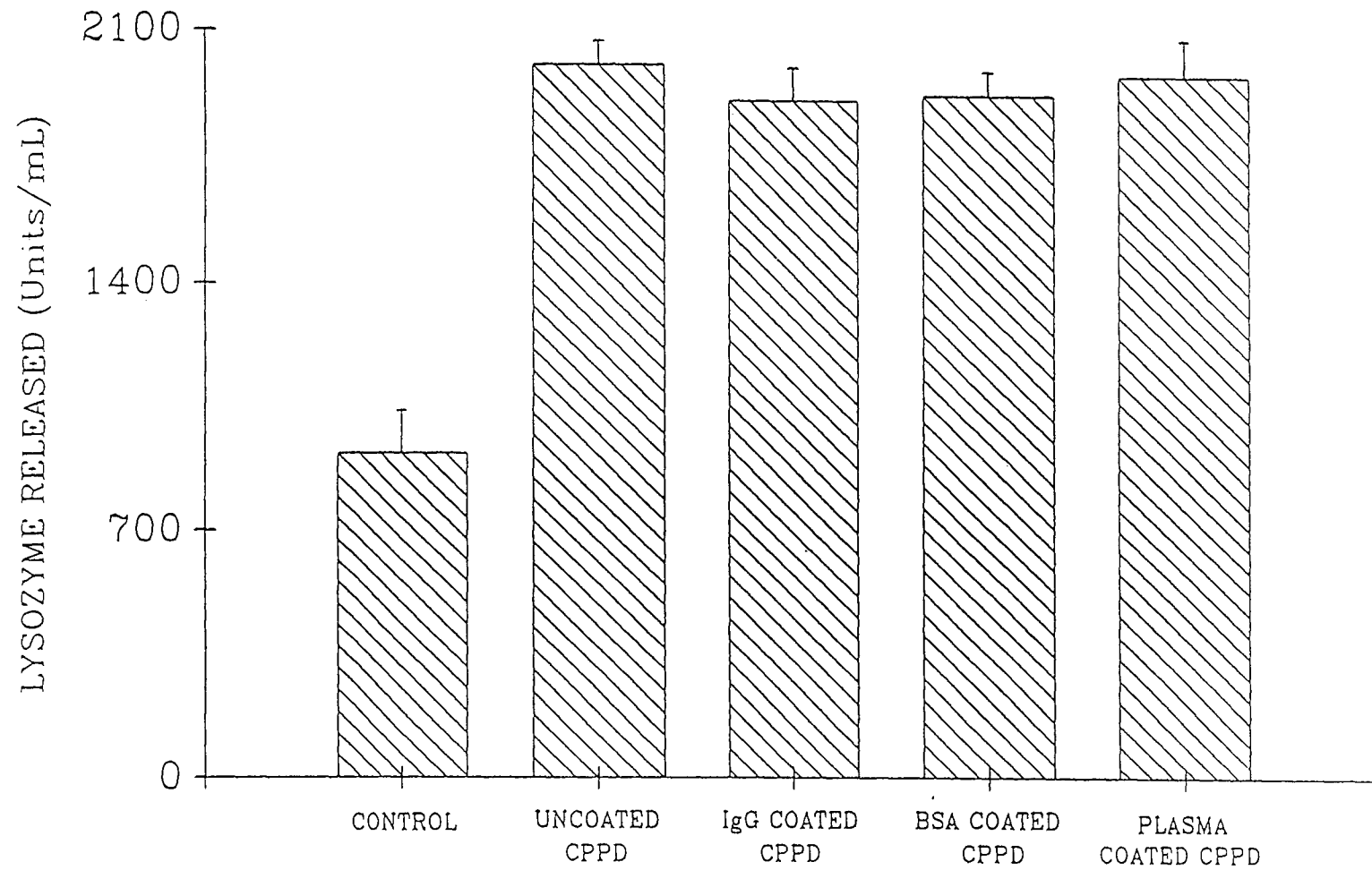


FIGURE 43

neutrophil responses to the uncoated and protein coated MSUM and CPPD crystals could also be studied.

SUMMARY AND CONCLUSIONS

1. The method of preparation of MSUM was successfully modified to yield crystals of a smaller and more uniform size range.

2. MSUM and CPPD crystals at concentrations of 5 mg/mL and 50 mg/mL respectively induced the generation of superoxide anion by neutrophils. The rate of superoxide production induced by CPPD crystals was slower than for MSUM crystals but there was no significant difference in the maximum amounts of superoxide anion generated from neutrophils stimulated by either MSUM or CPPD.

3. The reduction of ferricytochrome c was SOD inhibitable for CPPD/neutrophil incubations indicating that the reduction of ferricytochrome c was driven primarily by superoxide anions. However, the reduction of ferricytochrome c was not SOD inhibitable for MSUM/neutrophil incubations. This was attributed to inactivation of SOD probably due to adsorption onto the surface of MSUM crystals.

4. The precoating of CPPD and MSUM crystals with Ig G and plasma had no effect on superoxide generation. The absence of an enhanced responsiveness of neutrophils to crystals opsonized with Ig G may be because under the incubation conditions employed, the neutrophils stimulated by uncoated crystals were already producing maximum possible levels of

superoxide. There was no significant effect of BSA precoating of MSUM on MSUM induced superoxide release, whereas BSA-coated CPPD produced a significant increase in superoxide generated.

5. The CL generated by neutrophils in response to stimulation by MSUM was more rapid and extensive than that generated on stimulation by CPPD.

6. In general, the nature of the protein coating on the crystals strongly influenced the CL response with Ig G enhancing and plasma proteins inhibiting the CL generated. Adsorbed Ig G probably enhanced F_C receptor mediated crystal-neutrophil activation and CL generation. The major inhibitory component of plasma may be the low density lipoprotein fraction which binds strongly to MSUM and CPPD crystals probably interfering with the initial crystal-neutrophil membrane binding process.

7. CPPD crystals stimulate degranulation of cytochalasin B pretreated neutrophils as monitored by the release of MPO. MSUM induced MPO release could not be monitored due to adsorption of MPO by MSUM. Both MSUM and CPPD crystals stimulated degranulation as monitored by LYZ release but MSUM also adsorbed LYZ.

8. There was no significant effect of protein coating on CPPD induced LYZ release but precoating with Ig G significantly enhanced CPPD induced release of MPO. Protein

precoating of MSUM crystals did not have any effect on the MSUM induced LYZ release or MSUM induced MPO release.

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