STUDIES ON THE GRANULOCYTE CELL SURFACE OXIDASE

A thesis submitted for

the Degree of

Doctor of Philosophy

in the

University of Tasmania
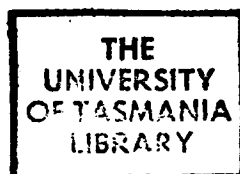

by

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SUMMARY

This thesis examined the possible role of the microfilaments and microtubules in controlling the activity of the granulocyte plasma membrane oxidase. There have not been any detailed investigations of this topic published to date.

The conflicting literature on the nucleotide specificity and location of the oxidase within the granulocyte was examined. *In vitro* techniques for isolating granulocytes from human peripheral blood and for quantitating the initial rate of plasma membrane oxidase activity were developed and used for these investigations. The involvement of microfilaments and microtubules in oxidase activity was studied by using pharmacological agents known to disrupt these structures *in vitro* (cytochalasin B, and colchicine, vinblastine and vincristine, respectively). Control experiments were also performed to ensure that microfilament and microtubule disruption by these agents provided the best explanation for the results presented herein. Correlative experiments were conducted to determine if a change in hexose monophosphate shunt activity was associated with the observed changes in plasma membrane oxidase activity brought about by the drug treatments.

Experiments on non-phagocytic granulocytes showed that microfilament disruption led to enhanced oxidase activity, while microtubule disruption produced a dual effect : a paradoxical enhancement (with low doses or brief exposures to the agents) and an impairment in oxidase activity. These results suggested that microfilaments act as a constraint against, while microtubules are required for plasma membrane oxidase activity. The requirement for microtubules was highlighted in experiments where the usual enhancement in oxidase activity with cytochalasin B was ablated when the

granulocytes were concomitantly incubated with a microtubule-disrupting
agent. (With phagocytic granulocytes qualitatively similar results were
observed. However, those experiments were not suitable for studying cell
surface oxidase activity because the presence of ingestable particles made
it impossible to determine whether plasma membrane and/or granule oxidase
activity was being measured.)

Based on the spectrophotometric and electron microscopic results
presented and the relevant findings from other investigators, a scheme
concerning the participation of the cytoskeletal elements in granulocyte
plasma membrane oxidase activity was devised. In this scheme the micro-
filaments physically constrain the oxidase within the plasma membrane,
and thereby hold the activity of the oxidase in check. Thus, microfilament
disruption is envisaged to result in enhanced oxidase activity by freeing the
enzyme. However, the oxidase is viewed as optimally active only as long as
the microtubules are present to maintain the plasma membrane topography
suitable for the enzyme's activity.

STATEMENT REGARDING ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or College, and to the best of my knowledge and belief contains no copy or paraphrase of material previously published or written by another person, except where due reference is made in the text.

Signed: _____

Date: June, 1979

ACKNOWLEDGEMENTS

TO MY PARENTS

PUBLICATIONS

1. Dewar CL, Wolowyk MW, and Hill JR (1976). A simple method for
    processing erythrocytes for scanning electron microscopy.
    Amer. J. Clin. Pathol. 66: 760-765.

2. Dewar CL (1978). An improved method for isolation of granulocytes
    from peripheral blood. J. Immunol. Methods 20: 301-310.

3. Dewar CL and Wolowyk MW (1979). Scanning electron microscopy of blood
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4. Lowenthal RM, Marsden KA, Dewar CL and Thompson GR. Congenital
    dyserythropoietic anaemia (CDA) with severe gout, rare Kell
    phenotype and erythrocyte, granulocyte and platelet membrane
    reduplication: a new variant of CDA Type II. Brit. J.
    Haematol. (in press).

5. Dewar CL, Lowenthal RM and Marsden KA. Ultrastructural studies
    of an unusual variant of congenital dyserythropoietic anaemia
    type II. (submitted for publication).

6. Dewar CL and Lowenthal RM. The role of microtubules and micro-
    filaments in granulocyte plasma membrane oxidase activity.
    (submitted for publication).

7. Dewar CL. The oxidase responsible for the granulocyte metabolic
    burst : a critical review. (submitted for publication).

ABBREVIATIONS USED IN THIS THESIS

ANS	8-anilino-1-naphthalene sulfonic acid
ATP	adenosine triphosphate
con A	concanavalin A
con A-FITC	concanavalin A, fluorescently labelled with fluorescein isothiocyanate
CFH	calcium-free physiological saline solution
CGD	chronic granulomatous disease
Ci	Curie
CMFH	calcium and magnesium-free physiological saline solution
colch	colchicine
cyto B	cytochalasin B
DAAO	D-amino acid oxidase
dextran	Dextran T 500
DMSO	dimethylsulfoxide
ELS	erythrocyte lysing solution
FAD	flavin adenine dinucleotide
G6PD	glucose-6-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMPS	hexose monophosphate shunt
HSS	HEPES physiological saline solution
IFG, IFGs	Isopaque-Ficoll gradient(s)

K_m — the kinetically defined Michaelis constant : the concentration of substrate at which the reaction proceeds half-maximally

KCN — potassium cyanide

LDH — lactate dehydrogenase

MPO — myeloperoxidase

NAD^+ — nicotinamide adenine dinucleotide (oxidized)

NADH — nicotinamide adenine dinucleotide (reduced)

$NADP^+$ — nicotinamide adenine dinucleotide phosphate (oxidized)

NADPH — nicotinamide adenine dinucleotide phosphate (reduced)

$NAD(P)^+$ — NAD^+ and/or $NADP^+$

$NAD(P)H$ — NADH and/or NADPH

NBT — nitroblue tetrazolium

O_2^- — superoxide

OD — optical density

PHA — phytohaemagglutinin

TH — transhydrogenase

Tris — Tris (hydroxymethyl) aminomethane

V_{max} — the maximal velocity of the reaction

VCR — vincristine

VBL — vinblastine

water — glass-distilled, deionized water

STATISTICS

P value = probability value between means (Student's paired t-test)

CHEMICALS

ANS	Sigma Chemical Co., St. Louis Mo., USA
Bovine albumin (fraction V)	Commonwealth Serum Laboratories, Melbourne, Victoria, Australia
Colchicine	Sigma, and more recently Boehringer Mannheim Australia Pty. Ltd., Mount Waverley, Vic., Australia
Con A-FITC	Calbiochem, La Jolla, Calif., USA
Cytochalasin B	Sigma
Dextran T 500	Pharmacia, Uppsala, Sweden
Dioxane (spectral grade 1, 4-dioxan)	Hopkin and Williams, Essex, England
DMSO	Sigma
Eosin Y (= eosin yellowish)	George T. Gurr, London, England
Ficoll (M. Wt. ~ 400,000)	Sigma
Folin and Ciocalteu's Phenol Reagent	Sigma
Glucose (^{14}C-1-glucose)	The Radiochemical Centre, Amersham, Bucks., England
Glutaraldehyde EM (25% aqueous solution)	TAAB Laboratories, Reading, England
HEPES	Calbiochem
Hyamine Hydroxide 10X (= Hyamine 10X)	Packard Instrument Pty. Ltd., Mount Waverley, Vic., Australia
Lead nitrate	Ajax Chemicals, Sydney, Australia
Methylene blue	George T. Gurr, London, England
NBT (grade III, crystalline, lemon yellow)	Sigma
Osmium tetroxide	Ferak, Berlin, W. Germany

Packard liquid scintillation fluid (Permablend III)	Packard
Polystyrene latex beads	Sigma
Propylene Oxide	B.D.H. (Australia), Boronia, Vic., Australia
Pyridine (analytic reagent grade)	B.D.H. (Australia)
Siliconizing solution (Siliclad)	Clay Adams, Parsippany, N.J. USA
Sodium cacodylate $[(CH_3)_2\ AsO_2\ Na \times 3\ H_2O]$	B.D.H. (Australia)
Sodium citrate $(Na_3C_6H_5O_7 \times 2\ H_2O)$	B.D.H. (Australia)
Sodium deoxycholate $(C_{24}H_{39}O_4Na)$	B.D.H. (Australia)
Sodium metrizoate (32.7% $^W/_V$)	Nyegaard and Co., Oslo, Norway
Spurr's resin (all components)	Ladd Research Industries, Burlington, Vermont, USA
Tris (Trizma)	Sigma
Tris maleate (Trizma maleate)	Sigma
Trypan blue	George T. Gurr, London, England
Uranyl acetate $[UO_2(C_2H_3O_2)_2 \times 2\ H_2O]$	B.D.H. (Australia)
Vinblastine (Velbe)	Eli Lilly and Co., Indianapolis, Indiana, USA
Vincristine (Oncovin)	Eli Lilly and Co.

MINOR EQUIPMENT USED

BEEM capsules	Better equipment for Electron Microscopy Inc., Bronx, N.Y., USA
Copper grids for electron microscopy	Athene-type by VECO, ordered from LADD Research Industries Burlington, Vt., USA
Millipore filters	Millipore Corporation, Bedford, Mass., USA
Mini-scintillation vials (plastic)	Filtrona Plastics, Thomastown, N.S.W., Australia
Petri dishes (plastic, 35 x 10 mm style)	Falcon, Oxnard, Calif., USA
Test-tubes (polycarbonate)	Nalge Sybron Corp., Rochester, N.Y., USA
Universal containers	Johns Professional Products, Cheltenham, Vic., Australia

MAJOR EQUIPMENT USED

Baird Atomic Fluorimeter

Hitachi HS-7S Electron Microscope

JEOL Scanning Electron Microscope

Leitz Orthoplan Fluorescence Microscope (with Ploemopak)

LKB III Utramicrotome

MSE Mistral 4L refrigerated centrifuge

Packard 2450 Tri-Carb liquid scintillation spectrometer

Unicam SP8-100 Ultraviolet spectrophotometer

SECTION 1

INTRODUCTION

1.1   <u>Introductory remarks</u>

This thesis is concerned with the human granulocyte

plasma membrane oxidase responsible for superoxide production.

Specifically it sets out to examine the possible role of the

cytoskeletal elements (the microfilaments and microtubules) in

the control of the activity of this enzyme.

1.2   <u>The Granulocyte</u>

The neutrophilic polymorphonuclear leucocyte (granulocyte)

is able to engulf and subsequently kill ingested micro-organisms

and for this reason these cells have been called "professional

phagocytes" (Rabinovitch, 1968) and recognized as an important

first line of defence against infection. The role played by

phagocytic cells in higher organisms was first recognized by

the Russian zoologist, Metchnikoff, in 1883 (cited by Stossel,

1974; Murphy, 1976). Phagocytosis by unicellular organisms had

been understood as a process whereby single cells obtained food,

but it was Metchnikoff who postulated that the same process in

eukaryotes could protect the higher organism from invasion by

pathogens.

Intense study of the granulocyte since Metchnikoff's

work has shown that the cell is capable of directed movement

towards a target, called chemotaxis (McCutcheon, 1946; Harris,

1954) and that phagocytosis begins as soon as the cell touches an

attractive particle.

It has also been shown that the granulocyte exhibits

discretion when it comes to ingestion: foreign but not autologous

cells are eaten, and encapsulated micro-organisms are not touched

while others are vigorously attacked (Mudd *et al* , 1934; Foley

and Wood, 1959; Rabinovitch, 1968; Maruta and Mizuno, 1971;

Richardson and Sadoff, 1977).

The target is identified as ingestable by (the

granulocyte's) recognition of a host-derived coating of immuno-

globulin and complement molecules, known as opsonin[1]. The role

of these proteins as molecules which interact with the surface

receptors on the granulocyte to promote ingestion has recently

been reviewed by Stossel, in 1975.

To engulf the micro-organism, the migrating granulocyte

extends hyaline ectoplasm to form pseudopodia on either side of

the particle and then the pesudopodia simply surround the particle

and fuse at its distal side. The micro-organism is then contained

within a phagocytic vacuole or phagosome, the boundary of which

is inverted plasma membrane. The phagosome pinches off from the

parent plasma membrane and moves towards the cell centre (Mudd

et al, 1934; Hirsch and Cohn, 1960).

At about this time, the cytoplasmic granules within the

vicinity of the phagosome move towards and fuse with the vacuole

membrane, discharging their cytotoxic contents into the phago-

some (Hirsch and Cohn, 1960). There is evidence that the smaller

specific granules discharge their contents first followed by the

azurophilic granules (Bainton, 1973). The act of degranulation

and lysosomal enzyme release from the cytoplasmic granules, in

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1. This term was coined by Wright and Douglas in 1903 and comes
    from the Greek opsono: I prepare for eating (cited by Koch,
    1978).

concert with an increase in metabolic activity in the phagocytos-
ing cell serves to kill the micro-organism.

1.3   Phagocytosis-associated metabolic events

The increased metabolic activity ("respiratory burst")
following contact and phagocytosis is characterized by an
increased consumption of oxygen, an increase in glucose oxidation
via the hexose monophosphate shunt, and an increase in the
production of hydrogen peroxide and superoxide radical (Sbarra
and Karnovsky, 1959; Iyer, Islam and Quastel, 1961; Rossi and
Zatti, 1964; Selvaraj and Sbarra, 1966; Baehner and Nathan,
1968; Babior, Kipnes and Curnutte, 1973). It was found that
the increased uptake of oxygen during phagocytosis and
phagocytosis itself were insensitive to inhibitors of mitochondrial
oxidative metabolism (Sbarra and Karnovsky, 1959) and that the
energy for phagocytosis was derived from anaerobic glycolysis
(McKinney et al, 1953; Beck, 1958; Marchand, Leroux and
Cartier, 1972). The oxygen taken up by the cells during
phagocytosis was shown, at least in part, to be reduced to
hydrogen peroxide (Iyer, Islam and Quastel, 1961). Iyer et al
were also the first to suggest that the H_2O_2 generated by
phagocytosing cells was used as a microbicidal agent. The
cellular pathway for the reduction of molecular oxygen to hydrogen
peroxide has been the subject of considerable debate for many
years.

1.4   The oxidase responsible for superoxide production

Three flavoproteins (NADH oxidase, NADPH oxidase and
D-amino acid oxidase) as well as myeloperoxidase have been
suggested as the enzyme responsible for the generation of H_2O_2

(reviewed by Gee and Cross, 1973). It is now accepted that the enzyme involved is a reduced pyridine nucleotide oxidase (Babior, 1978) but whether the hydrogen donor for the oxidase is NADPH or NADH has not been resolved (see Appendix I).

The oxidase catalyzes the reduction of oxygen by a one-electron transfer step and therefore the unstable and highly reactive intermediate, superoxide radical, is formed (Babior et al, 1973; Babior et al, 1976). The stoichiometry for this reaction, with NADPH for example, is:

$$2O_2 \quad + \quad NADPH \longrightarrow 2O_2^- \quad + \quad NADP^+ \quad + \quad H^+$$
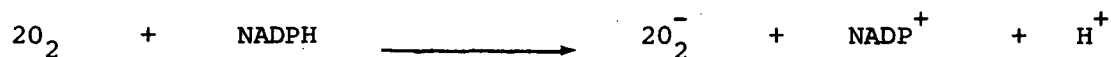
(Babior, 1978)

Using methods which allowed precise quantitation of the fate of the oxygen taken up during the metabolic burst, Root and Metcalf (1977) demonstrated that all of the oxygen taken up was converted to O_2^- and then 80 percent of this superoxide was converted to H_2O_2 by dismutation. They showed as well that the dismutation of two molecules of superoxide was the only important source of the H_2O_2 generated by the granulocyte. H_2O_2 is formed from the superoxide radical intermediate in the following reaction:

$$2H^+ \quad + \quad O_2^- \quad + \quad O_2^- \longrightarrow O_2 \quad + \quad H_2O_2$$

(Fridovich, 1972)

This dismutation reaction can occur spontaneously or it can be catalyzed by the enzyme superoxide dismutase. Interestingly, the enzyme catalyzes the reaction at a faster rate than the spontaneous dismutation (Fridovich, 1978).

The microbicidal potential of the superoxide radical was first suggested by McCord, Keele and Fridovich in 1971 because of the presence and distribution of superoxide dismutase in certain microbes. Anaerobic microbes are relatively deficient in superoxide dismutase compared to aerobes, and thus anaerobes are more sensitive to the cytotoxic effects of superoxide. The granulocyte's oxygen-dependent mechanisms for killing micro-organisms involving H_2O_2, superoxide radical, and other metabolites of oxygen such as hydroxyl radicals and singlet oxygen have been reviewed by Klebanoff (1975), Koch (1978) and Babior (1978).

1.5 The plasma membrane oxidase

The oxidase responsible for catalyzing the formation of superoxide has been found in the cytoplasmic granules (see Appendix I). Recent evidence indicates that in addition to the granular location of the oxidase, a plasma membrane location also exists (Briggs et al, 1975; Segal and Peters, 1976; Tsan et al, 1976b; Goldstein et al, 1977). Teleologically, it is sensible to have the enzyme located in the plasma membrane because with ingestion the plasma membrane containing this oxidase (Briggs et al, 1975) is internalized around the particle and forms the limiting intracellular boundary of the phagosome. It is self-evident that maintenance of close contact between this enzyme in the membrane and the ingested particle would contribute to the efficiency of superoxide and hydrogen peroxide delivery to the phagosome (see Appendix I).

1.6 The fluid mosaic model of the membrane

In the light of the recent reports describing a plasma

membrane location for the oxidase, it seemed an opportune time to
study this enzyme. To begin this study of the plasma membrane
oxidase it was necessary to review the structure of the cell
membrane. The accepted model for plasma membrane organisation is
the fluid mosaic model of Singer and Nicolson (1972). In this
model of the membrane, proteins are situated in a bilayer mixture
of lipids, and the non-polar portions of both the lipids and
proteins are found within the hydrophobic interior of the bilayer.
The hydrophobic interior is sandwiched between an outer and inner
layer of hydrophilic molecules - the polar head groups of the
phospholipid molecules and the polar groups of proteins. The
proteins can extend into the membrane to different depths depend-
ing upon the distribution of the polar and non-polar groups
within the particular protein (Figure 1.1). Thus proteins are
classed as integral (embedded) or peripheral (surface-associated).
The integral proteins can be further sub-divided into ecto- and
endoproteins. Ectoproteins have a large mass projecting outside
the membrane, beyond the cell surface, while endoproteins project
away from the membrane on its cytoplasmic surface. Examples of
ectoproteins are the glycoproteins, histocompatibility antigens,
immunoglobulin molecules, and drug and hormone receptors
(Rothman and Lenard, 1977; Smith, 1978). An example of an
endoprotein is the erythrocyte membrane protein, spectrin (Smith,
1978).

The fluid mosaic model for membrane organisation
proposed that proteins would be mobile within the lipid matrix
of the membrane, and therefore with such movements the topographi-
cal organisation of membranes would be random (Singer and

Nicolson, 1972). It has often been found, however, that the
associations (lipid-lipid, lipid-protein, and protein-protein)
between components render most if not all membranes non-random,
or heterogeneous, in short-range topography (Nicholson, 1977).
Nevertheless, many membrane proteins are free to move within
the plane of the membrane and assume a homogeneous or random
distribution. Definitive evidence for the mobility of proteins
within the membrane came from the experiments of Frye and
Edidin (1970). In their experiments they labelled the surface
antigens of mouse and human cells with different fluorescent dyes,
and then fused the cells with Sendai virus to form a hybrid.
They found that with time the human and mouse antigens, made
visible by fluorescence microscopy, had spread over the surface
of most of the hybrid cells. Their work validated the Singer-
Nicolson model for membranes by showing that these proteins were
able to translocate within the plane of the membrane.

1.7   Movement of proteins within the membrane

It is certain that free diffusion alone cannot
explain the ordering of cell-surface topography, especially the
long-range movements of proteins in the membrane (Nicolson, 1976).
Major factors affecting the movement of proteins within the plane
of the membrane include:-

1) the binding of a ligand to its cell membrane receptor. Such
binding can induce movement, or redistribution, of the ligand-
receptor complex within the membrane. The ligands most extensive-
ly studied are the lectins (plant proteins which bind to specific
carbohydrates on the cell surface) and antibodies to leucocyte
surface immunoglobulins (Schreiner and Unanue, 1976).

Redistribution of leucocyte surface immunoglobulin has been

envisaged to be of some importance *in vivo* in that it could be a

mechanism whereby leucocytes (especially lymphocytes) redistribute

immunoglobulins to present a dense cluster of these molecules to

other immuno-competent cells, thereby facilitating cell signalling,

recognition and contact phenomena (Taylor *et al*, 1971;

Nicolson, 1977).

2) the cytoskeletal elements of the cell, in particular the

microtubules and microfilaments. It is now established that

cells can maintain a degree of topographic order within their

fluid membranes by virtue of trans-membrane associations

between proteins in the membrane and cytoplasmic cytoskeletal

elements (Berlin *et al*, 1974; Edelman, 1976; Nicolson, 1976).

These elements are located underneath the plasma membrane and

include microtubules, microfilaments, intermediate filaments

and possibly other filament-like structures, as illustrated in

Figure 1.2. The interaction between the plasma membrane

proteins and the cytoskeletal elements has been studied by

exposing cells to drugs known to impair the function of

microtubules or microfilaments, and observing the effect such

treatment has upon the movement of anti-immunoglobulin antibody

or lectin-binding proteins within the plane of the membrane.

These drugs can inhibit or stimulate to various degrees, and

sometimes even reverse the redistribution of such surface

receptors (Schreiner and Unanue, 1976).

Similar studies with such drugs have shown that during

ingestion granulocytes can, through the cytoskeletal elements,

confer a topographical heterogeneity upon the plasma membrane.

This then allows the granulocyte to determine phagocytosable (internalizable) and transport (non-internalizable) sites (Ukena and Berlin, 1972; Berlin, 1975).

The work to be discussed in this thesis was prompted by the discovery of a plasma membrane location for the oxidase involved in the metabolic burst of granulocytes. In the light of reports of a role for the cytoskeletal elements in the control of plasma membrane phenomena, it seemed an opportune time to study their possible role in the control of plasma membrane oxidase activity. This thesis describes those studies.

1.8   Outline of the Thesis

As explained above, the aim of this work was to examine the possible role of the cytoskeletal elements in the control of granulocyte plasma membrane oxidase activity. To begin these studies it was first necessary to devise an improved method for the isolation of granulocytes from peripheral blood. This method is described in Section 2. Similarly, *in vitro* techniques for quantitating the activity of the plasma membrane oxidase in intact cells had to be devised and standardized (Section 3). To prevent interference from the cytoplasmic (granule-associated) oxidases in these experiments, the cells were not provided with phagocytosable material. In Section 4 the techniques described in Sections 2 and 3 were used to examine the effects of microtubule and/or microfilament disruption on the activity of the plasma membrane oxidase. Similar experiments were conducted with phagocytosing granulocytes, and the effects of microtubule and/or microfilament disruption on the enzyme activity under these circumstances are outlined in Section 5.

The effects of microtubule or microfilament disruption on phagocy-
tosis itself are also described in that section. In Section 6, the
effects of microtubule disruption on concanavalin A receptor re-
distribution were examined. Studies with the fluorescent membrane
probe, 1-anilino-8-naphthalene sulphonate, are detailed in Section
7. HMPS activity in resting (non-phagocytic) granulocytes after
exposure to agents disrupting microtubules or microfilaments was
examined and is described in Section 8. Electron microscopy was
used to study the location of the plasma membrane oxidase after
microtubule or microfilament disruption (Section 9). Finally,
in Section 10 the results and conclusions from these experiments
are discussed in relation to the published literature from
other investigators.

ECTOPROTEINS

EXTRACYTOPLASMIC SIDE

CYTOPLASMIC SIDE
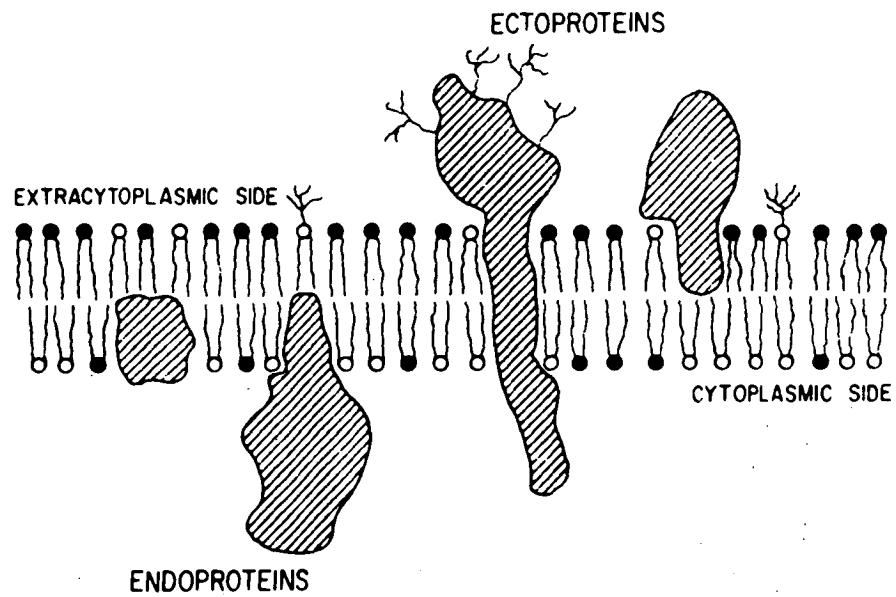
ENDOPROTEINS

FIG. 1.1.   The arrangement of ectoproteins and endoproteins

within the membrane (adapted from Rothman and

Lenard, 1976).   All of the proteins shown are

integral (embedded).   The circles represent the

polar head groups of the phospholipid molecules.
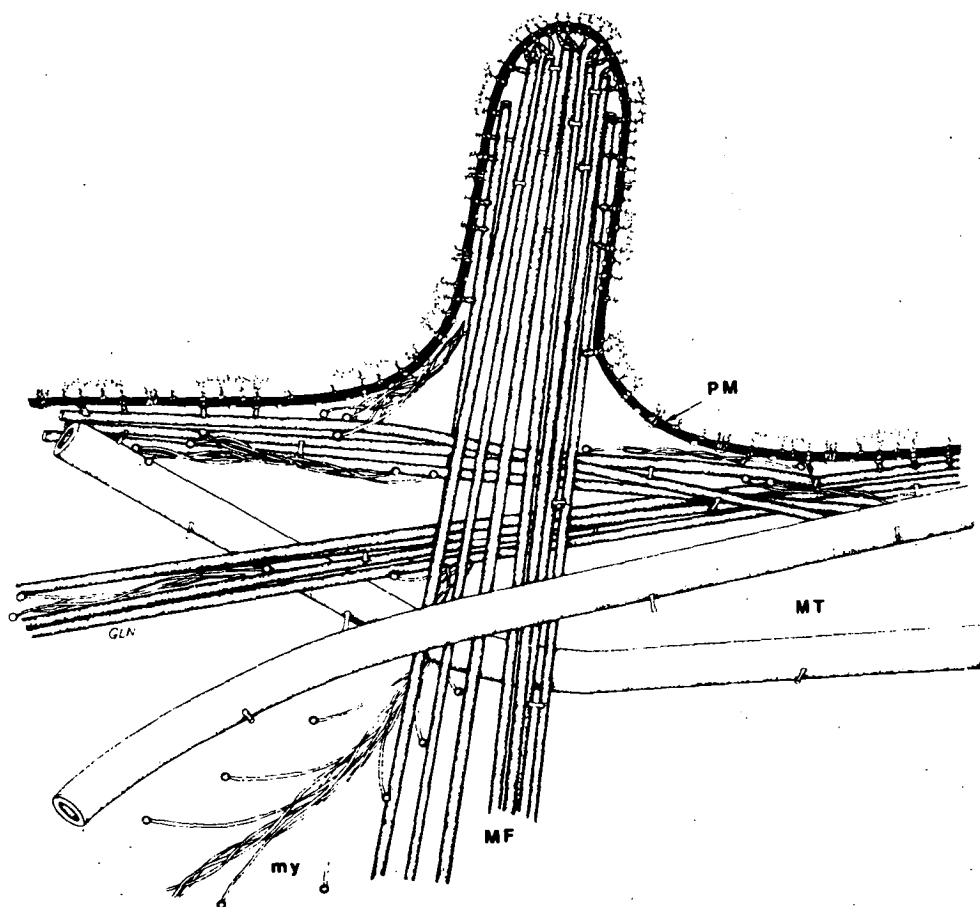
FIG. 1.2. A model depicting opposite but coordinated roles
for the microfilaments (contractile) and micro-
tubules (structural) in controlling cell surface
topography (taken from Nicolson, 1977). Bridging
molecules (such as myosin and α-actinin, see
text) between the microfilaments (MF) and micro-
tubules (MT) and between the cytoskeletal elements
and the plasma membrane (PM) components allow for
indirect interactions. Myosin (my) is shown
existing both as small bundles and as larger
filaments.

SECTION 2

SEPARATION OF GRANULOCYTES FROM

PERIPHERAL BLOOD

2.1   INTRODUCTION

Granulocytes are the most difficult of the leucocytes to separate from peripheral blood. Their density is the greatest of the leucocytes and it increases with the degree of maturation of the cell (Zipursky *et al*, 1976). The density of the more mature segmented granulocytes is similar to that of the erythrocyte (Tullis, 1952), making separation of the granulocytes from the erythrocytes the most difficult step in their isolation from peripheral blood.

Relatively good yields of granulocytes can be obtained from peripheral blood by simple sedimentation of whole blood admixed with dextran or methylcellulose to aggregate the erythrocytes (Skoog and Beck, 1956). The sedimentation rate of the erythrocytes is increased by their aggregation but the sedimentation of leucocytes is largely unaffected, and the leucocytes can be harvested from the upper part of the tube after erythrocyte sedimentation is complete. However, the gain in yield using this method is outweighed by the impurity of the final leucocyte preparation.

In 1964 Böyum employed, in a different way, the concept of sedimenting erythrocyte aggregates to isolate the leucocytes. In his modification the erythrocyte-aggregating agent was mixed with a non-toxic compound of high density, sodium metrizoate. The blood was then carefully layered on top of this mixture, causing the erythrocytes to aggregate at the interface and sediment to the bottom of the tube. The leucocytes remained in the plasma layer above the interface because of the density barrier. The high density, non-toxicity and pH near neutrality

were the properties which made sodium metrizoate particularly suitable for this purpose (Day, 1970).

This work led into the best method to date for separating granulocytes from peripheral blood; a technique which was also devised by Böyum. In this method the mononuclear leucocytes were first removed from the blood by one centrifugation of diluted blood layered onto a mixture of sodium metrizoate and a sucrose polymer (Ficoll). Ficoll was chosen for this purpose because of its relatively low viscosity and high solubility. The density of the sodium metrizoate-Ficoll gradient was 1.077 g/ml, which allowed the granulocytes to sediment to the bottom of the tube under one centrifugation. In a second step the granulocytes were separated from the erythrocytes by sedimentation employing dextran as an erythrocyte-aggregating agent. The disadvantages of this technique are its relatively low yields of granulocytes, especially in blood anticoagulated with heparin, and the erythrocyte contamination of the final granulocyte preparation. This feature (as discussed in section 2.4) makes the granulocyte preparation unsuitable for functional studies. The aim of the experiments described in this section was to devise an improved method of isolating granulocytes from peripheral blood. In addition the functional capacity of the isolated granulocytes was examined with phagocytic, candidacidal and NBT-dye reduction assays (see Dewar, 1978).

2.2   METHODS

2.2.1   Blood

Venous blood anticoagulated with heparin (10 IU/ml)

was collected from 16 healthy adults for these studies.

2.2.2   Plasma

AB-Rh-negative plasma was obtained from the Red Cross

Blood Transfusion Service, Hobart, Tasmania. It was divided into

5 ml aliquots and stored at -20°C.

2.2.3   Isopaque-Ficoll density gradients (IFGs)

IFGs were prepared by mixing 30 ml Isopaque with 70 ml

Ficoll 0.09 g/ml, giving a gradient with a final density of

1.077 g/ml at 20°C.

2.2.4   Dextran solution

Dextran was prepared as a 0.040 g/ml solution in

0.15 M sodium chloride.

2.2.5   HEPES-buffered physiologic saline solutions

A HEPES-buffered physiologic saline solution

containing Ca^{+2} and Mg^{+2} (HSS) was prepared for these experiments.

In addition, Ca^{+2} free (CFH) and Ca^{+2} and Mg^{+2} free (CMFH)

solutions were prepared and used where indicated (Appendix II).

2.2.6   Erythrocyte lysis

In preliminary experiments the technique of Craddock *et al*

(1974) was used to lyse the contaminating erythrocytes in the

final step of granulocyte isolation. However this method

resulted in too much stroma from incompletely lysed erythrocytes,

and the stroma was impossible to separate from the granulocytes.

Therefore the erythrocyte lysing solution (ELS) of Goyle

(1968) was adopted and proved satisfactory. The ELS consisted

of isotonic NH_4Cl in Tris buffer, pH 7.4.

2.2.7   <u>Total and differential leucocyte counts</u>

Counts were performed according to the methods out-
lined in Dacie and Lewis (1968). At least 200 cells were counted
for total and 500 cells for differential leucocyte counts. In
addition, differential leucocyte counts of the final granulocyte
preparations, using slides prepared according to Hirsch and Cohn
(1960), were performed.

2.2.8   <u>Buffy coat leucocytes</u>

Buffy coat leucocytes were prepared by centrifuging
whole blood at 1000 x g for 15 min, collecting the buffy coat
and lysing the erythrocytes in ELS (Goyle, 1968). The granulocytes
were then washed in CFH once and resuspended in HSS to 25 x 10^6
cells/ml.

2.2.9   <u>Phagocytic assay</u>

Phagocytosis of polystyrene latex beads was measured
in a system consisting of 1.25 x 10^6 cells, 10% plasma,
and 50 x 10^6 polystyrene latex beads in a total volume of 500 µl
HSS. Phagocytosis was initiated by the addition of the beads and
promoted by end-over-end rotation (30 rpm) at $37^{\circ}C$ for 20 min.
It was terminated by adding 100 µl of 0.009 g/ml EDTA in 0.15 M
sodium chloride at $4^{\circ}C$ and centrifuging at 100 x g x $4^{\circ}C$ for
5 min to separate the uningested beads from the cells. The cell
pellet was used to prepare slides which were used to calculate
the number of cells containing 0, 1-5, 6-10, or >10 beads per
cell. At least 200 cells were counted from each slide.

2.2.10   <u>Candidacidal assay</u>

The candidacidal assay developed by Lehrer and Cline

(1969), employing methylene blue to distinguish between viable and nonviable intracellular yeast was used. The strain of *C. albicans* used was originally obtained from the vaginal swab of a patient. During the incubation the ratio of granulocytes: *Candida* was 1:1, the volume 1.0 ml, and the final concentration of added AB-Rh-negative plasma 25% v/v. Incubations were done in triplicate using sterile technique. The reaction was stopped after 60 min by the addition of 0.25 ml of sodium deoxycholate (2.5%) which lysed the granulocytes without disturbing the *Candida*. Methylene blue was added to facilitate the haemocyto-meter counts of the number of viable and non-viable (blue-stained) micro-organisms. At least 200 *Candida* were counted from each sample tested and the percentage of stained micro-organisms was determined. In addition, the ingestion and killing of the *Candida* was monitored by electron microscopy (section 9.2.6.3).

2.2.11   NBT dye reduction test

         A modification of Baehner and Nathan's technique (1968) was used to quantitate NBT dye reduction as a measure of cyanide-insensitive oxidative metabolism (Fridovich, 1974). In this test all solutions were Millipore-filtered (0.45 μm) to ensure a 'spontaneous' dye reduction test (Baehner and Nathan, 1968). The final test system contained between 6.25×10^5 and 1.25×10^6 cells in a solution of 0.49 mM NBT, 1 mM KCN, 6.0 mM glucose, 0.06 mM Ca^{2+} and Mg^{2+} in HSS to a final volume of 200 μl with a pH of 7.2.

2.2.12 Calculating the recoveries of isolated granulocytes

2.2.12.1 Recoveries using the quotient G_1/G_0

The recoveries were calculated by dividing the number of granulocytes isolated from the gradients (G_1) by the number of granulocytes applied to the gradients (G_0) and multiplying by 100.

G_0 was determined from the total and differential leucocyte counts and the volume of blood applied to the gradients. At least 200 cells were counted in each half of the haemocytometer chamber in calculating recoveries.

2.2.12.2 Recoveries calculated using Boyum's method

Boyum expressed his yield of granulocytes as the number of granulocytes in the erythrocyte/granulocyte pellet as a percentage of the total number of granulocytes found in the tubes after separation (Boyum, 1968). The formula was not given in his paper, but the formula for similarly calculating the yield of mononuclear cells was supplied, and using the latter as a guide the following formula was devised to express Boyum's method of estimating yields:

$$100 \times \frac{g_2}{g_0} \times \frac{m_0 - m_2}{m_1 - m_2}$$

where g_2 = percent granulocytes in the bottom fraction

g_0 = percent granulocytes in whole blood

m_1 = percent mononuclear cells in the top fraction

m_0 = percent mononuclear cells in whole blood

and m_2 = percent mononuclear cells in the bottom fraction

2.2.13 Isolation of granulocytes

Using Böyum's method (1968) as a starting point, a three-step procedure was devised for the isolation of granulocytes from peripheral blood. In the first step, 2 ml of blood was mixed with 4 ml of isotonic saline in siliconized test tubes (tubes 'A'). This blood plus 1 ml of saline washings from 'A' was layered onto each 3 ml IFG at 20°C in siliconized polycarbonate test tubes ('B'). The loaded gradients were then centrifuged for 30 min at 20°C with a force of 400 x g at the blood - IFG interface.

The supernatant, containing plasma diluted with saline, mononuclear cells, and IFG, was removed to within 1-2 mm of the pellet (Fig. 2.1). The pellets containing the granulocytes and erythrocytes were washed in 5 ml of CFH twice, and then mixed with 2 ml AB-Rh-negative plasma. Dextran solution (3.0 ml) at 4°C was then added and the tubes gently mixed with a 5 ml pipette, sealed, and allowed to sediment for 50 min at 4°C. After this time, the granulocyte-rich upper layer (Fig. 2.2) was removed and the cells harvested by centrifugation in tubes 'C'. The cells were washed once in CFH, and the erythrocytes lysed in ELS (Goyle, 1968). The granulocytes were then collected by centrifugation, washed in CFH, and resuspended in HSS at 25 x 10^6 cells/ml. The method is shown diagrammatically in Figure 2.3.

2.3 RESULTS

Sections 2.3.1 - 2.3.5 inclusive outline the results of experiments preliminary to the adoption of the separation procedure outlined in section 2.2.13.

2.3.1 Yield after 30 min or 40 min centrifugation

In Böyum's procedure the diluted blood was centrifuged for 40 min on the IFGs, but it was suggested by Böyum that 30 min may be sufficient if the procedure were to be used for granulocyte isolation. Böyum also suggested however, that the change from 40 to 30 min would cause some erythrocyte and granulocyte contamination of the mononuclear layer, and thus a slight drop in the yield of granulocytes from the lower layer. In two experiments where this was examined it was found that 30 min was just as efficient in sedimenting the granulocytes as 40 min. [Exp't 1: 2.5 ± 0.2 x 10^6 granulocytes (40 min) versus 2.4 ± 0.3 x 10^6 granulocytes (30 min) per IFG (3 IFGs). Exp't 2: 3.2 ± 0.1 x 10^6 granulocytes (40 min) versus 3.2 ± 0.2 x 10^6 granulocytes (30 min) per IFG (3 IFGs)]. Therefore in subsequent work a 30 min centrifugation of blood on IFGs was adopted as the standard procedure.

2.3.2 Volume of plasma used in the dextran sedimentation step

Böyum used 1 ml of the heparinized, autologous plasma found at the top of the tube after the centrifugation step as the source of plasma for the dextran sedimentation (Fig. 2.1). When 1 ml of this diluted plasma was compared with 1 ml of AB-Rh-negative plasma, it was found that the latter provided a more efficient separation of granulocytes. Furthermore, 2 ml of AB-Rh-negative plasma gave an improved yield over 1 ml (Table 2.1). Increasing the volume of AB-Rh-negative plasma to 3 ml was of no benefit (Table 2.1), therefore 2 ml of plasma was routinely used.

2.3.3   Autologous versus AB-Rh-negative plasma

          On finding that whole AB-Rh-negative plasma provided

a better separation of granulocytes than diluted autologous plasma

(section 2.3.2) it was reasoned that whole autologous plasma

might be more efficient than AB-Rh-negative plasma. Centrifuging

the blood (as in section 2.2.8) to obtain the undiluted autologous

plasma necessitated replacing the plasma with heparinized isotonic

saline (10 IU/ml) to dilute the blood before separation on IFGs.

This caused clumping of the reconstituted blood which interfered

with the efficiency of the centrifugation (Table 2.2), as

measured by the numbers of granulocytes isolated. Therefore

AB-Rh-negative plasma was used in all subsequent work.

2.3.4   Dextran

2.3.4.1   Volume of dextran in the sedimentation step

          In Böyum's procedure 0.4 ml of dextran, 0.045 g/ml

in isotonic saline, is used with 1 ml of diluted autologous

plasma in the sedimentation step. In two experiments where the

volume of dextran was altered, it was found that the optimal

volume for the dextran was 3.0 ml (Table 2.3).

2.3.4.2   Concentration of dextran

          It was found that 0.040 g/ml and 0.045 g/ml were

equally effective while 0.035 g/ml dextran was not as

efficient in aggregation and sedimentation of the erythrocytes

(Table 2.4). Therefore, 0.040 g/ml dextran was used in

subsequent experiments.

2.3.4.3   Erythrocyte contamination of the granulocytes in the sedimentation

step

Following Böyum's procedure, 0.4 ml dextran was used

in the sedimentation step and the erythrocyte contamination of

the upper granulocyte-rich layer varied from 32:1 to 66:1. When

3.0 ml dextran was used however, the erythrocyte contamination

was reduced to between 10 and 13 erythrocytes per granulocyte

(Table 2.5).

2.3.5   Inner diameter of the sedimentation step tube

Böyum suggested that performing the dextran sedimentation

in an 8.5 mm versus 13 mm inner diameter tube increased the yield

of granulocytes. In two experiments with 9 mm versus 15 mm inner

diameter tubes, no difference was found in the yield of

granulocytes [$3.6\pm0.1 \times 10^6$ granulocytes (15 mm) versus 3.5 ± 0.1

$\times 10^6$ granulocytes (9 mm) per IFG].

2.3.6   Viability

Viability of the isolated granulocytes as assessed by

Trypan Blue or Eosin Y dye exclusion was always > 98%.

2.3.7   Granulocyte functional tests

2.3.7.1   Phagocytic assay

The percentage of granulocytes which had ingested 0,

1-5, 6-10 or >10 polystyrene latex beads after 20 minutes was

the same whether the granulocytes had been isolated by the

procedure described in section 2.2.13 or as buffy coat (2.2.8)

leucocytes (Table 2.6).

2.3.7.2   Candidacidal assay

Buffy coat and isolated granulocytes were identical in their ability to kill ingested *C. albicans* after 60 minutes incubation (Table 2.7). Electron microscopical studies of the assay confirmed that ingested *Candida* were being killed (Figure 2.4).

2.3.7.3   NBT dye reduction assay

The NBT dye reduction capacity of isolated granulocytes was compared to that of buffy coat leucocytes from 4 samples. Reduction was identical in all cases except at 90 min, where the O.D. of reduced dye from the buffy coat of sample 3 (Fig. 2.5) was significantly different from that of the isolated granulocytes from the same sample. When this test was repeated with cells from the same individual 3 weeks later (3+, Figure 2.5), no significant difference between granulocytes obtained from either method could be found.

2.3.8   Differential counts of whole blood and isolated granulocytes

Differential counts of the peripheral blood (Table 2.8) showed that the granulocytes were mainly composed of neutrophils ($95.5\pm0.7\%$) with only $4.3\pm0.7\%$ and $0.2\pm0.1\%$ eosinophils and basophils respectively. The isolated granulocytes showed $94.9\pm0.7\%$ neutrophils which was not significantly less than in the peripheral blood. Similarly the percentage of eosinophils in the final preparation ($3.8\pm0.6\%$) was not significantly less than in the peripheral blood. However the percentage of basophils in the preparation ($1.1\pm0.3\%$) was significantly greater than in peripheral blood ($p < 0.01$).

2.3.9   Recoveries of isolated granulocytes

2.3.9.1 Recoveries using the quotient G_1/G_0 x 100

A mean of 70.9% of granulocytes applied to 8 gradients was recovered using the technique in section 2.2.13 (Table 2.8).

2.3.9.2 Recoveries calculated using Böyum's method

Using the formula G_1/G_0 x 100 (section 2.2.12.1) a mean of 69.5±2.4% of the granulocytes applied to 6 (of the original 8) IFGs was recovered. For these 6 samples full data was obtained and therefore the formula

$$100 \times \frac{g_2}{g_0} \times \frac{m_0 - m_2}{m_1 - m_2} \quad \text{(section 2.2.12.2)}$$

was also used to calculate the recovery of granulocytes. With this formula the recovery rose to 79.1±10.4% (Table 2.9).

2.3.10   Isolated granulocytes

The final granulocyte preparation is shown in Figure 2.6

2.4   DISCUSSION

Using the two-step procedure Böyum was able to isolate 48.7% of the granulocytes from peripheral blood anticoagulated with heparin (Böyum, 1968). He estimated the yield of granulocytes by taking the number of granulocytes in the erythrocyte/granulocyte fraction as a percentage of the total number of granulocytes found in all fractions after separation. Using his method to estimate the yield (section 2.2.12.2), and the improved separation procedure described in section 2.2.13, a mean of 79.1±10.4% of the granulocytes was isolated from 6 peripheral blood samples. Employing the alternative formula G_1/G_0 x 100 (section 2.2.12.1) to

calculate the recovery of granulocytes from the same 6 samples,
the yield was 69.5±2.4%. The discrepancy in recoveries is most
likely due to Boyum's inaccuracy of assuming that the total number
of granulocytes found in the tubes after separation is equal to
the number applied to the gradients. This assumption does not
take into account losses of granulocytes other than to the mono-
nuclear layer, for example, losses to glassware. Therefore
with Boyum's formula the yields can exceed 100% (Table 2.9). It
can be seen that Boyum's formula describes the efficiency of the
separation more accurately than it reflects the yield. Neverthe-
less, with the isolation procedure described in Section 2.2.13
a significant improvement in yields of granulocytes has been
achieved.

Several modifications of Boyum's technique were made
which may have contributed to the increased yields of granulo-
cytes. Firstly, collecting the saline washings from the tubes
used for dilution of whole blood ('A') minimized losses of cells
to glassware at that point. Secondly, washing the pellet from
hypertonic IFGs restored the density of the granulocytes and
may have made the sedimentation step more efficient. Thirdly, the
use of whole AB-Rh-negative plasma rather than diluted autologous
plasma (collected after separation on IFGs) was clearly advantage-
ous in the second step. The major losses of granulocytes occurred
during this step, when sedimenting aggregates of erythrocytes
trapped the granulocytes and prevented them from moving upward
into the plasma layer (Boyum, 1968). As the yields obtained with
whole plasma (70.9%) were similar to those obtained from separat-
ing unfractionated leucocytes from whole blood-dextran mixtures

(Skoog and Beck, 1956), it was reasoned that undiluted plasma may somehow have prevented the majority of granulocytes from being sedimented with the erythrocytes. An attempt to further increase the yield of granulocytes by using autologous plasma (saved from centrifuging the blood) met with failure, however. The reason for this is not clear, but it may be due to the observed clotting of the centrifuged and then reconstituted blood. It can therefore be concluded, in agreement with Böyum, that the separation is hampered by using blood altered in any way other than by dilution.

In the second step, the increased volume, decreased density and lowered temperature (4°C) of dextran had a net effect of slowing the sedimentation by 10 min and decreasing the erythrocyte contamination. The lower erythrocyte contamination was much easier to remove by lysis. Finally, allowing sediment- ation to occur in the same tubes that were used for the IFGs rather than transferring to new tubes had the effect of eliminating another source of cell loss to glassware.

Examination of the final granulocyte preparation showed the proportion of basophils therein to be significantly greater than in peripheral blood. This discrepancy may be due to the inaccuracy of determining the true percentage of the small numbers of basophils in the blood films as opposed to the concentrated final cell preparation. Alternatively, the procedure may effectively isolate basophils, resulting in a higher percentage of basophils in the final granulocyte preparation.

The erythrocytes were lysed in the final step of

this procedure because in early tests of granulocyte function the erythrocytes were found to interfere. Granulocytes tended to clump around crenated erythrocytes during extended incubations, and this introduced inaccuracies in phagocytic and candidacidal assays where granulocyte contact with beads or yeast, respectively, was required.

Analysis of the results of granulocyte function testing showed the NBT dye reduction activity of buffy coat granulocytes in sample 3 was significantly greater than that of isolated granulocytes at 90 min. At the time of testing, monocytes comprised >15% of the buffy coat leucocytes in sample 3 and it was assumed that the difference in dye reduction was due to the monocytes which have been shown able to reduce the dye (Humbert *et al*, 1973). This was confirmed when a repeat of the test 3 weeks later showed no significant difference between buffy coat and isolated granulocytes. The monocyte count at retesting was <4%.

2.5   SUMMARY

In this section, a method is described by which granulocytes may be isolated from human peripheral blood. The yield of granulocytes using this technique (70.9%) is considerably greater than that obtained by alternative methods. The isolated cells are mainly composed of neutrophils, but the ratio of eosinophils to neutrophils is not significantly different from that of the peripheral blood. The isolated cells are comparable to buffy coat granulocytes with respect to phagocytic, candidacidal and NBT dye reduction capacity and are representative of the granulocyte population of whole blood. The purity of the final cell preparation makes it suitable for critical work.

FIG. 2.1   Blood which has been separated by centrifugation on

(top)   an IFG.   The fractions shown contain:-

        (1) diluted autologous plasma

        (2) mononuclear cells (lymphocytes, monocytes)

        (3) IFG

        (4) the pellet of granulocytes and erythrocytes

FIG. 2.2   The appearance of the blood after the erythrocyte-

(bottom)   granulocyte pellet has sediminted for 50 minutes

            in a mixture of AB-Rh-negative plasma and dextran.

            The layers shown contain:-

        (1) granulocyte-rich plasma/dextran with moderate

            erythrocyte contamination

        (2) erythrocytes
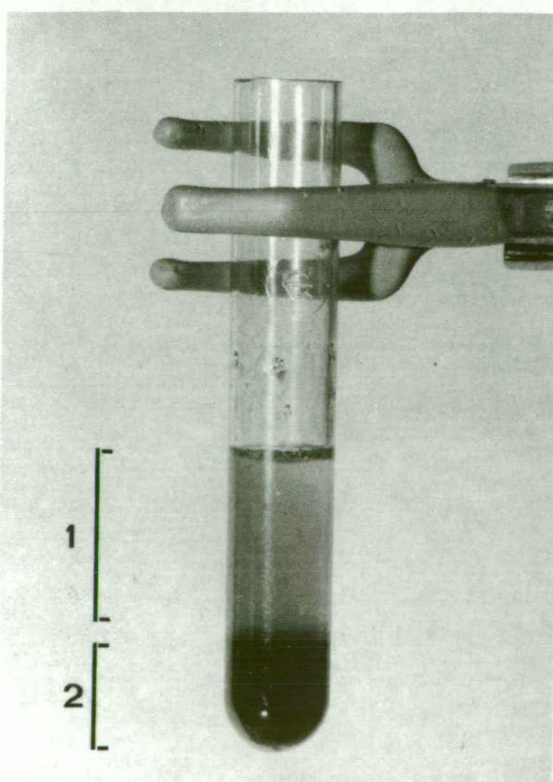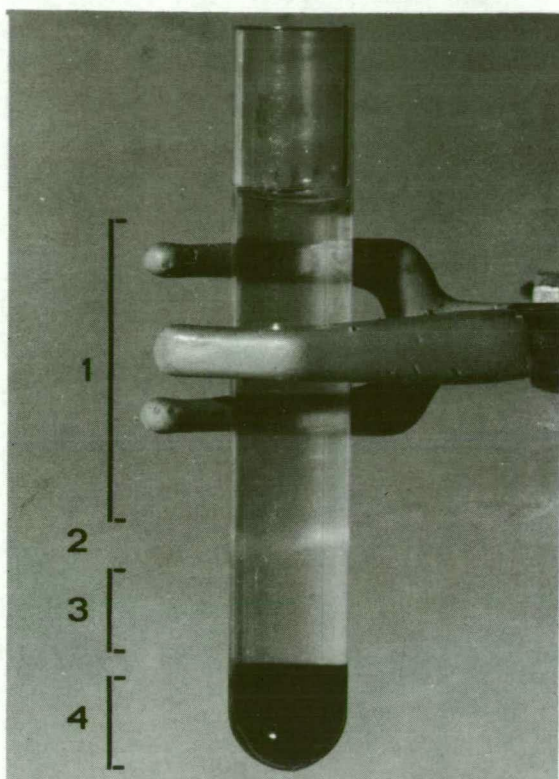
Diluted blood and washings from tubes 'A'
    layered onto IFGs in tubes 'B'

STEP 1   centrifugation 400g x 30 min x 20°C

Pellets washed twice, then plasma and dextran
    added and tubes mixed by vortex

STEP 2   sedimentation 1g x 50 min x 4°C

Plasma removed into tubes 'C', then cells
    washed once and pelleted

STEP 3   lysis of erythrocytes 10 min x 4°C

Final pellet washed once and resuspended
    to 25 x 10^6 granulocytes/ml

Fig. 2.3 The three step procedure for the isolation
         of granulocytes from peripheral blood, as
         described in detail in section 2.2.13,
         and in Dewar (1978).

FIG. 2.4 Electron micrographs of ingested *Candida albicans*

within the phagocytic vacuoles of isolated granulo-

cytes. Fusion of the granules with the membrane

of the vacuole is visible in the uppermost

micrograph (asterisks). The thick cell wall

surrounding the microorganism is evident in the

top and middle views (arrows). The bottom micro-

graph shows the remnants of a partially digested

microorganism, identifiable again by the thick

cell wall (arrow). Magnifications : top,

19,300x, middle 11,400x, bottom 14,300x.
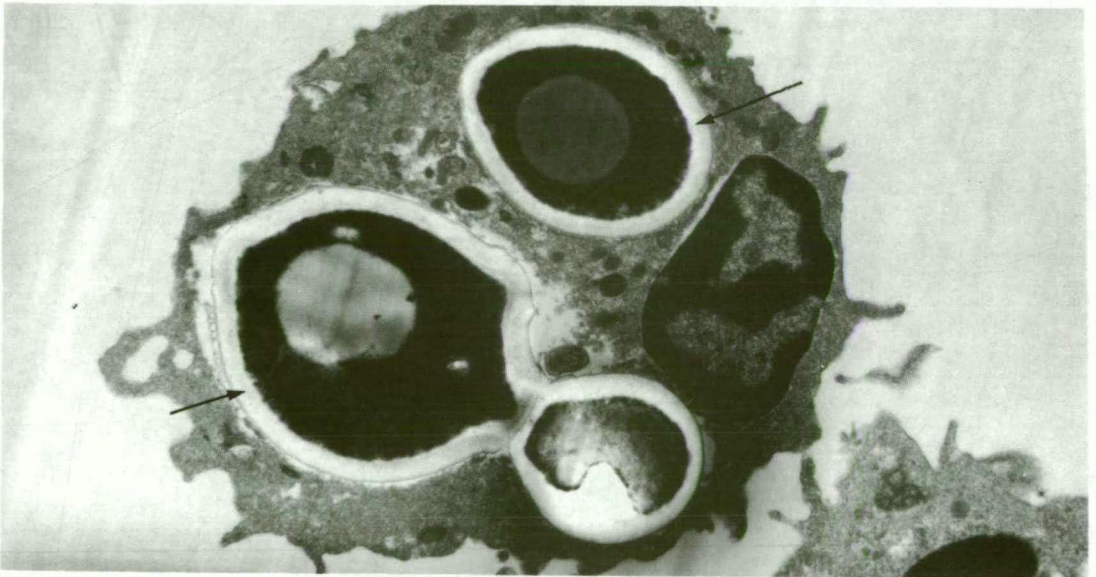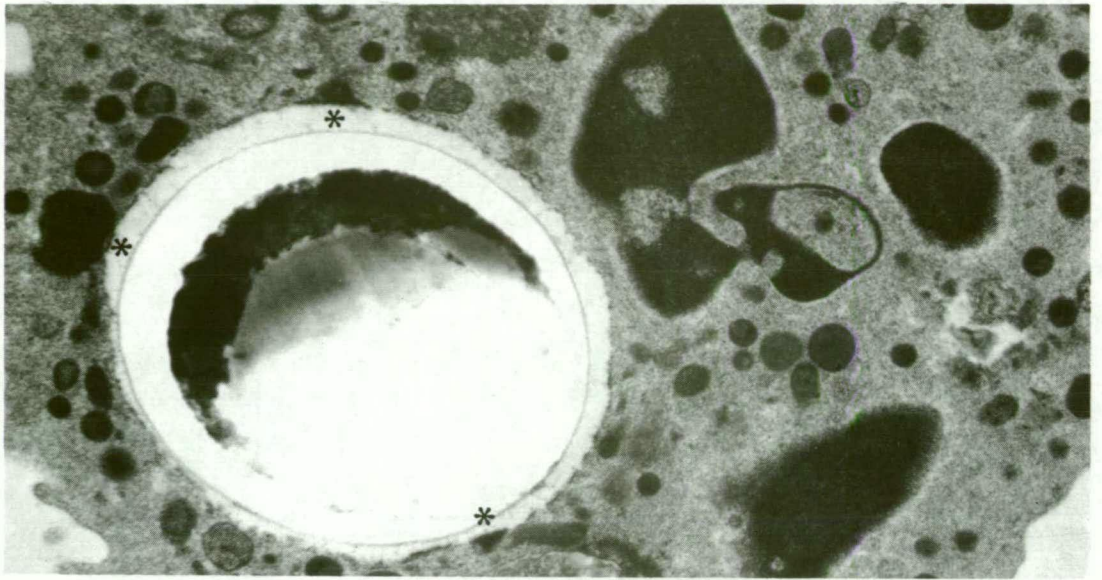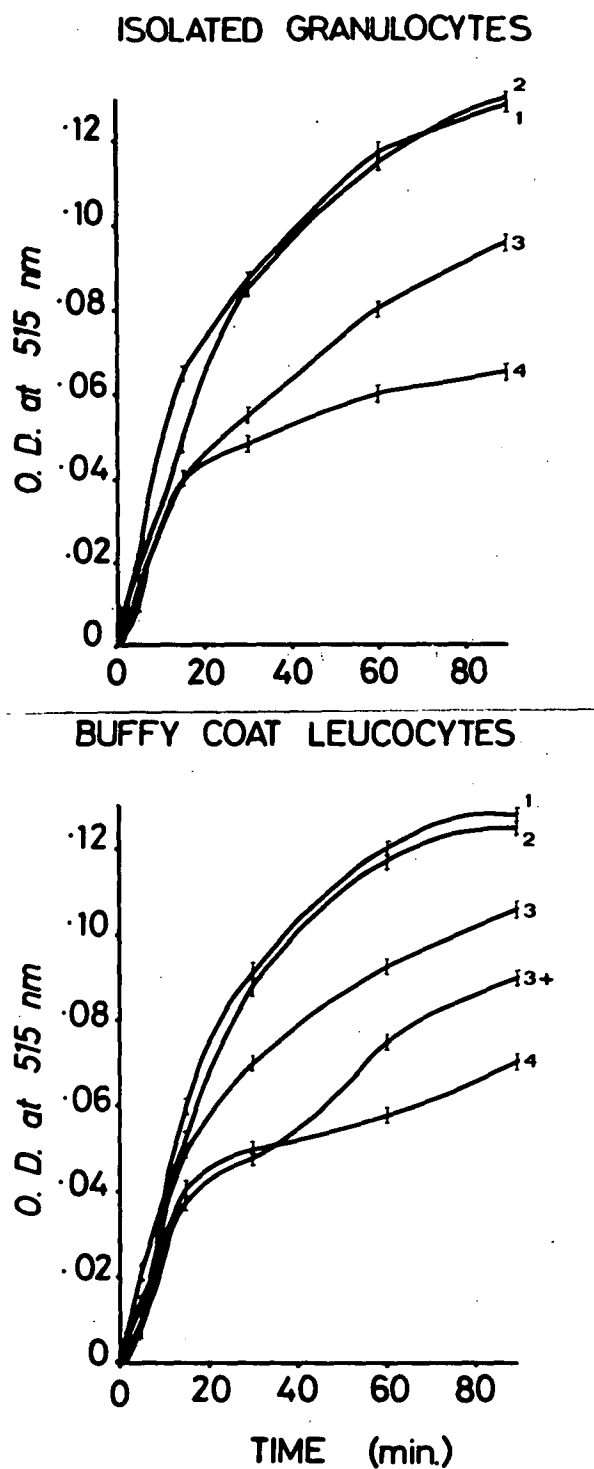
FIG. 2.5. The NBT dye reduction capacity of isolated (top) versus buffy coat (bottom) leucocytes. Sample 3 † was a repeat (3 weeks later) of sample 3. Each point represents the $\bar{X}$ ± SEM of 3 determinations.

FIG. 2.6   The final isolated granulocyte preparation (two

views).   Magnification top 1,250x, bottom

2,100x.
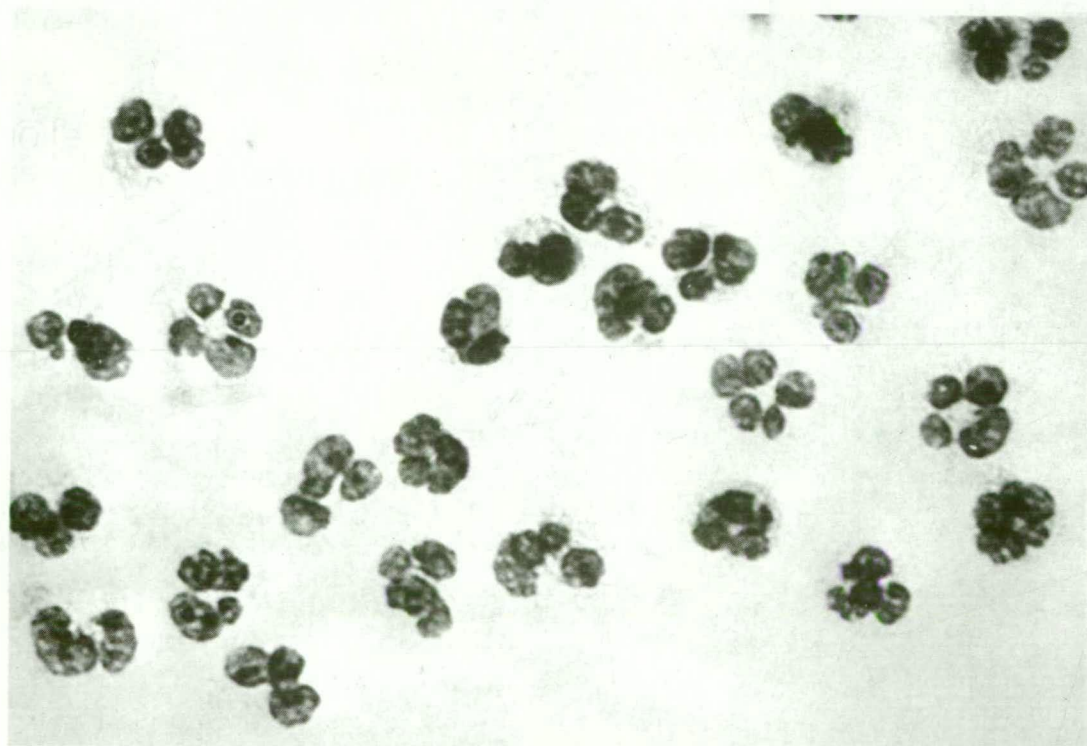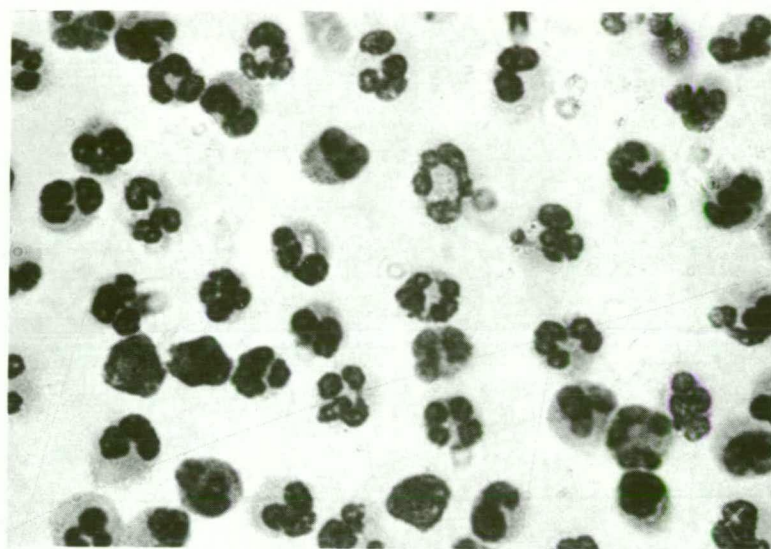
TABLE 2.1   Volume of plasma used in the dextran sedimentation step

Volume (ml)	Number of granulocytes ($\times 10^6$) recovered from IFGs				$\bar{X} \pm$ SEM
	Experiment 1		Experiment 2		
1.0 [a]	2.4	2.3	2.4	2.0	2.3 ± 0.1
1.0 [b]	2.8	2.5	2.1	2.6	2.5 ± 0.1
2.0 [b]	3.5	3.1	3.0	3.6	3.3 ± 0.1
3.0 [b]	3.7	3.6	3.0	3.1	3.4 ± 0.2

[a] 1.0 ml diluted autologous plasma

[b] 1.0, 2.0, or 3.0 ml AB-Rh-negative plasma

TABLE 2.2   Autologous versus AB-Rh-negative plasma

	Autologous		AB-Rh-negative	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Granulocytes	2.9	2.2	3.4	3.2
isolated (x 10^6)	2.6	2.4	3.5	3.6
per IFG				
$\bar{X} \pm$ SEM	2.5 $\pm$ 0.1		3.4 $\pm$ 0.1*	

* The difference between the two means is statistically significant
  (p < 0.01)

TABLE 2.3   Volume of dextran used in step 2

Volume (ml)	Number of granulocytes ($\times 10^6$) isolated per IFG				$\overline{X} \pm$ SEM
	Experiment 1		Experiment 2		
0.4	0.2	0.9	1.5	1.2	1.0 ± 0.3 [a]
1.0	3.4	4.5	2.0	1.5	2.9 ± 0.7 [b]
3.0	3.5	3.9	2.5	2.3	3.1 ± 0.4 [c]
4.0	3.6	3.4	2.3	2.8	3.0 ± 0.3 [d]

Values b), c), d) are significantly greater than a), with $p < 0.01$. However, values c) and d) are not significantly different from each other.

TABLE 2.4   Concentration of dextran used in step 2

Concentration (g/ml)	Number of granulocytes ($\times 10^6$) isolated per IFG					$\bar{X} \pm$ SEM
	Experiment 1		Experiment 2			
0.035	2.4	2.7	2.3	2.1	2.5	2.4 ± 0.1 [a]
0.040	2.7	2.8	2.5	2.3	2.6	2.6 ± 0.1
0.045	2.8	2.7	2.8	2.5	2.3	2.6 ± 0.1

[a]
   Not significantly lower than the value 2.6 ± 0.1.

TABLE 2.5   Erythrocyte contamination of the granulocyte fraction

(after step 2) with 0.4 or 3.0 ml dextran

Volume (ml)	Cells x 10^6 per IFG		Ratio
	Erythrocytes	Granulocytes	Erythrocytes: granulocytes
0.4 [a]	175.0	5.4	32:1
0.4 [b]	680.0	10.2	66:1
3.0 [a]	155.0	14.9	10:1
3.0 [b]	280.0	21.6	13:1

[a] Experiment 1   [b] Experiment 2

TABLE 2.6   Phagocytic assay.   Percent granulocytes containing 0, 1 to 5, 6 to 10 or >10

beads per cell after 20 minutes

| Sample | Number of latex beads per granulocyte | | | | | | | |
| | 0 | | 1 - 5 | | 6 - 10 | | 10 | |
	Buffy coat	Isolated cells	Buffy coat	Isolated cells	Buffy coat	Isolated cells	Buffy coat	Isolated cells
1	32.2	23.4	2.9	5.3	5.1	15.0	59.8	56.3
2	22.6	19.8	4.4	4.9	7.3	6.3	65.7	69.0
3	29.8	28.5	4.5	5.9	4.7	5.7	61.0	59.9
4	20.4	27.6	5.2	4.3	5.2	5.9	69.2	62.2
$\bar{X}$ ± SEM	26.3±2.8	24.8±2.0	4.3±0.5	5.1±0.3	5.6±0.6	8.2±2.3	63.9±2.2	61.9±2.7
P value	N.S		N.S		N.S		N.S	

Symbols

P value = probability value for each pair of means

N.S.   = the difference between the two means is not statistically significant

TABLE 2.7   Candidacidal assay.   Percent *Candida* organisms

killed after 60 minutes

Sample	Buffy coat leucocytes	Isolated granulocytes
5	27.5	29.4
6	19.7	16.5
7	31.2	38.2
8	45.4	42.4
$\bar{X} \pm$ SEM	31.0±5.4	31.6±5.7
P value		N.S.

Symbols

P value = probability value between the means

N.S.   = the difference between the means is not

statistically significant.

TABLE 2.8   Differential counts of whole blood and isolated granulocytes

and percent recoveries of granulocytes

Sample	% Granulocytes in whole blood (A)	% Neutrophils in A	% Neutrophils in isolated granulocytes	% Eosinophils in A	% Eosinophils in isolated granulocytes	% Basophils in A	% Basophils in isolated granulocytes	% Recovery of Granulocytes
1	48.8	98.1	97.8	1.9	1.5	0	0.4	66.1
2	55.8	94.4	94.9	5.6	4.2	0	0.9	70.2
3	32.0	95.5	93.9	4.4	3.2	0.1	2.7	63.0
4	41.6	94.0	93.0	6.0	5.1	0	1.9	70.3
5	47.2	93.4	94.4	6.5	5.2	0.1	0.4	80.2
6	41.1	94.5	93.4	5.2	6.0	0.3	0.5	67.2
7	52.3	95.0	93.5	4.1	4.3	0.9	1.8	77.9
8	35.3	99.1	98.3	0.7	1.2	0.2	0.5	72.2
$\bar{x}$ ± SEM	44.3±2.9	95.5±0.7	94.9±0.7	4.3±0.7	3.8±0.6	0.2±0.1	1.1±0.3	70.9±2.0
P value		0.10<P<0.20		0.10<P<0.20		P<0.01		

Symbols for Table 2.8

P value = probability value between the two means. Only the basophils are significantly
elevated in the final preparation compared to whole blood.

Symbols for Table 2.9

G_0 = total number of granulocytes ($\times 10^6$) in the blood

G_1 = total number of granulocytes ($\times 10^6$) isolated

g_2 = percent granulocytes in the bottom fraction

g_0 = percent granulocytes in whole blood

m_0 = percent mononuclear cells in whole blood

m_2 = percent mononuclear cells in the bottom fraction

m_1 = percent mononuclear cells in the top fraction

a) using the quotient $G_1/G_0 \times 100$ (section 2.2.12.1)

b) using the formula from section 2.2.12.2:-

$$100 \times \frac{g_2}{g_0} \times \frac{m_0 - m_2}{m_1 - m_2}$$

TABLE 2.9   Calculating recoveries of granulocytes using the formulae
given in sections 2.2.12.1 and 2.2.12.2

Sample	G_0	G_1	g_2	g_o	m_o	m_2	m_1	percent[a] recovery	percent[b] recovery
1	36.9	24.4	98.8	48.8	40.0	1.2	95.0	66.1	83.0
2	97.4	68.4	99.0	55.8	33.6	1.0	97.0	70.2	60.3
3	28.4	17.9	99.6	32.0	40.3	0.4	98.0	63.0	127.6
4	60.7	42.7	99.3	41.6	25.7	0.7	92.0	70.3	64.4
5	43.0	34.5	98.4	47.2	35.6	1.6	93.0	80.2	77.1
6	25.9	17.4	98.1	41.1	26.8	1.9	98.0	67.2	62.1
$\bar{X} \pm SEM$								69.5 ± 2.4	79.1 ± 10.4

SECTION 3

ASSAY OF PLASMA MEMBRANE OXIDASE

ACTIVITY

3.1   <u>INTRODUCTION</u>

The aim of these experiments was to devise a method for studying the plasma membrane oxidase of isolated granulocytes. NBT dye reduction was chosen as a suitable method for examining the activity of the oxidase via the production of superoxide radical (Baehner *et al*, 1976 ; see also Babior, Curnutte and Kipnes, 1975).

NBT (nitroblue tetrazolium) is a yellow, water soluble quaternary ammonium salt (Rust, 1955) which is transformed to a highly coloured (dark blue) water-insoluble dinitroformazan once reduced. Its structure and the structure of the dinitroformazan are shown in Figure 3.1. The low redox potential of -0.05 volts for NBT (Hooper, 1969) makes it a useful indicator for biological work (Rust, 1955). There have been several reviews of the chemical nature and use of the tetrazolium salts, including NBT, as biological redox indicators (Rust, 1955; Hooper, 1969; Eadie *et al*, 1970).

Baehner and Nathan (1967) first described the reduction of NBT by isolated granulocytes and pointed out that the reduction was enhanced during phagocytosis. In addition, they noticed that the granulocytes from patients with chronic granulomatous disease (CGD) reduced dye very slowly, even when phagocytosing. They took advantage of this fact in designing a qualitative screening test for CGD, and later, a quantitative test which was sensitive enough to pick up the carrier state (Baehner and Nathan, 1968). About this time also, Park *et al* (1968) suggested that because phagocytosis enhanced NBT reduction, the presence of infection should be detectable with the NBT test if the granulocytes were ingesting pyogenic bacteria. However, intense study of the possible clinical useful-

ness of the NBT test as an indicator of infection showed that

there were many false positives and false negatives with the

test, and therefore this application of the NBT test has fallen

into disrepute. The pitfalls of using the NBT test in this

way have been reviewed by Segal (1974) and Lace *et al* (1975).

Fortunately the controversy regarding the use of

NBT reduction as a guage of the presence of infection has not

detracted from the well-established value of the test in

diagnosing CGD, and in studying the oxidase responsible for

superoxide production. Among the many workers who have used NBT

reduction to study the function of the granulocyte are Johnston

et al, 1969; Nathan *et al*, 1969; Gifford and Malawista, 1970;

Segal and Levi, 1973; Stossel, 1973; McCall *et al*, 1974;

Baehner *et al*, 1975 and 1976; Briggs *et al*, 1975; Segal and

Peters, 1976 and 1977.

For these studies of the plasma membrane oxidase,

non-phagocytic granulocytes have been used because in the

absence of phagocytosis NBT dye cannot enter the granulocytes

(Nathan *et al*, 1969; Segal and Levi, 1973; Briggs *et al*,

1975)[1]. Therefore dye reduction will occur at the plasma membrane

in non-phagocytic granulocytes, as Briggs *et al* (1975) have shown.

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[1]
    In phagocytosing granulocytes, NBT dye enters the cell along
    with the particles being ingested and reduction occurs intra-
    cellularly as well, in the phagocytic vacuoles. The superoxide
    source in this case can be the granule or plasma membrane
    oxidase (see Nathan *et al*, 1969; Johnston *et al*, 1969; Segal
    and Levi, 1973; and Appendix I).

With this in mind, an assay designed to measure the initial rate of superoxide production (NBT reduction) from the plasma membrane oxidase of isolated granulocytes was developed, and is detailed in this section.

3.2 METHODS

3.2.1 Physiological saline solutions

3.2.1.1 HEPES physiological saline (HSS)

HSS was prepared daily for experiments, as described previously (Dewar *et al*, 1976, Appendix II). The final composition of HSS was 136.9 mM Na^+, 6.0 mM K^+, 1.2 mM Mg^{+2}, 2.4 mM Ca^{+2}, 150.1 mM Cl^-, 11.9 mM glucose and 9.0 mM HEPES, with a pH of 7.4.

3.2.1.2 Ca^{+2} and Mg^{+2} free HEPES physiological saline (CMFH)

CMFH was also prepared daily as before (Dewar *et al*, 1976, Appendix II). The final composition of CMFH was 142.9 mM Na^+, 6.0 mM K^+, 148.9 mM Cl^-, 11.9 mM glucose and 9.0 mM HEPES with a pH of 7.4.

3.2.2 Granulocyte monolayer preparations

Granulocytes were isolated as described in section 2.2.13 and kept at $4^{\circ}C$ in CMFH at 25 x 10^6/ml until used [2].

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[2] The cells used in these and all other experiments described in this thesis were as fresh as possible, that is, they were used on the same day as they were isolated from the blood. In no experiments were the granulocytes aged more than 6 (and in most cases they were 1-2) hours post-isolation. Viability as assessed by Trypan Blue or Eosin Y dye exclusion was always ≥ 98%, even in granulocytes at 6 hours post-isolation.

To prepare the monolayers 200 μl of HSS was placed onto a 22 mm square glass coverslip seated in a round plastic Petri dish or its lid. Then 50 μl of the granulocyte suspension (1.3 x 10^6 cells) was injected into the HSS and gently mixed with the microlitre pipette (Fig. 3.2). The dishes were then incubated 10 min at 37°C in an humidified incubator (Clemco, Australia) to form the monolayers.

3.2.3   Nitroblue tetrazolium solution (NBT)

NBT was prepared as a saturated solution by adding 100 mg of NBT to 50 ml of HSS. [The actual concentration of NBT in solution was 1.05 mg/ml, as determined by Stossel's procedure (Stossel, 1973a)]. The NBT solution was prepared at least 24 hours in advance of an experiment to ensure that dissolution was complete. Before use the NBT solution was centrifuged and then filtered (Millipore, 0.45 μm) to ensure clarity. For tests, 2 volumes of this solution was mixed with 1 volume of KCN, 0.010 M in HSS, giving a final KCN concentration at testing of 1.5 mM.

3.2.4   Experiments to establish the initial rate of NBT reduction

3.2.4.1   Time course of NBT reduction by monolayers

Granulocyte monolayers were prepared as described in section 3.2.2. To the cells in HSS was added 200 μl of NBT test solution (section 3.2.3). The monolayers were then incubated at 37°C for fixed time periods between 1 and 60 min. At the allotted time the reduction of NBT was terminated by immersing the monolayers rapidly several times in a beaker of CMFH at 4°C. The last drop of liquid was removed and the monolayers dried in air. The NBT reduced by each monolayer was quantitated by

extracting each monolayer into 0.8 ml pyridine (10 min x 100°C),

and then reading the OD of reduced dye at 515 nm (Baehner and

Nathan, 1968).

3.2.4.2   Effects of granulocyte concentration on NBT reduction

Monolayers of granulocytes were prepared which con-

tained 0.25 - 3.8 x 10^6 cells per dish of 200 µl. Then by using

the assay described in section 3.2.4.1 with a 30 min incubation

period, the effect of cell concentration on NBT dye reduction

was determined.

3.2.4.3   Effect of NBT concentration on reduction

Solutions of varying NBT concentration were prepared

by diluting the NBT solution (section 3.2.3) with HSS. The NBT

concentrations tested were 0.08, 0.15, 0.23, 0.30, and 0.38 mM.

The assay of NBT reduction as described in section 3.2.4.1 was

performed with these solutions and a fixed time period of 5 min

incubation to determine the effect of NBT concentration on NBT

reduction. The data from these experiments was plotted as

the rate of NBT reduction versus the concentration of NBT.

3.2.4.4   The kinetics of the initial rate of NBT reduction

The data from the experiments on the effect of NBT

concentration (section 3.2.4.3) was used to construct double

reciprocal plots (1/rate of reduction versus 1/NBT concentration).

From these plots an 'apparent K_m' and V_{max} for the initial rate

of NBT reduction by intact granulocytes was determined

(Appendix III).

3.3   RESULTS

3.3.1   NBT dye reduction by granulocyte monolayers

          Light microscopical examination of unstained monolayer

preparations which had been exposed to NBT dye for 5 min (as in

section 3.2.4.1) showed a ring-like appearance of reduced dye at

the edges of the cells (Fig. 3.3).

3.3.2   Experiments to establish the initial rate of NBT reduction

3.3.2.1   Time course of NBT reduction

          From the plot of the reduction of NBT with time (Fig.

3.4), it was apparent that the rate of reduction was maximal, and

linear (with respect to time) for the first 5 min at least.

After the initial 5 min, the reduction of NBT showed a slower

increase with time until the capacity for reduction was complete,

at approximately 30 min.

3.3.2.2   The effect of granulocyte concentration on NBT reduction

          As shown in Fig. 3.5, the reduction of NBT was

directly proportional to the number of granulocytes (= the cell

concentration) on the monolayer.

3.3.2.3   The effect of NBT concentration on reduction

          Sixteen plots of the rate of reduction of NBT dye

versus the concentration of NBT were hyperbolic. Two of the

16 plots are shown in Fig. 3.6. These curves showed that the

rate of reduction of NBT dye was proportional to the dye concen-

tration, and that the rate of reduction reached a maximum at the

highest concentrations of NBT tested. With all 16 plots it

was apparent that there was very little difference in the rate

of reduction with the two highest concentrations of NBT tested

(0.30 and 0.38 mM NBT).

3.3.2.4 The kinetics of the initial rate of NBT dye reduction

The 16 hyperbolic plots of the rate of reduction versus the NBT concentration (section 3.3.2.3) were transformed into straight lines, or Lineweaver-Burk plots, by plotting 1/reduction rate against 1/NBT concentration (Appendix III). Two of these plots are illustrated in Fig. 3.7. For each Lineweaver-Burk plot, the y-intercept ($1/V_{max}$) and x-intercept (1/apparent K_m) values were used to determine the V_{max} and apparent K_m, respectively, for NBT reduction by intact granulocytes (Appendix III). As indicated in Table 3.1, the $\bar{X} \pm$ SEM values for these two kinetic parameters were:-

0.102 ± 0.011 (apparent K_m, in units of mM NBT)

0.081 ± 0.003 (V_{max}, in units of $OD_{515}/1.3 \times 10^6$ granulocytes/5 min)

3.4 DISCUSSION

In this section a method for studying the plasma membrane oxidase of isolated granulocytes has been presented. To ensure that plasma membrane superoxide production was being detected, non-phagocytic granulocytes were used, and superoxide production was quantitated by NBT reduction (Baehner *et al*, 1976). NBT cannot cross the granulocyte plasma membrane (Nathan *et al*, 1969; Segal and Levi, 1973; Briggs *et al*, 1975) and therefore dye reduction occurs at the plasma membrane in non-phagocytic granulocytes[1]. Once reduced to formazan, NBT is lipid-insoluble and therefore remains *in situ* at the site of reduction (Nachlas *et al*, 1957). Briggs *et al* (1975) were able to exploit this

property of NBT to demonstrate with electron microscopy that NBT

is reduced at the plasma membrane of non-phagocytic granulocytes.

The light microscopical studies presented in this section (and the

electron microscopical studies of section 9.3) confirm that dye

reduction occurs at the plasma membrane of these cells. It is

extremely unlikely that the NBT reduced at the cell surface of non-

phagocytic granulocytes is reduced by granule-produced superoxide

which has diffused throughout the cytoplasm because superoxide is

a highly reactive radical which would be consumed within the

cytoplasm[3] very near to its site of production (Fridovich,

1978; see also Goldstein *et al*, 1977).

Thus with NBT dye reduction as an index of plasma

membrane oxidase activity, the necessary conditions for measuring

the initial rate of superoxide production (NBT reduction) could

be established. There was a need to develop such an assay of NBT

reduction by non-phagocytic granulocytes. In almost all previous

studies, quantitation of granulocyte NBT reduction was performed

using a modification of the original method of Baehner and Nathan

(1968). Unfortunately, there were two problems with this assay.

Firstly, it was not firmly established that the concentration of

NBT used for the test was saturating, and secondly a relatively

long incubation period (15 min) with the NBT was used. This meant

that towards the end of the incubation period, the supply of NBT

dye was becoming exhausted, and therefore there was a concomitant

decrease in the rate of reduction. Hence with such an assay, only

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[3] For example, by dismutation via cytoplasmic superoxide
dismutase to produce H_2O_2 and O_2 (see section 1.4).

the granulocyte's *capacity* for reduction was being measured and the assay could not distinguish between variations in reduction *rate*, or *capacity*, or both. Thus if a particular drug treatment were to alter the rate (but not the capacity) of reduction, then only at high doses of this drug might a significant effect on NBT reduction have been detected by the assay of Baehner and Nathan.

In developing a more sensitive assay of NBT reduction by intact, non-phagocytic granulocytes, the papers of Weisman and Korn (1967) and Michell *et al* (1969) were consulted. These authors developed sensitive assays of the optimum (or initial) rate of ingestion by intact phagocytes. Following their methods, the reduction of NBT by intact granulocytes was treated as if it were a single enzymatic reaction (see Appendix III). It was found that (i) the initial rate of reduction was independent of the concentration of NBT used. That is, the reduction of dye exhibited first order and saturation kinetics with respect to the NBT concentration (Fig. 3.6), and therefore it was valid to consider the granulocyte as containing a finite number of enzyme (oxidase) molecules capable of being saturated by substrate (NBT). As well, (ii) the initial rate of reduction was constant with time as long as the incubation period with dye was $\leq$ 5 min (Fig. 3.4). Finally, (iii) reduction was found to be a linear function of the granulocyte (= enzyme) concentration (Fig. 3.5).

To confirm that this was a suitable assay of the initial rate of NBT reduction, irrespective of the granulocyte donor, the data from 16 individual plots of the rate of reduction versus the NBT concentration was analyzed. As these

particular plots yielded curved lines (Fig. 3.6), the data was hard to compare between donors. Therefore the same data was plotted as Lineweaver-Burk (or double reciprocal) plots (Appendix III). With these plots of 1/rate of reduction versus 1/NBT concentration, straight lines were obtained. This facilitated comparisons between the donors as to the values for the 'apparent K_m' (the concentration of NBT at which reduction proceeded half maximally) and V_{max} (the maximal velocity) of NBT reduction (Appendix III). There was good agreement in these values from the 16 normal donors (Table 3.1) indicating that this assay of the initial rate of NBT reduction was reproducible with human granulocytes collected from different donors.

SUMMARY

In this section, an assay designed to measure the initial rate of NBT reduction (O_2^- production) from the plasma membrane oxidase of isolated granulcoytes has been presented. The two kinetic parameters, apparent K_m and V_{max}, were determined for the reduction of dye by granulocytes isolated from 16 individuals. As these values showed good agreement in the 16 samples, it was concluded that this assay of the initial rate of NBT reduction was reproducible with human granulocytes collected from different donors. The assay procedure is shown diagrammatically in Figure 3.8.
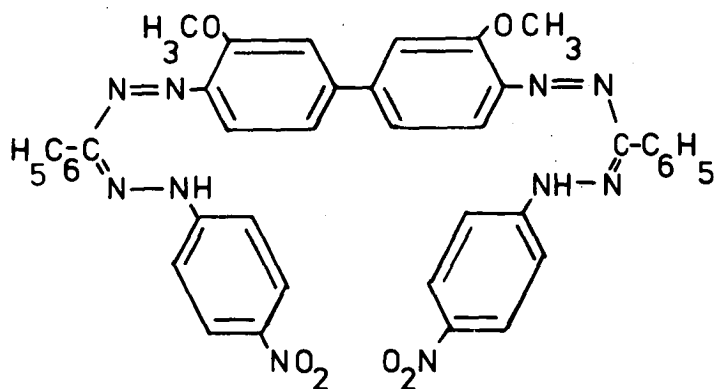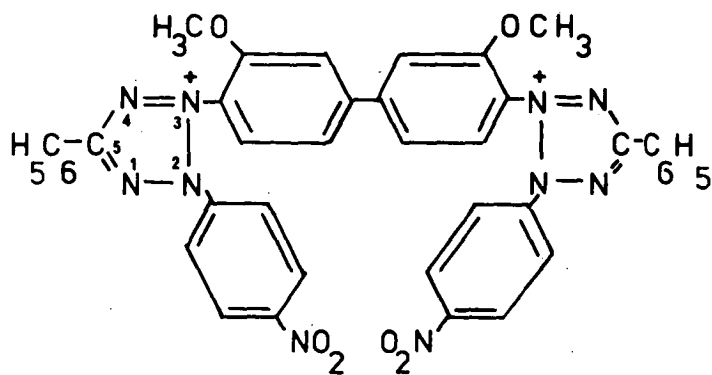
FIG. 3.1. The structures of NBT (top) and its dinitroformazan (bottom), taken from Nachlas et al (1957). Since tautomerism can occur, the structure of the tetrazole (the numbered ring, above left) is tautomeric with a form having double bonds between N-2 and N-1, and between C-5 and N-4, and with a positive charge at N-2 rather than N-3.

FIG. 3.2   Injecting the granulocyte suspension into HSS

on the glass coverslips.   (For the purposes of

this photograph the granulocytes were at a much

higher concentration than the standard

25×10^6 cells/ml.)

Magnification ~ 1.4 x.
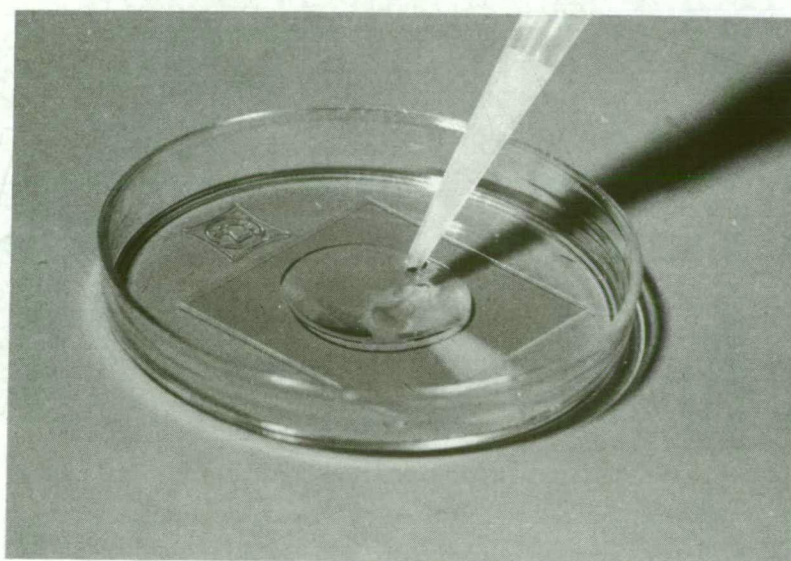
FIG. 3.3 NBT dye reduction at the cell surface of isolated

granulocyte.   Granulocyte monolayers were incubated

with NBT dye for 5 min, using the procedure outlined

in Fig. 3.8.   These granulocytes were then fixed in

methanol, stained briefly with May-Grünwald (2 min)

and Giemsa (4 min) stains, and mounted with Depex

(Dacie and Lewis, 1968, p58).   Dye reduction is evident

as a ring at the periphery of the cells and as a

heavy deposit where two granulocytes touch (top and

middle photographs).   The eosinophil granules are

prominent in the middle photograph.   The pictures

were taken with a Leitz Orthoplan light microscope

using Kodak Plus-X film and a yellow and green filter

combination.   May-Grünwald/Giemsa stain did not stain

the plasma membrane of these cells (cf. Fig. 2.6).

Magnification 2,800 x.
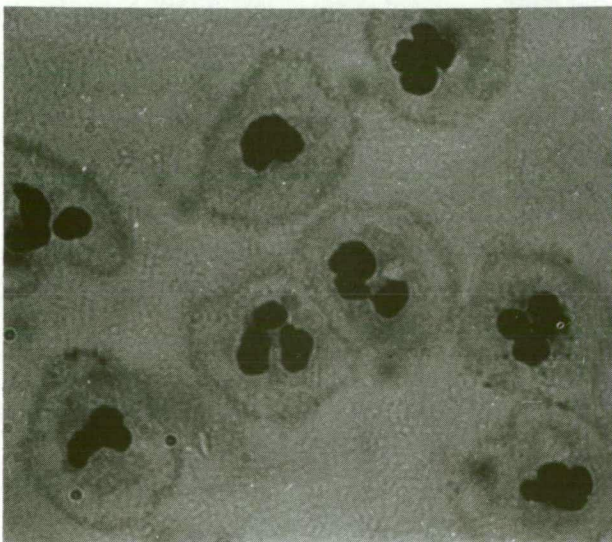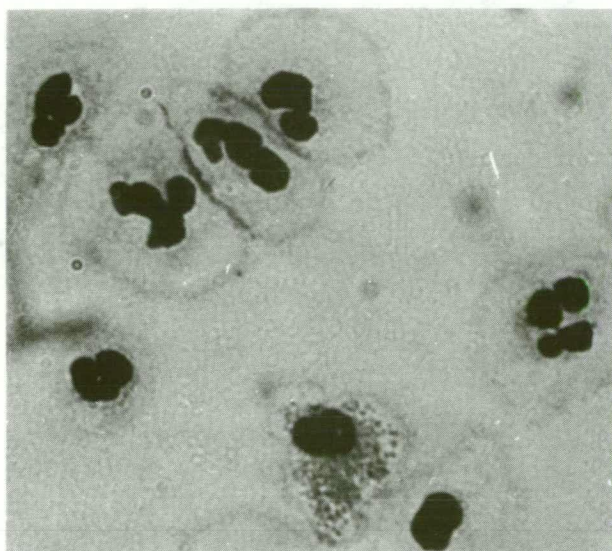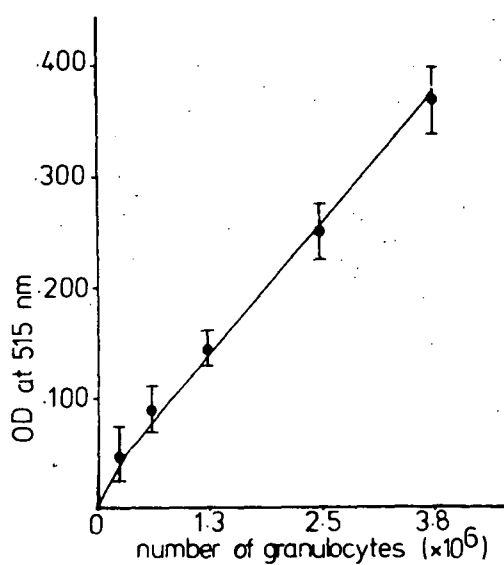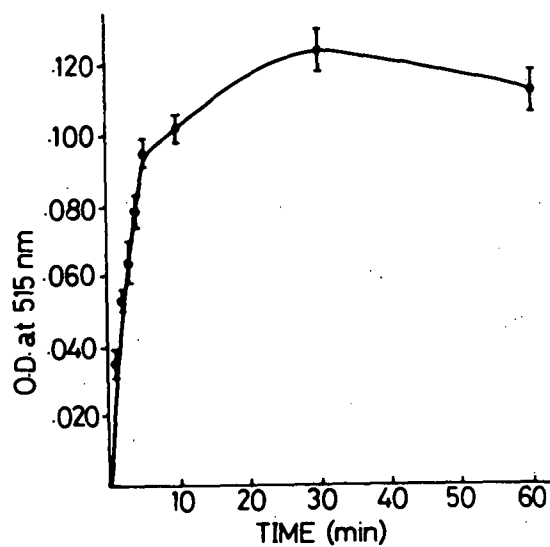
FIG. 3.4.   The plot of the reduction of NBT with time (top).
            Points shown are the $\bar{X}$ ± SEM of 3 determinations.

FIG. 3.5.   The reduction of NBT dye as a function of the number
            of granulocytes (bottom). Each point gives the
            $\bar{X}$ ± SEM of 3 determinations.
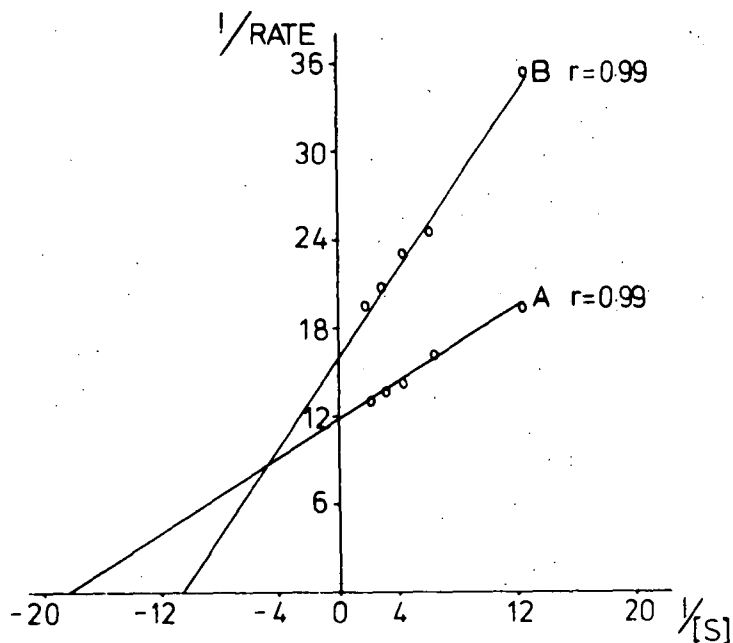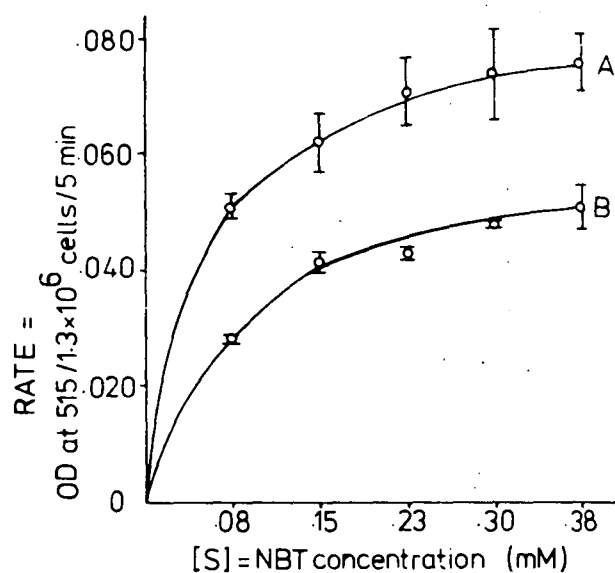
FIG. 3.6. Two plots (from 2 of 16 experiments) of the rate of NBT reduction versus the concentration of NBT dye (top). Each point is the $\bar{X}$ ± SEM of 3 determinations.

FIG. 3.7. Replotting the data from FIG. 3.6 (above) as Lineweaver-Burk plots (^{1}/reduction rate versus ^{1}/NBT concentration) to obtain straight lines. Line A corresponds to sample 8, and line B to sample 3 in Table 3.1 (see also Appendix III).

granulocyte monolayers prepared
on glass coverslips (section 3.2.2)

NBT test solution added (section 3.2.3)

dye reduction allowed to proceed for 5 min

reduction terminated by repeated immersion
of monolayers in cold CMFH

monolayers allowed to dry

reduced dye on each monolayer extracted into
pyridine at 100°C

OD of reduced dye on each monolayer determined
at 515 nm

Fig. 3.8 The assay of the initial rate of
        NBT reduction by intact, non-phagocytic
        granulocytes.

TABLE 3.1   Kinetic parameters for the reduction of
            NBT by intact granulocytes*

Sample	K_m [a]	V_{max} [b]	r
1	.083	.078	.99
2	.054	.075	.99
3[c]	.094	.063	.99
4	.068	.052	.93
5	.071	.075	.91
6	.050	.085	.90
7	.118	.093	.94
8[d]	.054	.086	.99
9	.103	.083	.94
10	.185	.102	.99
11	.105	.087	.92
12	.192	.069	.91
13	.102	.081	.95
14	.146	.093	.93
15	.127	.089	.92
16	.087	.090	.81
$\bar{X} \pm$ SEM	.102±.011	.081±.003	.94±.01

a)   apparent K_m for NBT, in mM concentration units

b) V_{max} in units of $OD_{515}/1.3 \times 10^6$ granulocytes/5 min

c)   corresponds to line B in Fig. 3.7

d)   corresponds to line A in Fig. 3.7

* See Appendix III

r = correlation coefficient

SECTION   4

MICROFILAMENT AND MICROTUBULE DISRUPTION

AND OXIDASE ACTIVITY

(NON-PHAGOCYTIC GRANULOCYTES)

4.1   INTRODUCTION

In this section, the possible role of cytoplasmic microtubules and microfilaments in the control of the activity of the plasma membrane oxidase was examined. For these studies, the granulocytes were incubated with drugs at doses known to disrupt microtubules or microfilaments (VBL, VCR, colchicine, and cytochalasin B, respectively; see Appendix IV). Drug incubations were followed by measurement of plasma membrane oxidase activity, using the procedure outlined in Section 3 and illustrated in Figure 3.8.

4.2   METHODS

4.2.1   Preparation of the drugs in HSS

The drugs (VBL, VCR, colch) were prepared as stock solutions of 1 mM in water and then dilutions of these stock solutions were used in the preparation of HSS. The only exception was cyto B, which was prepared as a stock solution in DMSO. However all subsequent dilutions of the drugs (including cyto B) were made with water.

Successive dilutions as outlined below were made to obtain concentrations of the drugs which, when used to prepare HSS, would result in a final drug concentration at testing of 1, 0.1, 0.01, or 0.001 µM. These doses have been shown to disrupt microfilaments (cyto B) or microtubules (colch, VBL, VCR) in vitro (see Appendix IV).

$$\text{stock solution (1 mM)} \quad = \quad 1000 \quad \mu M \quad (a)$$

$$\text{0.1 ml (a) + 2.9 ml water} \quad = \quad 33 \quad \mu M \quad (b)$$

$$\text{0.1 ml (b) + 0.9 ml water} \quad = \quad 3 \quad \mu M \quad (c)$$

$$\text{0.1 ml (c) + 0.9 ml water} \quad = \quad 0.3 \quad \mu M \quad (d)$$

$$\text{0.1 ml (d) + 0.9 ml water} \quad = \quad 0.03 \quad \mu M \quad (e)$$

To obtain HSS with 1 μM drug, 1 volume of dilution (b) was used to replace 1 volume of the water used in preparing HSS (Appendix II). HSS containing drug at 0.1 μM, 0.01 μM, or 0.001 μM was prepared in the same way, using drug dilutions (c), (d), and (e) respectively.

4.2.2   <u>DMSO control for cyto B-treated granulocytes</u>

Cytochalasin B was dissolved in DMSO at 1 mM to give stock solution (a), in section 4.2.1. Therefore in those experiments where the effects of cytochalasin B were being studied, control HSS containing DMSO at the appropriate concentration had to be prepared. DMSO was used as the control stock solution, and using the dilution format outlined in section 4.2.1, the DMSO concentrations were:-

$$\text{100 \% v/v} \quad = \quad (a)$$

$$\text{(a) x 1/30 = 3.3 \% v/v} \quad = \quad (b)$$

$$\text{(b) x 1/10 = 0.33 \% v/v} \quad = \quad (c)$$

$$\text{(c) x 1/10 = 0.03 \% v/v} \quad = \quad (d)$$

$$\text{(d) x 1/10 = 0.003 \% v/v} \quad = \quad (e)$$

Dilutions (b), (c), (d) and (e) were then used in the preparation of control HSS for the cytochalasin B experiments. Following the method discussed in section 4.2.1, the final DMSO

content (% v/v) during incubation of the granulocytes was

therefore:-

for 1 µM cytochalasin B or its control   ...   0.25   % v/v DMSO

for 0.1 µM   "   "   "   ...   0.03   % v/v DMSO

for 0.01 µM   "   "   "   ...   0.003   % v/v DMSO

for 0.001 µM   "   "   "   ...   0.0003 % v/v DMSO

4.2.3   The initial rate of NBT reduction by drug-treated granulocytes

4.2.3.1   The effect of drug dose on reduction

Monolayers of granulocytes were prepared as described
in section 3.2.2. Drug-treated granulocytes were exposed to the
drugs on the monolayers, while control cells were incubated in
water or DMSO. After a 10 min exposure to the agents or controls,
the granulocyte monolayers were assayed for the initial rate of
plasma membrane NBT reduction (Section 3, Figure 3.7). During
the 5 min reduction of dye, the drugs were present at a concen-
tration of 1 µM, 0.1 µM, 0.01 µM, or 0.001 µM. Dye reduc-
tion was terminated and quantitated spectrophotometrically as
described in section 3.2.4.1. As before, the initial rate of
NBT reduction was expressed as $OD_{515}/1.3 \times 10^6$ granulocytes/5
min.

4.2.3.2   The effect of incubation time with the agents

Granulocyte monolayers were exposed to each of the
different drugs (VBL, VCR, colch, or cyto B) or controls for
5, 10, 15, or 30 min. These monolayers were then assayed for
the initial rate of NBT reduction. The drug concentration
tested (and present) during dye reduction was 1 µM for each
agent. Reduction was quantitated as described in section 3.2.4.1.

4.2.3.3 Simultaneous cyto B and colch (or VBL) incubations

Granulocyte monolayers were incubated with cyto B and colch (or VBL) simultaneously and at the same dose, for 10 min prior to assaying the initial rate of NBT reduction. The drugs were present at three different concentrations (0.1, 0.01, and 0.001 µM) during these experiments. Control experiments were conducted in parallel with granulocyte monolayers exposed to either agent alone, or no drugs (water, DMSO).

4.2.3.4 Colch (or VBL) pre-incubation followed by cyto B

Granulocytes were pre-incubated with colch (or VBL) for 5 min followed by a 5 min incubation with cyto B at the same dose and in addition to the colch (or VBL). These granulo-cytes were then assayed for the initial rate of NBT reduction. At the time of reduction, the drugs were present either singly or together, at 0.001, 0.01, 0.1, or 1 µM. Control experiments were conducted in parallel with granulocyte monolayers exposed to either agent alone, or no drugs (water, DMSO).

4.2.3.5 Cyto B pre-incubation followed by colch (or VBL)

Granulocytes were pre-incubated with cyto B for 5 min, followed by a 5 min incubation with colch (or VBL), at the same dose and in addition to the cyto B. These granulocytes were then assayed for the initial rate of NBT reduction. At the time of reduction of dye, the drugs were present at 0.001, 0.01, 0.1, or 1.0 µM. Control experiments were conducted in parallel with granulocyte monolayers exposed to either agent alone, or no drugs (water, DMSO).

4.2.4   Incubation of NBT dye with the drugs

         The complete medium for assaying the initial rate of

NBT dye reduction, except for the granulocytes, was placed into

test tubes. To these tubes was then added cyto B, colch, VBL or

VCR at a final concentration of 1 μM. The tubes were then incu-

bated at 37°C for 30 min, centrifuged and the supernatant removed.

The contents of the tube were then extracted with pyridine and

NBT reduction quantitated (section 3.2.4.1).

4.2.5   Generation of lumicolchicine

         The suggestions of Wilson and Friedkin (1966) were

followed in preparing lumicolchicine from colchicine by ultra-

violet (UV) irradiation. Colchicine was prepared as a solution

of ~ 0.025 M in 95% ethanol. This stock solution was then diluted

1000-fold (three serial 10-fold dilutions), to give a solution of

~ 2.5 x 10^{-5} M. Matched quartz spectrophotometer cuvettes of

4 ml capacity and 10 mm path length were used to hold the

dilution of colchicine, which was then irradiated with long-wave

UV light having a peak at ~ 360 nm. The cuvettes were situated

10 cm from the source during irradiation. The absorbance of the

irradiated solution at 350.5 nm was checked periodically to

monitor the progress of the reaction. Irradiation was continued

until the OD at 350.5 nm had reached a low value and was no

longer decreasing.

         The irradiated sample and a control sample of

colchicine which had not been exposed to UV light were compared

(by performing a wavelength scan between 220 and 400 nm) to ensure

that lumicolchicine had been generated. The lumicolchicine solution

thus prepared was used for experiments after evaporating off the ethanol and resuspending the drug in water.

4.2.6   The reduction of NBT by lumicolchicine-treated granulocytes

Granulocyte monolayers were exposed to colchicine or lumicolchicine for 10 min prior to NBT reduction. Dye reduction was allowed to proceed for 5, 20, or 30 min in the presence of either agent at 2 μM. Control monolayers reduced dye for 5, 20, or 30 min in the absence of either drug. Reduction was terminated and then quantitated spectrophotometrically as described in section 3.2.4.1.

4.2.7   Protein measurements of granulocyte monolayers

Protein measurements of drug-treated and control monolayers were determined by the method of Lowry et al (1951) All measurements were carried out in duplicate, using the following procedure:-

Standard solutions of bovine albumin fraction V, containing ~ 2.5 - 50 μg protein/ml were prepared. The exact protein content of these reference solutions was calculated by using the conversion factor provided by Peters and Blumenstock (1967). That is, a 10 mg/ml solution of bovine albumin fraction V has an OD of 6.61 at 278.5 nm. To 0.5 ml of each standard solution was added 0.5 ml of 4% NaOH. Blanks were prepared by mixing 0.5 ml of water with 0.5 ml of 4% NaOH. The granulocyte monolayers were dissolved in 0.5 ml of 20% NaOH, and then diluted to 5.0 ml with water.

To 0.5 ml of the above solutions in NaOH (standard solutions, blanks or granulocyte monolayers in solution) was

added 2.5 ml of fresh copper reagent (section 4.2.7.1). The tubes were then incubated 30 min at 37°C.

After this time the tubes were agitated with a vortex mixer while 0.25 ml of dilute F-C reagent (section 4.2.7.2) was added. The colour was then allowed to develop for exactly 30 min at room temperature before the OD at 540 nm of the granulocyte and reference protein solutions was read against the blank. A standard curve of OD_{540} versus µg protein/ml was constructed from the data obtained.

The OD values at 540 nm for the granulocyte monolayers were then converted to µg protein/ml values with the aid of the standard curve.

4.2.7.1   Copper reagent

The copper reagent was prepared by mixing 20 ml of 10% Na_2CO_3 (anhydrous) with 1 ml of 2% sodium potassium tartrate ($NaKC_4H_4O_6$ x $4H_2O$), and then adding sufficient water for 100 ml of solution. To this solution was then added 2 ml of 0.5% $CuSO_4$ x $5H_2O$. The copper reagent was prepared fresh daily for protein determinations.

4.2.7.2   Dilute Folin-Ciocalteu (F-C) reagent

By the method of Oyama and Eagle (1956) it was determined that 5 ml of the purchased 2 M F-C phenol reagent should be diluted with 11 ml water to prepare a dilute F-C reagent suitable for protein determinations by the method outlined above. The F-C reagent was therefore prepared fresh daily in this manner.

4.3   RESULTS

4.3.1   The initial rate of NBT reduction by drug-treated granulocytes

4.3.1.1 The effect of drug dose upon reduction

As indicated in Fig. 4.1, cytochalasin B produced a dose-dependent stimulation in the initial rate of dye reduction from the plasma membrane. The stimulation with reference to the control varied from 22% (with a dose of 0.001 μM) to 83% (with 1 μM cyto B), and was statistically significant (Fig. 4.1). The DMSO used to dissolve the cyto B had no effect on dye reduction, even at the highest concentration tested (0.25% v/v DMSO, the control for 1 μM cyto B incubations).

A dual effect on NBT reduction was seen with the microtubule-disrupting agents colch, VBL or VCR. At the lowest dose tested (0.001 μM), a slight but statistically significant stimulation above the control value was seen. This stimulation varied between 13% (colch) and 45% (VCR) above the control (Fig. 4.1). With the other three doses tested, a trend towards a dose-dependent inhibition of the initial rate of dye reduction by VBL, VCR or colch was observed. The maximal inhibition was produced with the highest dose (1 μM), and varied between 15% (colch) and 18% (VBL, VCR) of the control value. As indicated in Fig. 4.1, the inhibition of dye reduction with higher doses was statistically significant with reference to the control.

4.3.1.2 The effect of incubation time with the agents

As illustrated in Fig. 4.2, granulocytes incubated for varying periods of time with cyto B showed a time-dependent stimulation of the initial rate of dye reduction. The

stimulation with reference to the control was 50% after 5 min, and rose to a high of 99% after 30 min incubation with the drug.

When granulocytes were incubated with the microtubule-disrupting agents (colch, VBL or VCR) for varying periods of time, an initial (slight) stimulation of reduction after 5 min was followed by a time-dependent decrease in the rate of dye reduction (Fig. 4.2). There was very little difference between the three agents in these experiments. The stimulations seen with very short incubation times were ˜ 12% above the control value. In contrast, the inhibition of the initial rate of dye reduction was as great as 38% with colchicine.

4.3.1.3   Simultaneous cyto B and colch (or VBL) incubations

The effects of incubating granulocytes with cyto B and colch (or VBL) simultaneously for 5 min before measuring the initial rate of dye reduction are illustrated in Fig. 4.3. A dose-dependent stimulation of the initial rate of dye reduction was observed, but the maximal stimulation seen (when the two agents were at a dose of 0.1 µM) was far less than the maximal stimulation seen with cyto B alone, at 0.1 µM. At 0.1 µM as well as 0.001 µM, the magnitude of the stimulation observed fell between that seen with cyto B or colch alone at the same dose. At 0.01 µM however, the stimulation with both agents together was greater than the stimulation seen with either agent alone. As indicated in Fig. 4.3, not all of these stimulations were statistically significant.

4.3.1.4   Colch (or VBL) pre-incubation followed by cyto B

When granulocytes were pre-incubated with colch (or VBL) for 5 min followed by a 5 min incubation with cyto B

(at the same dose and in addition to the colch or VBL), a marked

depression in the initial rate of NBT reduction was observed.

That is, the dye reduction curve seen with cyto B-treated

granulocytes was replaced by a curve which ran parallel but

showed a significant drop in the rate of dye reduction (Fig. 4.4).

4.3.1.5   Cyto B pre-incubation followed by colch (or VBL)

When granulocytes were pre-incubated with cyto B for

5 min followed by a 5 min incubation with colch (or VBL) in

addition to the cyto B, the usual dose-dependent stimulation

observed with cyto B treatment was lost. Instead the dye

reduction curve seen with the doubly-incubated granulocytes

was virtually parallel to the curve for cyto B, but with a

significant drop in the rate of dye reduction (Fig. 4.5).

4.3.2   Incubation of NBT dye with the drugs

When the medium for assaying NBT reduction was

incubated (in the absence of granulocytes) with either VBL, VCR,

colch or cyto B, no dye reduction was observed visually. Further-

more, no dye reduction could be detected spectrophotometrically

at 515 nm. The OD_{515} values were between 0.000 and 0.003 in

each case.

4.3.3   Generation of lumicolchicine

The OD at 350.5 nm of the colchicine dilution

($\sim$ 2.5 x 10^{-5} M) was found to be 0.464. After 165 min

irradiation with long-wave UV light, the $OD_{350.5}$ had fallen to

a value of 0.015 and was not decreasing any further with continued

irradiation.

When the irradiated sample and a control (not irradiated) sample of colchicine were compared by performing a wavelength scan between 220 and 400 nm, it was found that (i) the $OD_{350.5}$ of the irradiated sample was minimal (0.015), and (ii) two isosbestic points were apparent, at 255 and 305 nm (Fig. 4.6). These findings confirmed that lumicolchicine had been generated from colchicine (Wilson and Friedkin, 1966).

As the OD, or absorbance, of the irradiated colchicine at 350.5 nm was 0.464, the true concentration of the colchicine (and lumicolchicine) solution could be calculated, as shown:-

$\log \Sigma = $ 4.22 for colchicine in ethanol at 350.5 nm

(The Merck Index, 1958, p.278)

then $\Sigma = 16,600$

and as $\Sigma = A/bc$ (Beer's Law, see Meloan, 1968)

where Σ = molar absorptivity

A = OD (or absorbance) at 350.5 nm

b = path length (= 1 cm)

and   c = molar concentration of colchicine

then $16,600 = 0.464/1 \times c$

and $c = 2.8 \times 10^{-5} M$

Thus the true concentration of the irradiated colchicine sample was 2.8×10^{-5} M. As 1 mole of colchicine breaks down under UV irradiation to yield 1 mole of lumicolchicine (Wilson and Friedkin, 1966) it was concluded that the lumicolchicine solution thus generated was also 2.8×10^{-5} M.

4.3.4   The reduction of NBT by lumicolchicine-treated granulocytes

As indicated in Figure 4.7, incubation of granulocytes

for 5, 20 or 30 min with lumicolchicine at 2 μM had no effect on

NBT reduction. In parallel experiments with colchicine, at the

same dose, however, a significant depression in NBT reduction

was seen with incubations longer than 5 min. After 30 min

incubation with colchicine, for example, there was a 23% drop

in the rate of NBT reduction compared with the control.

4.3.5   Protein measurements of granulocyte monolayers

As detected by measuring the OD at 540 nm, there

were no differences in the protein content of drug-treated or

control granulocyte monolayers (Table 4.1). The OD_{540} values

of drug-treated and control monolayers were all approximately

0.035, representing a protein content of ~ 35 μg/ml (Figure

4.8) per monolayer.

4.4   DISCUSSION

Cytochalasin B produced a time and dose-dependent

stimulation of the initial rate of NBT dye reduction from the

plasma membrane oxidase of non-phagocytic granulocytes. The

marked stimulation in dye reduction was seen with incubation

periods as brief as 5 min, and doses as low as 0.001 μM cyto B.

The greatest stimulation seen was 99% above the control value,

and this occurred after a 30 min incubation with 1 μM cyto B

(Fig. 4.2).

Three other groups of workers also found that treat-

ment of non-phagocytic granulocytes with the cytochalasins

stimulated the production of superoxide. Using cytochalasin E,

Nakagawara *et al* (1975, 1976a) demonstrated a stimulation
in superoxide production with both NBT and cytochrome C
reduction. In addition they showed that this stimulation could
be enhanced if the granulocytes were incubated with concanavalin
A along with the cytochalasin (Nakagawara, Nabi and Minakami,
1977). Interestingly, they found that neither the cytochalasin
or cytochalasin/concanavalin A - induced stimulation in
superoxide production could be elicited from the granulocytes
of chronic granulomatous disease (CGD) patients (Nakagawara *et al*
1976a; Nakagawara, Nabi and Minakami, 1977). It is known that
binding of concanavalin A to the cell surface (even in the
absence of phagocytosis) is sufficient to stimulate the oxidative
metabolism of the granulocyte by perturbing the plasma membrane
(see for example, Goldstein *et al*, 1977). Thus it is not
unreasonable to conclude that the experiments of Nakagawara and
his associates suggest that the activity of a plasma membrane
oxidase is defective in CGD granulocytes (see also Appendix I).

The other two groups of investigators to find a
cytochalasin-induced enhancement in oxidase activity from non-
phagocytic granulocytes were Okuda *et al* (1976) and Zabucchi
et al (1978). The report from Okuda *et al* described a
dramatic increase in the number of granulocytes reducing NBT
dye (histochemically) when cytochalasin B was present. In
contrast, Zabucchi *et al* (1978) used the uptake of oxygen as a
guage of oxidase activity, and described a very slight stimulation
of this activity by cytochalasin B.

It must be mentioned that the cytochalasin-induced
stimulation of oxidase activity was not observed by Curnutte and
Babior (1975), Johnston and Lehmeyer (1976) and Roos and his

colleagues (Goldstein, Roos *et al*, 1975; Roos, Homan-Müller
and Weening, 1976; Roos *et al*, 1976). In fact, Curnutte and
Babior (1975) and Johnston and Lehmeyer (1976) found that
cytochalasin B inhibited the production of superoxide from non-
phagocytic granulocytes. In contrast, Roos *et al* found that
cytochalasin B was without effect. These discrepancies
cannot easily be explained, but initially it was thought that
adherent granulocytes may respond differently to cytochalasin B
than granulocytes in suspension. However, a closer examination
of the techniques employed by the various investigators revealed
that technical differences in measuring oxidase activity do not
provide the explanation. For example, adherent granulocytes may
(Okuda *et al*, 1976; and this thesis) or may not (Johnston
and Lehmeyer, 1976) show the enhancement in oxidase activity.
Similarly, even when the same method for quantitating oxidase
activity was used (cytochrome C reduction), it was found that
cytochalasin-treated granulocytes did or did not show increased
activity (Nakagawara *et al*, 1976a; Curnutte and Babior, 1975;
respectively). As many of these investigators were also
conducting experiments on cytochalasin-treated phagocytic
granulocytes, their work will also be discussed in section 5.4 of
this thesis.

In addition to the experiments with cyto B, the
results of studies with the microtubule-disrupting agents
(colch, VBL, VCR) were also presented in this section. As shown
in Figs. 4.1 and 4.2, a dual effect of these agents on plasma
membrane NBT dye reduction was observed. At low doses of,
or brief incubation periods with these agents, a slight but

statistically significant stimulation in the initial rate of
dye reduction was seen. Longer incubations (> 5 min) or higher
doses (>0.001 µM) with any one of these agents resulted in a
trend towards a dose-dependent inhibition of the initial rate of
dye reduction. The latter finding is in agreement with the results
of experiments conducted by Malawista and Bodel (1967) and Curnutte
and Babior (1975). By measuring the superoxide-dependent reduction
of cytochrome C, Curnutte and Babior found that colchicine or
vinblastine produced an inhibition in superoxide production from
non-phagocytic granulocytes. The rate of cytochrome C reduction
was 45% below that produced by untreated granulocytes when a
dose of 2.5 µM colchicine was employed. In contrast to the
studies of Curnutte and Babior, Malawista and Bodel (1967) and
Lehrer (1973) quantitated oxidase activity by measuring oxygen
consumption. Malawista and Bodel reported a 25% drop in
oxygen uptake in 8 experiments with colchicine-treated
granulocytes, but stated that the fall was not consistently
observed. Lehrer, on the other hand, and in a similarly brief
account maintained that the oxygen consumption of non-phagocytic
granulocytes was not affected by colchicine. These workers have
also studied the oxidase activity of colchicine-treated
phagocytic granulocytes, and therefore those studies are
discussed in section 5.4.

Some interesting results were seen when granulocytes
were (i) incubated with the two agents simultaneously (cyto B
with colch, or VBL), or (ii) pre-incubated with one agent
followed by an incubation with both agents together. In both of
these situations the overall effect was an inhibition of the

usual response seen with cyto B. That is, the usual stimulatory response with cyto B was largely ablated when the granulocytes were incubated with colch (or VBL) prior to, during, or after cyto B incubation. Assuming that microfilaments and microtubules were disrupted by the drugs, these results suggest that the stimulatory response seen with cyto B was bi-phasic, consisting of an initial disruption of the microfilaments by cyto B followed by a phase (characterized by the stimulation) which was dependent upon the presence of intact microtubules.

Several control experiments were conducted and must be discussed. In the absence of granulocytes, no dye reduction occurred even when NBT was incubated at $37^{O}C$ for 30 min with any particular agent. Thus any stimulations in dye reduction observed were not due to a direct effect of the agents on NBT dye. As well, the inhibition of dye reduction seen after colch, VBL, or VCR incubations (for longer than 5 min or at doses above 0.001 μM) was not an artefact of cell losses from the monolayer preparations, as the protein content of drug-treated and control granulocyte preparations was virtually identical.

Control experiments with lumicolchicine were conducted also. (Lumicolchicine is a photoproduct of colchicine which is unable to bind to tubulin and disrupt microtubules, and is therefore used as a control for experiments with colchicine, as discussed in Appendix IV). As lumicolchicine was without effect in experiments where an inhibitory response was observed with colchicine, it can be assumed that the inhibition in NBT reduction was mediated by colchicine's binding to tubulin and disruption of microtubules. This conclusion is strengthened

by the finding that NBT reduction showed a similar sensitivity to all three microtubule-disrupting agents tested (VBL, VCR and colch, see Appendix IV). Furthermore it appears as though microfilament and microtubule disruption have nearly opposite effects on plasma membrane oxidase activity, as measured by NBT reduction. That is, microfilaments provide a constraint against plasma membrane oxidase activity and thus cyto B-treated granulocytes show enhanced reduction; while microtubules are required for activity and therefore colch, VBL or VCR treatment produces an inhibition of dye reduction.

It is reasonably certain that the agents used in these experiments were mediating their effects through microfilament (cytochalasin B) or microtubule (colchicine, VBL, VCR) disruption (see Appendix IV). However, to make certain that the cytoskeletal elements were indeed disrupted by the respective treatments, the additional control experiments described in Sections 5 - 9 of this thesis were performed.

4.5   SUMMARY

The possible role of cytoplasmic microtubules and microfilaments in the control of granulocyte plasma membrane oxidase activity has been studied. The experiments were designed to record changes in the initial rate of oxidase activity (NBT reduction) brought about by treatment of the granulocytes with appropriate doses of pharmacological agents known to disrupt microtubules (colchicine, VBL or VCR) or microfilaments (cytochalasin B).

It was observed that treatment of granulocytes

with cyto B led to a time and dose-dependent enhancement of plasma

membrane oxidase activity. A similar stimulation was also reported

by Nakagawara *et al* (1976a), Okuda *et al* (1976) and Zabucchi *et al*

(1978). On the other hand, incubation of granulocytes with a

microtubule-disrupting agent (VBL, VCR or colch) resulted in a

dual response. A paradoxical enhancement in NBT reduction (oxidase

activity) was seen when low doses or brief incubation periods

with the agents were employed. However, and in agreement with

Malawista and Bodel (1967) and Curnutte and Babior (1975), when

longer incubation periods (> 5 min) or higher doses (>0.001 μM)

were used, an inhibition of oxidase activity was observed.

Control experiments with lumicolchicine, a photoproduct which is

unable to bind to tubulin and disrupt microtubules, showed

lumicolchicine to be without effect in experiments where

colchicine produced an inhibition in the rate of dye reduction.

Incubation of cytochalasin B-treated granulocytes

with a microtubule-disrupting agent either before, during, or

after the incubation with cytochalasin B led to an inhibition

in the usual stimulatory response seen with cytochalasin B.

Assuming that microfilaments (cyto B) and microtubules (colch or

VBL) were disrupted by these drug treatments, it appears that

the stimulatory response seen with cyto B is bi-phasic, consisting

of an initial disruption of the microfilaments by cyto B followed

by a phase (characterized by the stimulation) which is dependent

upon the presence of intact microtubules. Furthermore, it

appears as though microfilament and microtubule disruption have

nearly opposite effects on plasma membrane NBT reduction. That

is, microfilaments provide a constraint against plasma membrane

oxidase activity and thus cyto B-treated granulocytes show enhanced NBT reduction, while microtubules are required for activity and therefore VBL, VCR or colchicine treatment produces an inhibition of dye reduction.

To make certain that microfilaments and microtubules were indeed disrupted by the respective drug treatments, the additional control experiments presented in Sections 5 - 9 of this thesis were performed.
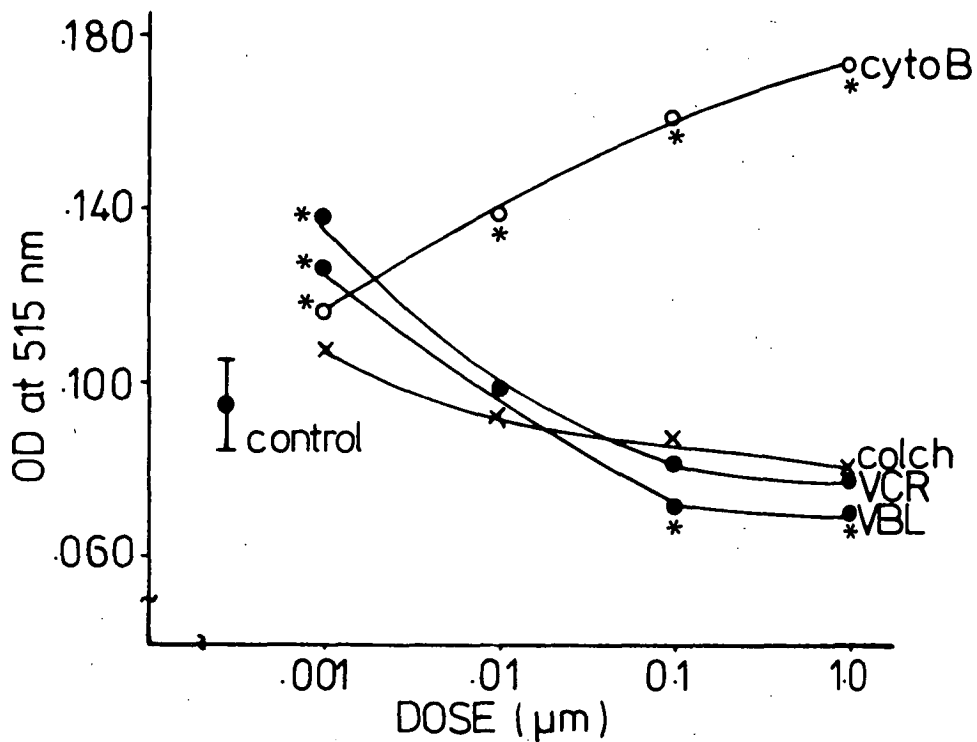
FIG. 4.1. Plot of the effect of drug dose upon the rate of NBT

reduction. Each point represents the mean of at least

3 determinations, and is the OD at 515 nm/1.3 x 10^6 cells/5 min.

SEM values were ± 10% of each individual mean. The control value

(0.095 ± .010) was obtained from experiments with 15 donors. In

all experiments the time of exposure to any agent was 10 min

Values significantly different from the control (p $\leq$ 0.05) are

indicated by an asterisk.
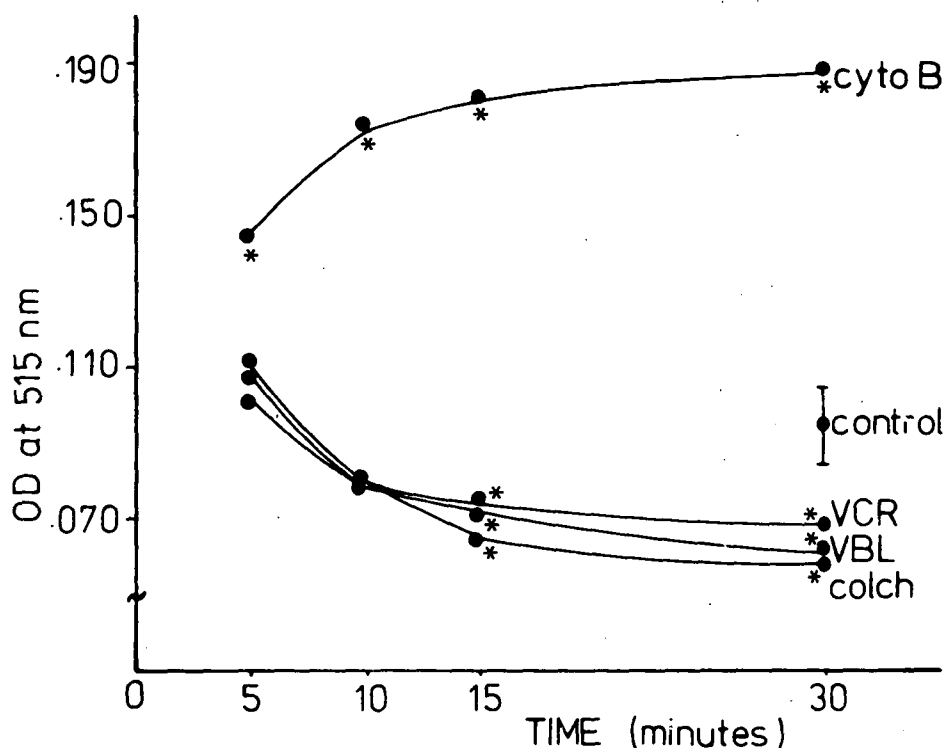
FIG. 4.2. Plot of the effect of incubation time with the agents
on the rate of NBT reduction. Each point represents
the mean of at least 3 determinations and is the OD at 515 nm/
1.3×10^6 cells/5 min. SEM values were ± 12% of each individual
mean. The control value (0.095 ± .010) was obtained from experi-
ments with 15 donors. In all experiments the dose of each agent
was 1 µM. Values significantly different from the control
(p ≤ 0.05) are indicated by an asterisk.
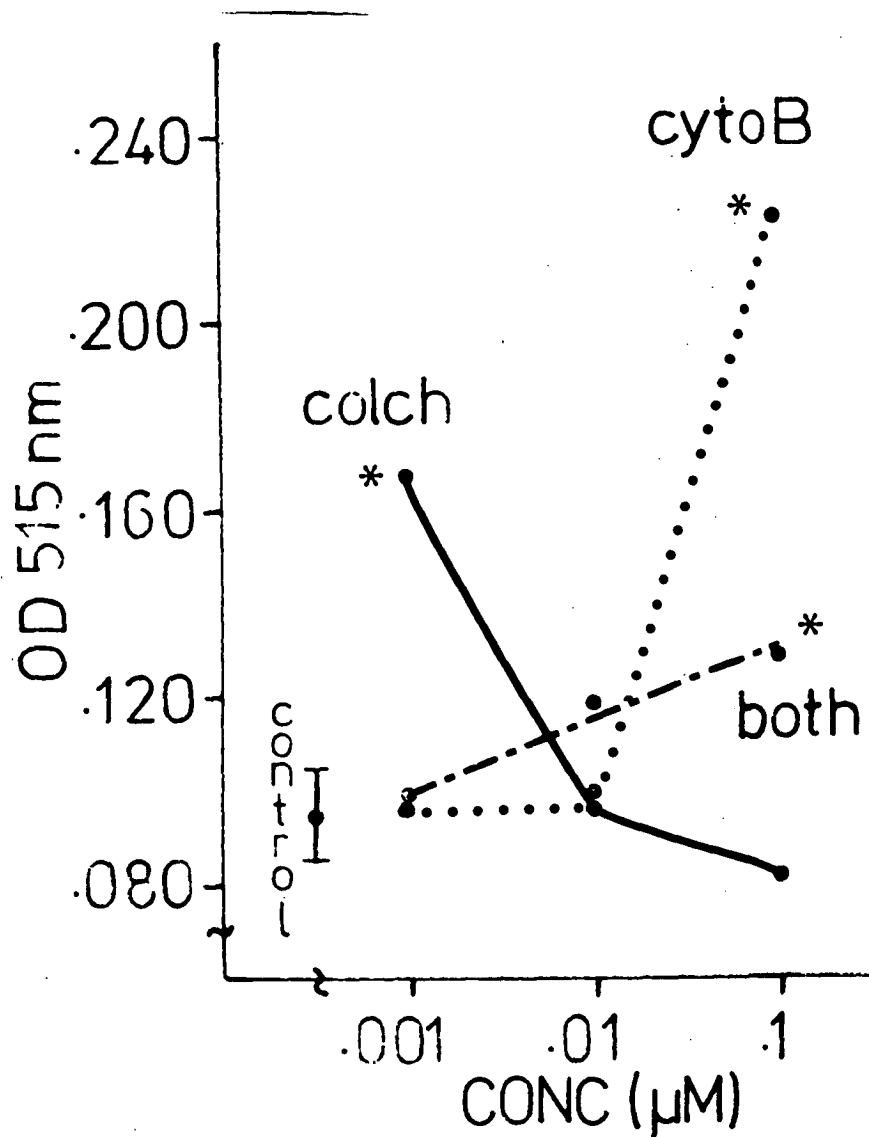
FIG. 4.3. Plot of the effects of incubating granulocytes with cyto B and colch simultaneously for 5 min (at 3 different doses) before measuring the initial rate of NBT dye reduction. Each point represents the mean of 3 experiments performed in triplicate, and is the OD at 515 nm/1.3×10^6 cells/ 5 min. SEM values were ± 11% of each individual mean. The control value (0.095 ± .010) was obtained from experiments with 15 donors. Values significantly different from the control ($p \leq 0.05$) are indicated by a (*).
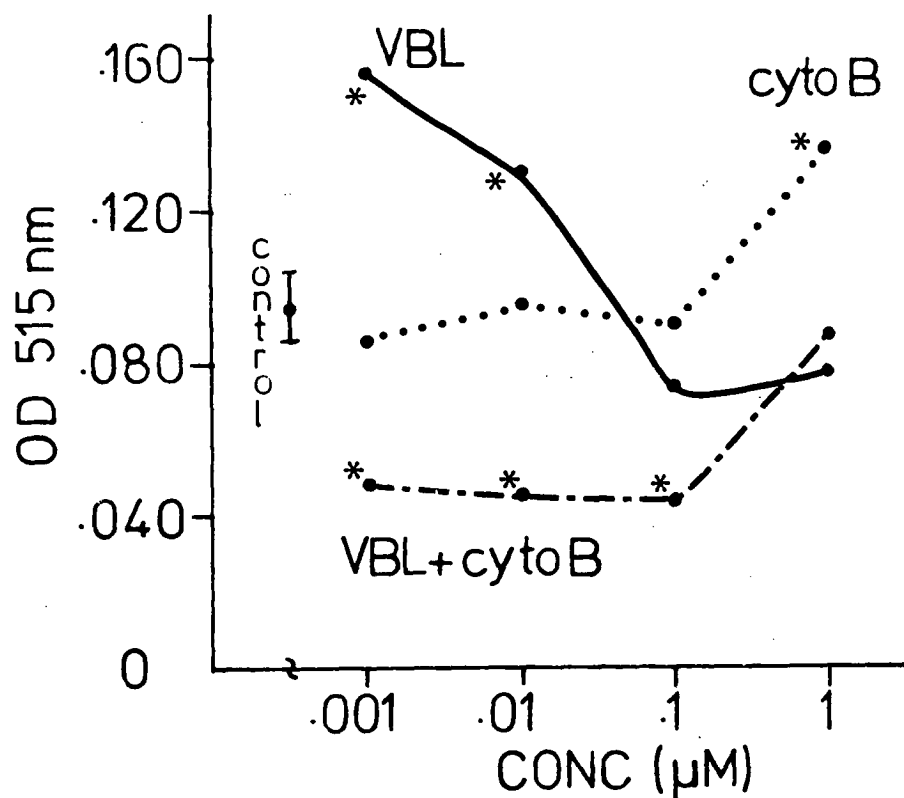
FIG. 4.4. The effects of pre-incubating granulocytes with VBL
for 5 min followed by a 5 min incubation with cyto
B (at the same dose and in addition to the VBL). Four doses were
tested, and each point represents the mean of 3 experiments
performed in triplicate. SEM values were ± 10% of each individual
mean. The control value (0.095 ± 0.010) was obtained from ex-
periments with 15 donors. Values significantly different from
the control (p ≤ 0.05) are indicated by a (*).
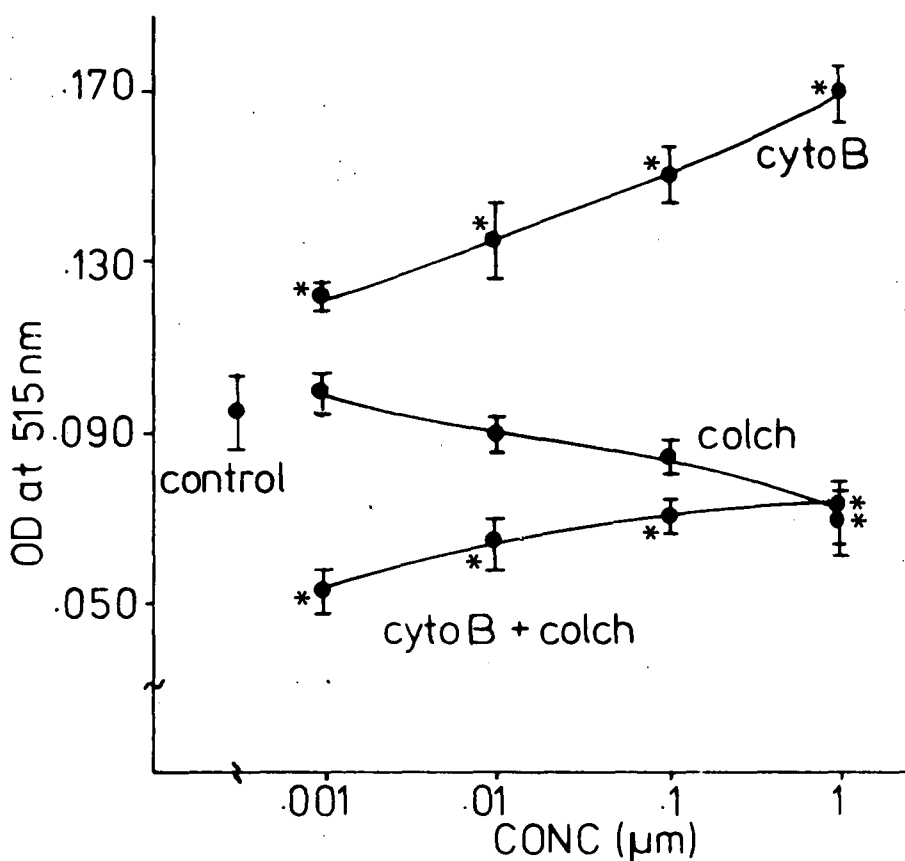
FIG. 4.5. The effects of pre-incubating granulocytes with
cyto B for 5 min followed by a 5 min incubation
with colch (at the same dose and in addition to the cyto B).
Four doses were tested, and each point represents the mean of
3 experiments conducted in triplicate. SEM values are shown
by the vertical bars. The control value (0.095 ± 0.010) was
obtained from experiments with 15 donors. Values significantly
different from the control ($p \leq 0.05$) are indicated by a (*).
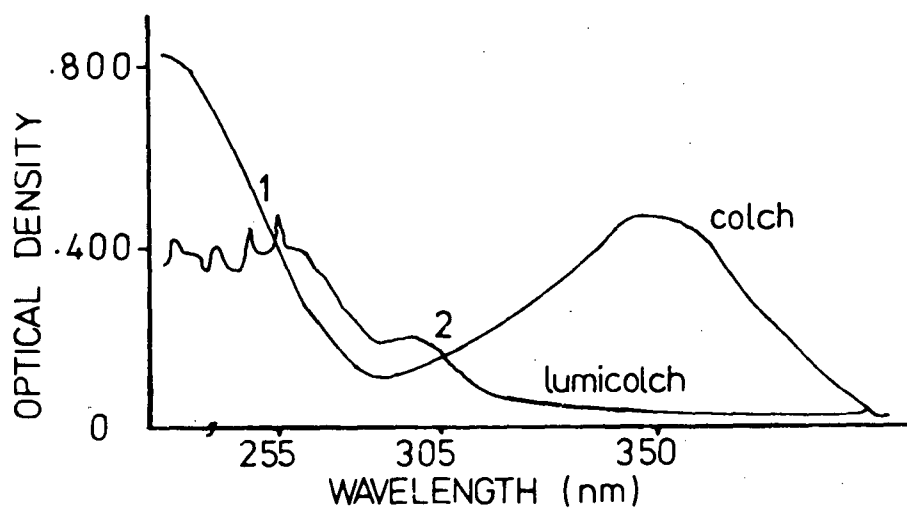
FIG. 4.6. A wavelength scan between 250 and 400 nm, confirming the generation of lumicolchicine from colchicine which has been irradicated with UV light. Two isosbestic points at 255 (1) and 305 nm (2) are apparent, as is the minimal absorption of the irradiated sample (lumicolchicine) at 350.5 nm.
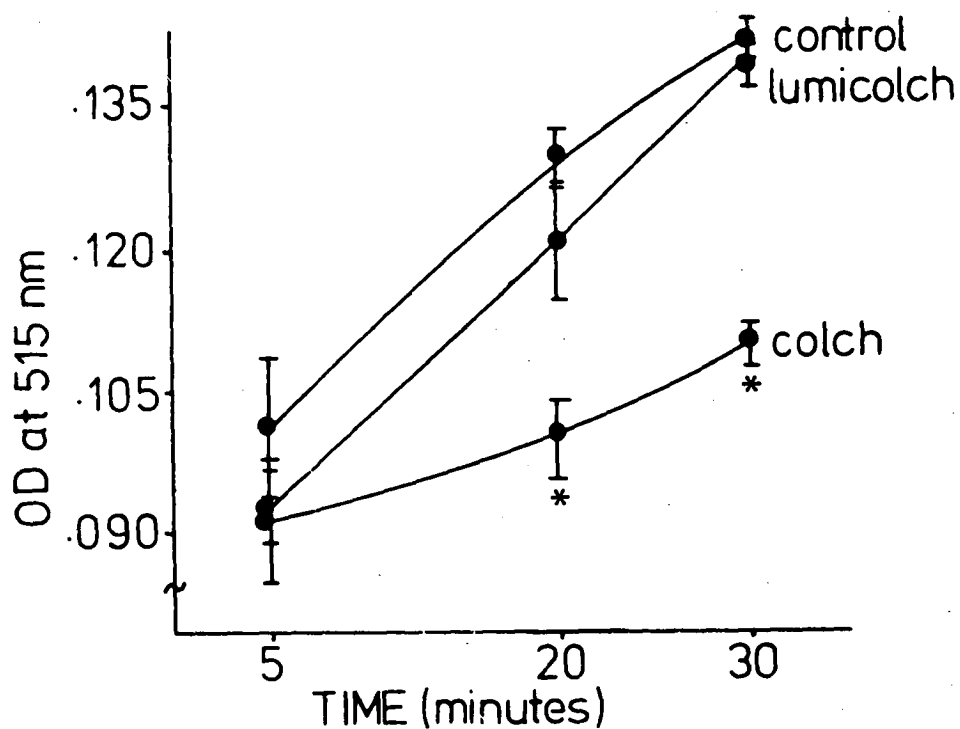
FIG. 4.7. The reduction of NBT dye by colch or lumicolchicine-
treated granulocytes. Granulocytes were incubated
with the agents at 2 μM for 10 min and then dye re-
duction was allowed to proceed for 5, 20 or 30 min
in the presence of the agent. Points are the $\bar{X}$ ± SEM
of 3 determinations, and are the OD at 515 nm/1.3 x 10^6
cells/5, 20 or 30 min. Values significantly different
from the control (p $\leq$ 0.05) are indicated by a (*).
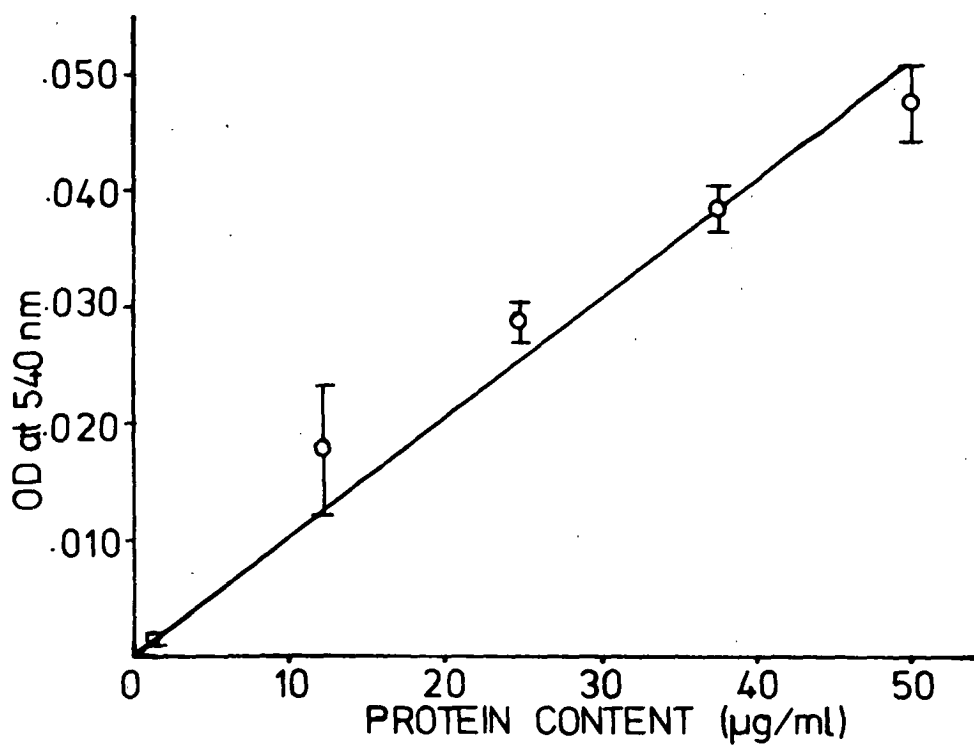
FIG. 4.8. The standard curve for protein determinations by the method of Lowry *et al* (1951).

TABLE 4.1   Protein content of drug-treated

and control monolayers

Drug Treatment	O.D. at 540 nm[*]
cytochalasin B	0.033 ± 0.002
colchicine	0.034 ± 0.004
vinblastine	0.036 ± 0.003
vincristine	0.033 ± 0.004
control (no treatment)	0.034 ± 0.004

* Representing the protein content of the mono-
layers (see section 4.3.5). Values are the
$\bar{X} \pm SEM$ of 2 experiments, each conducted in
duplicate. From Fig. 4.8, an O.D. of 0.034
is equivalent to a protein content of 32.5
µg/ml. None of the values for treated mono-
layers is significantly different from the
control value.

SECTION 5

MICROFILAMENT AND MICROTUBULE DISRUPTION

AND OXIDASE ACTIVITY

(PHAGOCYTIC GRANULOCYTES)

5.1   INTRODUCTION

          This section of the thesis describes experiments

monitoring the oxidase activity (NBT reduction) from phagocytic

granulocytes exposed to drugs known to disrupt microtubules or

microfilaments. It will be recalled that similar drug treatments

of non-phagocytic granulocytes resulted in changes in the initial

rate of NBT reduction (section 4.3). As discussed in section 4.4,

those experiments suggested that microtubules and microfilaments

may play opposite roles in controlling plasma membrane oxidase

activity. It was thought that comparable experiments with

phagocytic granulocytes might help clarify the mechanism behind

the changes in oxidase activity, and therefore such studies were

performed and are presented herein.

5.2   METHODS

5.2.1   Preparation of the drugs in HSS

          Colchicine, VBL and lumicolchicine were prepared as

stock solutions of 1 mM in water, while cyto B was dissolved at

the same concentration in DMSO. Dilutions of these stock

solutions were then carried out (as outlined in section 4.2.1)

to prepare HSS which contained any one drug at a concentration

(during tests) of either 1 or 2 µM. [The 2 µM concentration

was used where the drug was to be subsequently diluted, by half,

when mixed with polystyrene latex beads (section 5.2.2).]

Lumicolchicine was prepared as described in section 4.2.5 and used

as a control for the experiments with colchicine (see Appendix

IV).

As cyto B was dissolved in DMSO, the control experiments for cyto B-treated granulocytes were conducted using HSS which contained DMSO at the appropriate concentration of 0.25% v/v (see section 4.2.2).

The concentration of the agents (including lumicolchicine) during incubation was 1 μM.

5.2.2   Preparation of the polystyrene latex beads

Polystyrene latex beads with a mean diameter of 0.79 μM were dialyzed against three changes of water for 24 hours. A stock dilution was prepared by adding 50 μl of these beads to 2.0 ml of HSS. This was the standard bead dilution for virtually all procedures in which a phagocytosable stimulus was required. The exception was the bead dilution used for experiments where the beads were to be presented to granulocytes which had been preincubated with a drug. For these experiments it was necessary to prepare a mixture of beads with drug so that the cells would continue to be exposed to the drug at the same concentration during phagocytosis. The required mixture contained 1 volume of beads (at twice the standard dilution) and 1 volume of drug, at 2 μM. In this way the exposure to the particular drug was maintained at the desired concentration (1 μM) during phagocytosis.

5.2.3   Phagocytosis of polystyrene latex beads

Granulocyte monolayers of 1.3 x 10^6 cells were prepared by adding 50 μl of isolated granulocytes (section 2.2.13) to 100 μl of HSS (with or without drug at 1 μM). The monolayers were then incubated for 10 min at 37°C. After this time 100 μl of dialyzed polystyrene latex beads (section 5.2.2) was added to

each drug-free monolayer, whereas 100 μl of the bead/drug

mixture was added to each drug-containing monolayer.

Phagocytosis was allowed to proceed for 30 min at 37°C

and was then terminated by vigorously rinsing each monolayer in

excess CMFH at 4°C. The monolayers were then allowed to dry in

air before being extracted into 1.0 ml of spectral grade dioxane

for 2 hours at room temperature. Phagocytosis was then quantitated

by measuring the O.D. at 259 nm, which represents the polystyrene

content of the granulocyte monolayers (Weisman and Korn, 1967).

Parallel experiments were conducted with monolayers

which were taken to the stage of rinsing to remove uningested

beads, but were instead air-dried and extracted into NaOH. These

granulocytes were processed as described in section 4.2.7 and

were used to quantitate the protein content of the monolayer.

5.2.4   Preparation of monolayers for scanning electron microscopy

Glass adherent granulocytes which had been incubated

with or without drugs and then exposed to polystyrene latex

beads were processed for scanning electron microscopy as described

previously (Dewar and Wolowyk, 1979). These experiments were run

in parallel to those described in section 5.2.3. Briefly, the

monolayers were rinsed of uningested beads and then exposed to

0.1% buffered glutaraldehyde (section 5.2.5) and allowed to fix

for 30 min. The coverslips were then removed to fresh fixative

(at 2%) for another 30 min. After this time they were rinsed

in several changes of CMFH to remove any unreacted glutaraldehyde.

Dehydration was then started by exposure to increasing concen-

trations of ethanol (section 5.2.6). The ethanol concentrations

used were 1.8, 10, 20, 40, 50, 70, 80, 90% and absolute ethanol,

in that order. The coverslips were exposed to each ethanol for

2 min and twice to absolute ethanol. After the final ethanol

exposure the coverslips were air-dried and then glued onto a

scanning electron microscope stub. A thin coating of carbon and

gold was applied under vacuum before the cells were observed in

a JEOL scanning electron microscope at 25 kV accelerating voltage

and no tilt.

5.2.5   Buffered glutaraldehyde fixatives

            A 2% buffered glutaraldehyde was prepared by mixing

10 ml of Ca^{++} and Mg^{++}-free salt solution (Appendix II) with

10.6 ml glutaraldehyde EM (25%) and adding sufficient water for

100 ml. To this was added 10 ml of 155 mM glucose and 20 ml of

HEPES buffer (Appendix II).

            The 2% buffered glutaraldehyde was used to prepare

fixative at 0.1% concentration, in the following manner:

            5 ml of 2% fixative was mixed with 95 ml of CMFH (Appendix II).

We fixatives were both prepared on the day of use.

5.2.6   Ethanol solutions for dehydration

            Benzene-free absolute ethanol was mixed with water to

make 1.8, 10, 20, 40, 50, 70, 80, and 90% (v/v) solutions.

These solutions were always prepared on the day of use.

5.2.7   NBT reduction by drug-treated phagocytic granulocytes

            Granulocyte monolayers were prepared by adding 50 µl

of isolated granulocytes (section 2.2.13) to 100 µl of HSS, with

or without drug at 1 µM, and incubating for 10 min at $37^{o}C$. After

this time the monolayers were incubated for 5 or 20 min with

beads (± drugs) to give the following experimental conditions:-

- monolayers incubated with beads

- monolayers incubated with drug

- monolayers incubated with beads and drug simultaneously

For those monolayers incubated with beads or drug only, 100 µl of the standard bead dilution (section 5.2.2) or 100 µl of HSS containing drug at 1 µM (section 5.2.1) was added. For those monolayers exposed to beads and drug simultaneously, 100 µl of the 1:1 mixture of beads and drug was added (section 5.2.2). By this procedure all monolayers were made comparable. As before, the concentration of drug during the incubation was 1 µM and the control HSS for those experiments where cyto B was present contained DMSO at 0.25% v/v (see section 4.2.2).

After the 5 or 20 min incubation period, 200 µl of NBT solution (section 3.2.3) was added to each monolayer and reduction was allowed to proceed for 5 min. Therefore the total exposure to drug and/or beads was 10 or 25 min, respectively. Reduction was terminated by repeatedly rinsing each monolayer in CMFH at 4°C. The reduction of NBT was quantitated spectrophotometrically as described in section 3.2.4.1, and results were expressed as OD at 515 nm/1.x10^{6} granulocytes/5 min.

Parallel experiments were conducted with monolayers which were taken through the incubation period but not presented with NBT dye. Instead, these monolayers were rinsed in CMFH, air-dried, and used for protein determinations as described in section 4.2.7.

5.3   RESULTS

5.3.1   Phagocytosis of polystyrene latex beads

As shown in Table 5.1, granulocytes exposed to any one of the drugs (colch, VBL, cyto B) while phagocytosing showed a marked impairment in phagocytosis. Control incubations with DMSO at 0.25% (for those monolayers exposed to cyto B) or lumicolchicine (for those exposed to colch) did not display an inhibition in polystyrene uptake. The inhibition of phagocytosis was not complete with any of these agents, but was most pronounced with cyto B, which gave a 55% inhibition when compared with the control. The protein content of comparable drug or bead-treated monolayers was not significantly different from untreated (control) mono-layers (Table 5.2, groups 1 and 2).

5.3.2   Scanning electron microscopy

The majority of untreated granulocytes were observed adhering to the glass coverslips with cytoplasmic extensions projecting in all directions. The surface of these cells was relatively smooth, with only a few surface undulations or ruffles (Fig. 5.1). Some of the untreated granulocytes displayed an elongated pseudopod as well as the rounded cell body (Fig. 5.2). In contrast, no such pseudopodia were seen in the granulocytes incubated with cyto B, colch or VBL. Otherwise, the general surface ultrastructure of the drug-treated granulocytes was not remarkably unlike that of the control cells, as both populations exhibited a variety of surface features. It was notable however, that a proportion (15 of 175 examined) of the granulocytes exposed to cyto B had more marked surface undulations than the control cells (Fig. 5.3).

At first glance, granulocytes exposed to polystyrene latex beads presented the same general appearance as non-phagocytic granulocytes. However, closer examination at higher magnifications revealed areas of surface protrusion containing engulfed beads. These ingested beads were identifiable (by their smooth profiles and size) only when situated very close to the surface of the cell (Fig. 5.4). When compared with non-phagocytic granulocytes, none of the granulocytes presented with beads displayed pronounced pseudopodia adherent to the glass coverslip. Granulocytes exposed to colch, VBL, DMSO (0.25%) and lumicolchicine while phagocytosing did not appear to differ markedly from the drug-free control cells in surface morphology. In contrast, cyto B-treated granulocytes showed many adherent and incompletely ingested beads on the cell surface (Fig. 5.5).

5.3.3   NBT reduction by phagocytic, drug-treated granulocytes

5.3.3.1   Cytochalasin B-treated granulocytes

As shown in Table 5.3, granulocytes exposed to cyto B while ingesting polystyrene latex beads displayed a significant stimulation in NBT reduction when compared with untreated (phagocytic) granulocytes. The stimulation was apparent in granulocytes incubated for 5 or 20 min with the agents (Table 5.3), but was greatest (at 23% above the appropriate control) after 5 min incubation. The solvent used to dissolve cyto B was without effect in these experiments (Table 5.3 , DMSO 0.25%). Phagocytosis itself was also stimulatory to NBT reduction by glass-adherent granulocytes (line 3, Table 5.3).

5.3.3.2 Colch and VBL-treated granulocytes

As shown in Tables 5.4 and 5.5, both colch and VBL
treatment of phagocytosing granulocytes resulted in a
significant depression in the rate of NBT reduction. The in-
hibition was most pronounced with granulocytes incubated with
VBL for 20 min, and in this case dye reduction was 38% below
the appropriate control (phagocytic, line 2, Table 5.5). Incubation
of non-phagocytic or phagocytic granulocytes with lumicolchicine
had no effect on dye reduction (Table 5.4). As before, phago-
cytosis itself resulted in a marked enhancement in the rate of
NBT dye reduction (Tables 5.4, 5.5).

5.3.4 Protein content of drug-treated phagocytic monolayers

Granulocyte monolayers exposed to beads and drug
(VBL, colch, cyto B) simultaneously showed the same protein
content as control (untreated) monolayers (Groups 3 and 4,
respectively, Table 5.2).

5.4 DISCUSSION

Micromolar doses of colchicine and VBL were found to
similarly impair the ability of granulocytes to phagocytose
polystyrene latex beads (Table 5.1). In parallel experiments
lumicolchicine had no effect, suggesting that microtubule dis-
ruption was responsible for this impairment in colchicine or
VBL-treated cells (see Appendix IV). Examination of the
literature reveals that there is some controversy over whether
microtubule-disrupting agents always inhibit ingestion in
granulocytes (see Table 5.6). For example, both Malawista
(1971) and Lehrer (1973) maintain that the ingestion of

Staphylococcus Aureus is unaffected by colchicine. Similarly Oliver, Ukena and Berlin (1974) state that the uptake of paraffin oil droplets containing Oil Red O is not inhibited by colchicine, but others provide evidence to the contrary (Stossel *et al*, 1972). The reason for these discrepancies is not known, but it is notable that the choice of particle may influence the outcome of tests of phagocytic function in drug-treated granulocytes (see Lehrer, 1973; Cannarozzi and Malawista, 1973). Furthermore, different results probably arise as a result of methodological differences in the phagocytic assays themselves. In this regard, it is certain that spectrophotometric methods (Stossel *et al*, 1972) or isotopic procedures (Root and Metcalf, 1977) for quantitating phagocytosis are far superior to techniques employing light microscopical counts of ingested particles, and yet many investigators use the latter (see Stossel, 1973a,b).

The impairment of phagocytosis in granulocytes treated with colchicine or VBL undoubtedly provides part of the explanation for the decreased oxidase activity (NBT reduction) also observed in these cells (see Tables 5.4 and 5.5, respectively). However, as discussed in the text to follow, several other factors need to be considered when studying the impairment in oxidase activity in phagocytic granulocytes. As shown in Tables 5.4 and 5.5, phagocytic granulocytes incubated for 20 min with colch or VBL showed a marked drop in the rate of NBT reduction (~ 40% below the drug-free control). Lumicolchicine had no effect on phagocytosis (Table 5.1) or on the oxidase activity of phagocytic granulocytes (Table 5.4) in experiments where colchicine and

VBL produced a similar inhibition, suggesting that these responses were mediated by microtubule disruption (see Appendix IV)   It is clear that the observed inhibitions in NBT dye reduction and phagocytosis were not artefacts due to loss of granulocytes from the coverslips, as the protein content of drug-treated and control monolayers was almost identical (Table 5.2).

The colchicine or VBL-induced inhibition of phagocytic oxidase activity has been observed by many other investigators, despite the fact that the methods for assaying oxidase activity have varied (see Table 5.7).   It is notable that the few workers who have not found an impairment of phagocytic oxidase activity by these drugs employed oxygen uptake as a guage of oxidase activity.   Two points can be made with respect to this type of assay:-

(i)   oxygen uptake should probably not be considered equivalent to measuring oxidase activity by other methods, notably cytochrome C reduction, and therefore the results of such assays must be interpreted with caution (see the discussion to follow with respect to Zabucchi's experiments)

(ii)   using oxygen uptake, Lehrer (1973) showed that the choice of particle would influence the outcome of tests of oxidase activity in colchicine-treated phagocytic granulocytes.   For example, he found little impairment of oxygen uptake by colchicine-treated granulocytes ingesting *Candida albicans*, even though colchicine markedly impaired phagocytosis of the

micro-organism itself. Conversely, he showed that the ingestion of

Staphylococcus aureus was not affected by colchicine while the

post-phagocytic increase in oxygen uptake was markedly diminished

under the same conditions. Lehrer concluded that oxidase activity

is not directly linked to phagocytosis in granulocytes, and this

has been confirmed by others (see for example Goldstein *et al*,

1975, 1977; Roos *et al*, 1976; Johnston and Lehmeyer, 1976;

DeChatelet *et al*, 1976; Stendahl *et al*, 1977). From such

experiments two conclusions can be drawn, namely that (1)

perturbation of the plasma membrane (and not phagocytosis

per se) is responsible for the enhancement in oxidase activity,

and that (2) impairment of phagocytosis cannot be considered as

the complete explanation for the diminished oxidase activity

observed in colchicine or VBL-treated granulocytes. Alternative

approaches to understanding this finding are discussed below, with

reference to the experiments of Jandl *et al* (1978).

In addition to the experiments with colchicine and

VBL, studies with cyto B were also presented in this section of

the thesis. The majority of investigators find (in agreement

with the results presented herein) that treatment of phagocytic

granulocytes with cyto B gives an enhancement in oxidase activity

(see Tables 5.3 and 5.8). The two dissenting reports are

remarkable in that:-

(i) one of these groups is not consistent in its findings. This

group has found the usual enhancement in superoxide production

from cyto B-treated phagocytic granulocytes (Goldstein, Roos

et al, 1975; Roos, Goldstein *et al*, 1976), as well as an

absence of effect with cyto B (Roos, Homan-Müller and Weening,

1976). Examination of the latter report indicates that insufficient cytochrome C was present in their assay to detect the release of superoxide. For example, Fig. 4 from their paper shows that a doubling of the cytochrome C concentration leads to the detection of enhanced superoxide release from cyto B-treated versus control granulocytes. Therefore, this group has not provided any strong evidence against the usual findings.

(ii) the report from Zabucchi *et al* (1978) measured phagocytic oxidase activity via oxygen uptake, finding this to be inhibited by cyto B. Two other groups have also detected the same drop in oxygen uptake (Roos, Homan-Müller and Weening 1976; Root and Metcalf, 1977). However, these two groups simultaneously monitored superoxide production as well, and found it to be enhanced [see (i) above]. Thus it seems that oxygen uptake and superoxide production (specifically, cytochrome C reduction) cannot be considered equivalent methods for measuring the oxidase activity of granulocytes. Therefore, the report by Zabucchi *et al* (1978) does not provide firm proof that phagocytic oxidase activity is inhibited by cyto B.

Some insight into the mechanism behind the observed stimulation in oxidase activity with cyto B is provided by the experiments of Jandl *et al*(1978). They showed that (in the absence of cyto B) the superoxide-generating activity of phagocytic granulocytes declines with time, and they likened this phenomenon to a termination of the respiratory burst. Jandl *et al* suggested that the termination arose when a plasma

membrane oxidase[1] was degraded and inactivated by the act of

phagocytosis. Furthermore, they found that phagocytosis was

impaired by cyto B and therefore suggested that in cyto B-

treated granulocytes the oxidase was not internalized with

subsequent termination of its activity.

It is relevant at this point to discuss the problems

of studying oxidase activity from cyto B-treated, intact,

phagocytic granulocytes. As shown in Table 5.1, the experiments

in this thesis indicate that cyto B markedly but not completely

inhibits phagocytosis of polystyrene latex beads. It was

initially thought that the remnant phagocytosis detected

spectrophotometrically in such cells was artefactual - a reflection

of beads binding to the cell surface. Although scanning electron

microscopy confirmed that bead adherence to cyto B-treated cells

was occurring (Fig. 5.5), transmission electron microscopy showed

that some beads were indeed ingested (see section 9.3). Many

other investigators have also reported that granulocytes treated

with cyto B show a marked impairment in phagocytosis, and that

this is a result of the disruption of microfilaments by this drug

(see Reaven and Axline, 1973; Boxer *et al*, 1974; Stossel, 1977;

and Appendix IV). A residual phagocytic activity has been found

even when very high doses of cyto B were employed, and is

hypothesized to occur through a microfilament-independent process

(Zigmond and Hirsch, 1972).

As indicated in Table 5.9, some investigators

argue that the impairment of phagocytosis by cyto B is absolute,

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[2] Subsequent work by others lends support to their reasoning that
a plasma membrane oxidase activity was being measured (see
Appendix I).

while others (in accord with the results in this thesis) provide

evidence that the inhibition is only partial. To add to the

disagreement one report maintains that cyto B can inhibit or

stimulate phagocytosis of the same micro-organism, depending

upon the dose used (Cannarozzi and Malawista, 1973). It is

very difficult to reconcile these divergent results, especially

when a difference of opinion exists despite the use of the same

particle (see Table 5.9). Interestingly, whether or not a

complete inhibition of phagocytosis was reported, almost all

investigators have reported that cyto B-treated granulocytes

show many adherent and incompletely ingested particles on the

cell surface. These granulocytes have been found to release

enhanced amounts of superoxide (discussed above) as well as

lysosomal enzymes into the external medium and have therefore

been called 'secretory' cells (Weissmann et al, 1973). This

term has been used because the granulocytes respond to the

adherent particles by mobilizing the cytoplasmic granules,

which fuse with the plasma membrane underneath the particle.

In effect these regions of the plasma membrane subtending

the adherent particles can be thought of as false (incompletely

internalized) phagosomal membranes. With granule fusion, the

lysosomal enzymes are extruded from the cell and are therefore

detectable in the external medium (see for example Henson,

1972; Weissmann et al, 1973; Zurier, Hoffstein and Weissmann

1973 a,b; Hawkins 1973; Zurier et al, 1974; Hoffstein et al

1974 and Henson and Oades, 1975). It has been stated by one

group of investigators in particular that granulocytes rendered

secretory by cyto B are completely unable to ingest the particles

used as a stimulus for secretion (Zurier, Hoffstein and Weissmann,

1973 a,b; Goldstein, Roos, Kaplan and Weissmann, 1975; Roos,

Goldstein, Kaplan and Weissmann, 1976; Roos, Homan-Müller and

Weening, 1976). On this basis, Roos, Homan-Müller and Weening

(1976) refer to the superoxide release as coming from a plasma

membrane oxidase which is stimulated by continual contact with

the particles without subsequent phagocytosis. However, it is

incorrect on their part to conclude that the superoxide originates

only from a plasma membrane oxidase in these cyto B-treated

granulocytes, for two reasons:-

(i)   these granulocytes have been shown able to ingest particles

        to some extent (see Table 5.9), and

(ii)   in these cells the granules fuse with the plasma membrane

        underneath adherent particles.

By both of these processes, some of the superoxide released would

have to be accredited to a granule-located oxidase (see Appendix

I). Thus in studying the oxidase activity of cyto B-treated

granulocytes, the presence of particles makes it impossible to

state that the detected superoxide comes only from the plasma

membrane. In contrast, non-phagocytic granulocytes do not

appear to present the same problem, as granule fusion with the

plasma membrane does not occur in cyto B-treated granulocytes

in the absence of particles (Henson, 1971 a,b; Henson, 1972;

Zurier, Hoffstein and Weissmann, 1973b; Henson and Oades, 1975).

For this reason the mechanisms behind the release of superoxide

from the plasma membrane are more easily studied and interpreted

when non-phagocytic granulocytes are employed.

5.5   <u>SUMMARY</u>

In this section of the thesis the results of experiments on phagocytic granulocytes are presented. These experiments were undertaken in an attempt to uncover the mechanism behind the observed changes in oxidase activity brought about by the treatment of non-phagocytic granulocytes with microtubule and microfilament-disrupting agents (see section 4.4).

It was found that agents known to disrupt microtubules (colch, VBL) or microfilaments (cyto B) were able to markedly impair phagocytosis of polystyrene latex beads. Cytochalasin B was the most potent in this regard. Treatment of granulocytes with cyto B also resulted in the phenomenon of beads adhering to the surface of the granulocytes without being ingested, although some beads were fully internalized.

NBT reduction (as a guage of oxidase activity) was also examined in phagocytic granulocytes exposed to these agents. Granulocytes incubated with cyto B while phagocytosing showed enhanced oxidase activity when compared to untreated phagocytic cells. In contrast, VBL and colch exposure resulted in an inhibition in NBT reduction from phagocytic granulocytes. Several groups of investigators have also observed these opposite results. As the microtubule and microfilament-disrupting agents gave a similar inhibition in phagocytosis yet opposite results in tests of oxidase activity, it is apparent that oxidase activity and phagocytosis are not linked in granulocytes. This point has also been made by others. It is therefore evident that the decrease in phagocytosis can provide some (but not all) of the explanation for the drop in NBT reduction seen in colch or

VBL-treated granulocytes.   However, an interpretation of the results of the experiments with cyto B is much more difficult. For example, other investigators have provided evidence that the cytoplasmic granules fuse with the plasma membrane subtending particles adhering to the surface of cyto B-treated granulocytes. Therefore it is likely that the superoxide present in the external medium surrounding such cells (and thus the enhanced oxidase activity detected herein by NBT reduction) could have arisen from a granule oxidase.   In conclusion it is evident that in studies of phagocytic granulocytes it cannot be concluded that the superoxide released is from the plasma membrane oxidase alone.   For this reason it seems that such studies are not as useful as experiments on non-phagocytic granulocytes when attempting to examine the role of microtubules and microfilaments in controlling the activity of a plasma membrane oxidase.

FIG. 5.1   Scanning electron micrograph of a glass-adherent

(top)   granulocyte.   The surface of the cell is relatively

                smooth, with two rounded projections (asterisks)

                which probably delineate underlying nuclear lobes.

                Magnification 7,500 x.

FIG. 5.2   A granulocyte migrating on glass.   The leading

(bottom)   lamellipodium (right) and rounded cell body

                regions are clearly seen.   This cell also dis-

                plays a relatively smooth surface profile.

                Magnification 10,400 x.
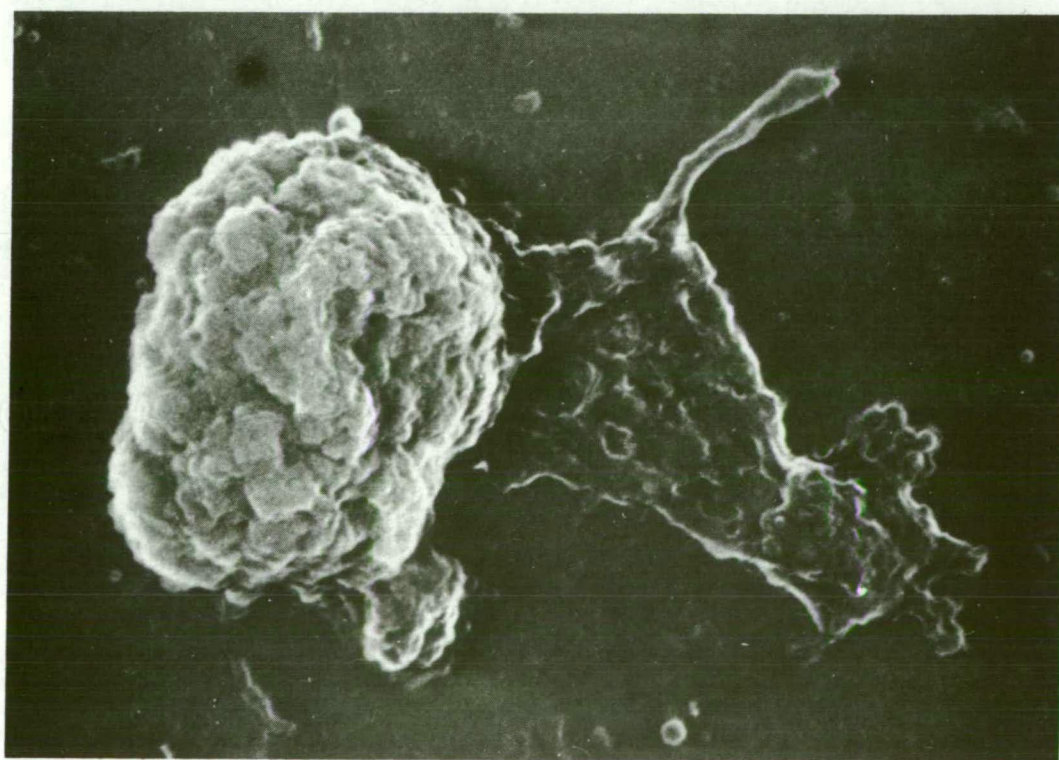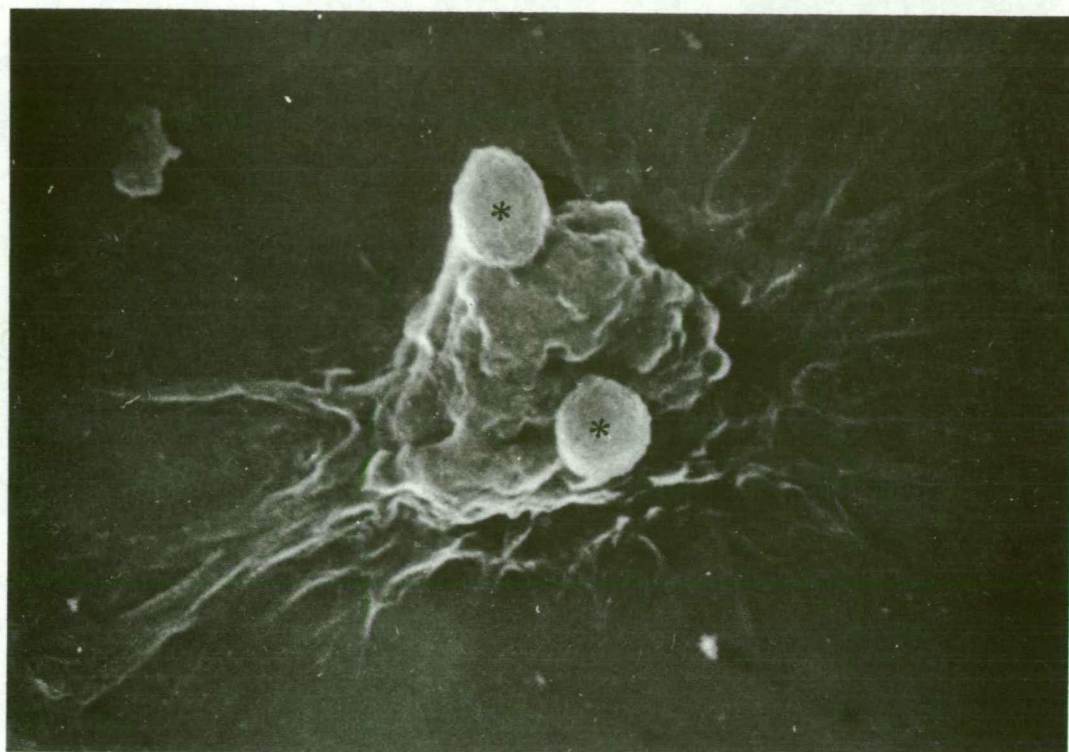
FIG. 5.3 A granulocyte which has been incubated 5 min with

cytochalasin B. In contrast to the granulocyte

cell surfaces in Figs. 5.1 and 5.2, the surface

membrane of this cell is markedly ruffled.

Long and short villous projections from the

granulocyte cell surface can be seen.

Magnification 5,900 x.

FIG. 5.4   A granulocyte which has been exposed to polystyrene

(top)   latex beads. As in Fig. 5.1, a rounded region which

            probably represents a nuclear lobe can be seen at

            'A'. The smaller round surfaces (asterisks) are the

            size of the latex beads (cf. Fig. 5.5). These four

            beads appear to be internalized, as a thin covering

            membrane can be seen to surround them.

            Magnification 11,700 x.

FIG. 5.5   A granulocyte which has been treated with cytochalasin

(bottom)   B and exposed to polystyrene latex beads. Many beads

            are adherent to the cell surface. One bead is

            attached to the surface of the cell by a stalk-like (*)

            projection. Magnification 11,300 x.
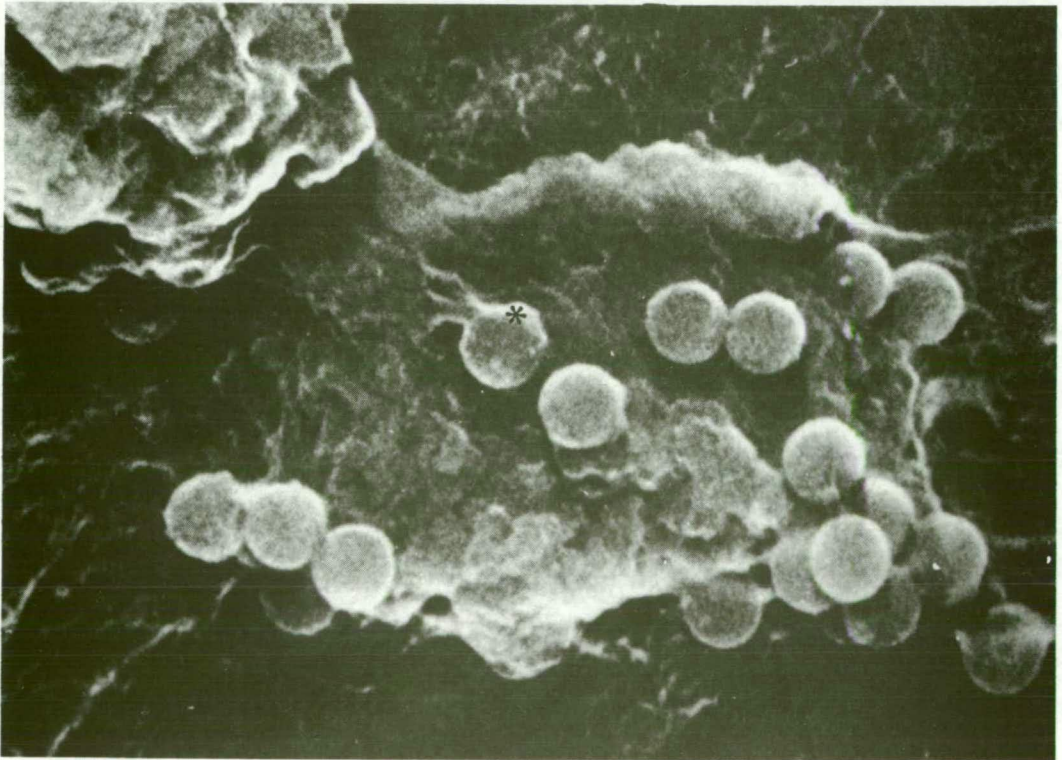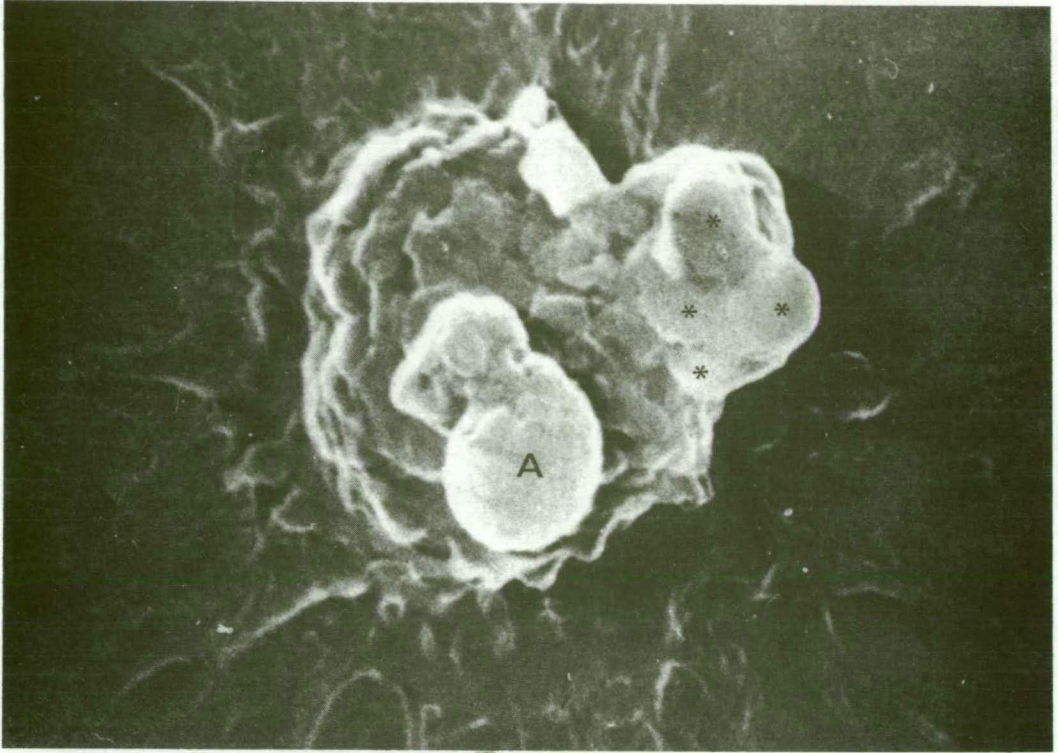
TABLE 5.1   Phagocytosis by drug-treated granulocytes

Treatment	OD at 259 nm (polystyrene)
Drugs - cytochalasin B	0.041±0.006
colchicine	0.068±0.004
vinblastine	0.058±0.002
Controls - no treatment	0.091±0.005
- lumicolchicine	0.092±0.007
- DMSO 0.25% v/v*	0.093±0.006

OD values shown are the $\bar{X}$ ± SEM of 6 determinations (3 experiments performed in duplicate), representing the polystyrene content of the granulocytes. All of the drugs produced a significant depression in phagocytosis with p <.01 compared to the untreated control.

* DMSO content of cyto B-treated monolayers.

TABLE 5.2   Protein content of treated granulocyte   monolayers

Group	Treatment	OD at 540 nm (protein)
1	cytochalasin B	0.032±0.002
	colchicine	0.036±0.002
	vinblastine	0.031±0.004
2	phagocytosis of polystyrene beads	0.035±0.005
3	cytochalasin B/phagocytosis	0.034±0.004
	colchicine/phagocytosis	0.033±0.002
	vinblastine/phagocytosis	0.039±0.007
4	control - no treatment	0.034±0.004

OD values shown are the $\bar{X}$ ± SEM of 4 determinations (2 experiments in duplicate), representing the protein content of granulocyte monolayers. Monolayers for groups 1 and 4 were exposed to the drugs at 1 µM (or no drug in the case of the control) for 40 min (section 5.2.3). Group 2 monolayers were incubated with polystyrene beads for 30 min (section 5.2.3). Group 3 were exposed to the drugs at 1 µM while ingesting beads, for 30 min (section 5.2.5).

TABLE 5.3   NBT reduction by cyto B-treated phagocytic granulocytes

Treatment	Incubation Time	
	5 min	20 min
1. control - no treatment	0.103±.008	0.097±.004
2. DMSO 0.25% v/v *	0.105±.004	0.102±.008
3. phagocytosis of beads	0.136±.008	0.171±.008
4. phagocytosis/DMSO 0.25% v/v	0.131±.005	0.168±.007
5. phagocytosis/cyto B	0.167±.004	0.198±.009

Values given are OD (of NBT, at 515 nm/1.3×10^6 granulocytes/5 min, and are the $\bar{X}$ ± SEM of 9 determinations (3 experiments in triplicate). All treatments (except for DMSO 0.25%) resulted in significantly enhanced NBT reduction (p <.01 for each treatment when compared with its 5 or 20 min control). In addition, granulocytes exposed to cyto B while phagocytosing (line 5) showed a significant stimulation in NBT reduction when compared with phagocytosing granulocytes (line 3). For both 5 and 20 min treatments in these cases, the probability was also <.01.

* DMSO content of monolayers exposed to 1 μM cytochalasin B.

TABLE 5.4   NBT reduction by colchicine-treated phagocytic granulocytes

Treatment	Incubation Time	
	5 min	20 min
1. control (no treatment)	0.092±.006	0.096±.003
2. lumicolchicine	0.094±.003	0.091±.008
3. phagocytosis of beads	0.142±.005	0.182±.006
4. phagocytosis/lumicolchicine	0.148±.003	0.178±.004
5. phagocytosis/colchicine	0.122±.004	0.110±.003

Values given are OD (of NBT) at 515 nm/1.3×10^6 granulocytes/
5 min, and are the $\bar{X}$ ± SEM of 9 determinations (3 experiments
in triplicate). All treatments (except for lumicolchicine)
resulted in significantly enhanced NBT reduction ($p < .01$ for
each treatment when compared with the appropriate 5 or 20 min
control). However, granulocytes exposed to colchicine while
phagocytosing (line 5) showed a significant depression in
reduction when compared with phagocytosing granulocytes (line 3).
For both 5 and 20 min treatments in these cases, $p < .01$.

TABLE 5.5   NBT reduction by VBL-treated phagocytic granulocytes

| | Treatment | Incubation Time | |
		5 min	20 min
1.	control (no treatment)	0.098±.004	0.102±.004
2.	phagocytosis of beads	0.137±.008	0.190±.007
3.	phagocytosis/VBL	0.113±.005	0.116±.003

Values given are OD (of NBT) at 515 nm/1.3×10^6
granulocytes/5 min, and are the $\overline{X} \pm$ SEM of 6
determinations (2 experiments in triplicate). All
treatments resulted in significantly enhanced NBT
reduction (p <.01 for each treatment when compared
with its 5 or 20 min control). However, granulocytes
exposed to VBL while phagocytosing beads (line 3)
showed a significant drop in NBT reduction when
compared with phagocytosing granulocytes (line 2).
The drop in both cases had a probability <.01.

TABLE 5.6   Phagocytosis by colch or VBL-treated granulocytes *

Phagocytosable particle	Slight or no inhibition of phagocytosis by drugs	Inhibition of phagocytosis by drugs
polyvinyltoluene beads	Ukena and Berlin (1972)	Lehrer (1973)
polystyrene beads		Lehrer (1973), and this thesis (Table 5.1)
heat killed yeast	Penny et al (1966)	
zymosan		Weissmann, Dukor and Zurier (1971)
C. albicans		Lehrer (1973)
starch seeds of Saponaria vaccaria		Chang (1975)
paraffin oil emulsion containing Oil Red O	Oliver, Ukena and Berlin (1974)	Stossel et al (1972)
Staph. albus	Malawista and Bodel (1967)	
Staph. aureus 502A	Malawista (1971), Lehrer (1973)	
Streptococcus	Malawista and Bodel (1967)	
uric acid crystals		Goldfinger, Howell and Seegmiller (1965)
monosodium urate crystals		Malawista and Seegmiller (1965)
sodium orotate crystals		Malawista and Seegmiller (1965)

* see section 5.4

TABLE 5.7   Oxidase activity of phagocytic granulocytes

exposed to colch or VBL[*]

Procedure for assaying oxidase activity	Phagocytosable particle	No effect from drugs	Decrease in activity from drugs
O_2 uptake	B. mycoides	Zabucci et al (1978)	
O_2 uptake	C. albicans	Lehrer (1973)	
cytochrome C reduction	E. coli C.		Curnutte and Babior (1975)
O_2 uptake	S. aureus 502A		Malawista (1971), and Lehrer (1973)
O_2 uptake	Staph. albus		Malawista and Bodel (1967)
O_2 uptake	polyvinyltoluene beads		Lehrer (1973)
O_2 uptake	polystyrene beads		Lehrer (1973)
NBT reduction (spectrophotometric)	polystyrene beads		this thesis (Tables 5.4 and 5.5)
NBT reduction (histochemical)	freeze-dried bacterial extract (Sigma Code 840 -15)		Toso, Chilosi and Antoci (1978)

*   see section 5.4

TABLE 5.8   Oxidase activity of phagocytic granulocytes

exposed to cyto B*

Procedure for assaying oxidase activity	Phagocytosable particle	Depression or no effect from cyto B	Stimulation from cyto B
cytochrome C reduction	*E. coli* C		Curnutte and Babior (1975)
O$_2$ uptake	latex beads	Zabucchi *et al* (1978)	
NBT reduction (spectrophotometric)	polystyrene beads		this thesis (Table 5.3)
cytochrome C reduction	opsonized zymosan		Goldstein, Roos, Kaplan and Weissman (1975), Roos, Goldstein, Kaplan and Weissmann (1976), and Jandl *et al* (1978)(see text)
cytochrome C reduction	opsonized zymosan	Roos, Homan-Müller and Weening (1976)	Roos, Homan-Müller and Weening (1976) (see text)
cytochrome C reduction	opsonized zymosan	Jandl *et al* (1978) (see text)	
O$_2$ uptake	opsonized zymosan	Roos, Homan-Müller and Weening (1976)	
O$_2$ uptake	opsonized zymosan	Zabucchi *et al* (1978)	
O$_2$ uptake	*Staph. aureus* 502A	Root and Metcalf (1977)	
cytochrome C reduction	*Staph. aureus* 502A		Root and Metcalf (1977)
O$_2$ uptake	*B. mycoides*	Zabucchi *et al* (1978)	
O$_2$ uptake	paraffin oil emulsion containing Oil Red O	Zabucchi *et al* (1978)	

*   see section 5.4

TABLE 5.9 (continued)

Phagocytosable particle	Inhibition of phagocytosis by cyto B	Incomplete inhibition of phagocytosis by cyto B
Staph. aureus 502A		Malawista, Gee and Bensch (1971)
		Zigmond and Hirsch (1972)
		Cannarozzi and Malawista (1973) (see text)
		Root and Metcalf (1977)
Staph. aureus 209P		Okuda *et al* (1976)
Staph. albus 'air'		Zigmond and Hirsch (1972)
E. coli K38		Zigmond and Hirsch (1972)
E. coli		Davies *et al*** (1973)
Latex beads		Zigmond and Hirsch (1972), and this thesis (Table 5.1)
S. typhimurium RIA		Zigmond and Hirsch (1972)

* see section 5.4   ** These authors do not state that the inhibition was incomplete, but their data points strongly in that direction.

TABLE 5.9   Phagocytosis by cyto B-treated granulocytes[*]

Phagocytosable particle	Inhibition of phagocytosis by cyto B	Incomplete inhibition of phagocytosis by cyto B
zymosan	Zurier, Hoffstein and Weissmann (1973 a,b)	Zigmond and Hirsch (1972)
opsonized zymosan	Goldstein, Roos, Kaplan and Weissmann (1975)	Skosey et al (1974)
	Roos, Homan-Müller and Weening (1976)	Jandl et al (1978)
	Roos, Goldstein, Kaplan and Weissmann (1976)	
starch granules from sweet potatoes	Tou and Stjernholm (1975)	
starch particles from Saponaria vaccaria		Cannarozzi and Malawista (1973) (see text)
monosodium urate crystals		Spillberg, Gallacher and Mendell (1975)
B. mycoides		Zabucchi et al (1978)

SECTION   6

ASSAY OF THE PRESENCE OR ABSENCE OF

MICROTUBULES IN DRUG-TREATED GRANULOCYTES

6.1   INTRODUCTION

Concanavalin A (con A) is a plant lectin which binds

to most plasma membrane glycoproteins bearing α-mannopyranosyl

molecules (Hunt and Marchalonis, 1974). Consequently there are

many different con A receptors on the cell surface. (Allan,

Auger and Crumpton, 1972; Henkart and Fisher, 1975), and these

are heterogeneous with respect to their affinity for the lectin

(Allan, Auger and Crumpton, 1972; Cuatrecasas, 1973).

Fluorescently labelled con A (con A-FITC) has been

used by many investigators to study the surface distribution of con

A binding sites. By incubating cells with con A-FITC and then

examining them under ultraviolet light, it has been shown that

the binding sites for the lectin are randomly distributed on most

cell types (reviewed by Schreiner and Unanue, 1976) including

granulocytes (Oliver, Zurier and Berlin, 1975; Oliver and Zurier,

1976; Oliver, 1976 a,b). With the passage of time the randomly

distributed bound con A induces its own surface redistribution

into caps (or single aggregates) localized to one end or pole of

the cell (Oliver, Zurier and Berlin, 1975). Similarly, bound con

A moves into a cap when cytoplasmic microtubules are disrupted by

colchicine or other anti-mitotic agents. This phenomenon has

been observed in many cell types (reviewed by Nicolson, 1976;

Schreiner and Unanue, 1976), including mouse and human

granulocytes (Oliver, Zurier and Berlin, 1975; Oliver and Zurier,

1976; Oliver, 1976 a,b) and leukaemic granulocyte precursors

(Lotem, Vlodavsky and Sachs, 1976).

Oliver and her colleagues have taken advantage of this

observation in designing an assay for the presence or absence

of microtubules in granulocytes (reviewed in Oliver, 1976 a,b).

The assay involves labelling the con A-binding sites on the plasma

membrane with con A-FITC and then examining the surface distribu-

tion of the bound con A by fluorescence microscopy. As detailed

by these authors, a marked increase in the proportion of

granulocytes which show a capped pattern of fluorescence provides

evidence that microtubules have been disrupted (reviewed in

Oliver, 1976 a,b).

The aim of the experiments presented herein was to use

the assay of Oliver and her colleagues to determine if cytoplasmic

microtubules are disrupted by drug treatment of the granulocytes

using the experimental conditions described in section 4.2 of

the thesis. Treatment of the granulocytes with the agents in

question (the microtubule-disrupting agents colchicine, VBL and

VCR) produced significant changes in the rate of NBT reduction

from the plasma membrane, and it was proposed that this effect

was mediated by microtubule disruption (section 4.4). Therefore

the experiments described in this section of the thesis were

designed to determine if microtubules were indeed disrupted by

these agents.

6.2   METHODS

The assay for the presence or absence of microtubules

in granulocytes (using con A-FITC) was taken from Oliver,

Albertini and Berlin (1976). The experiments presented herein

were comparable to those presented in section 4.2 with respect

to the doses and incubation times with the microtubule-

disrupting agents, colch, VBL and VCR.

6.2.1   The assay for con A receptor redistribution

Monolayers of granulocytes were prepared on glass coverslips by adding 10 µl of the granulocyte suspension (= 2.5×10^5 cells; isolated as described in section 2.2.13) to 50 µl of CMFH, and incubating at 37°C for 5 min. Then 50 µl of HSS (at 37°C) with or without drugs (section 6.2.3) was added to the monolayer and the granulocytes incubated 5 min. After this time 100 µl of con A-FITC at 20 µg/ml in CMFH at 37°C (section 6.2.4) was added and the monolayers incubated for fixed time periods of 2, 5 or 10 min. The final drug and con A-FITC concentrations for these experiments were 1 µM and 10 µg/ml, respectively. [The drugs were not removed during this incubation with the fluorescent lectin and therefore the concentrations reported refer to the concentrations present at the time of incubation with con A-FITC. This experimental procedure was also followed by Oliver, Albertini and Berlin (1976).] The incubations were terminated by repeated and vigorous rinsing of the monolayers in several changes of CMFH (at 4°C) containing sodium azide (0.1% w/v) to reduce pinocytosis of the con A-FITC (Preud'homme and Labaume, 1976)[1].

The monolayers were then air-dried, fixed in absolute ethanol for 5 min and dried again. The coverslips were inverted and mounted in 1:1 phosphate-buffered saline:glycerol (section

---

[1]At this stage, representative monolayers were used for post-incubation viability determinations using Eosin Y.

6.2.5), and then sealed with nail varnish (Preud'homme and

Labaume, 1976). Monolayers prepared in this fashion were examined

in a Leitz Orthoplan fluorescence microscope with Ploemopak[2] for

incident light illumination. The surface distribution of the

bound lectin was scored as described in section 6.2.2.

6.2.2   Scoring the surface distribution of the bound con A-FITC

The percentage of granulocytes showing a random, patched

or capped distribution of fluorescence was determined. As defined

by Oliver, Zurier and Berlin (1975), capped cells exhibited either

a polar shell of fluorescence or a more concentrated knob or pro-

trusion on the membrane; patched cells showed small clumped

aggregates which were often perinuclear; and random cells were

diffusely (= uniformly) labelled by the fluorescent con A.

The distribution of fluorescence was observed on at

least 100 cells in any one experimental group. All tests were

performed in duplicate, and the entire experiment was conducted

twice.

6.2.3   Preparation of the drugs in HSS

The drugs were prepared at 4 µM in HSS to provide a

final drug concentration of 1 µM during incubation with con

A-FITC (section 6.2.1). To prepare the drugs in HSS the

following dilution procedure was employed:-

---

[2] Filter block I_2, exciting filter BP 450-490, beam splitting
mirror RKP 510 (for blue excitation), and suppression filter
LP 515.

stock solution of drug in HSS = 1 mM = 1000 μM = (a)

0.1 ml (a) + 0.9 ml HSS   = 100 μM = (b)

0.1 ml (b) + 0.9 ml HSS   = 10 μM = (c)

0.1 ml (c) + 2.4 ml HSS   = 4 μM = (d)

Dilution (d) was used in the experiments. Parallel (control) experiments were performed using drug-free HSS.

6.2.4 Fluorescently labelled concanavalin A

Fluorescein isothiocyanate labelled con A (con A-FITC) with a fluorescein/protein ratio of 1.1 was used to prepare a stock solution of 6.0 mg/ml in CMFH. Aliquots (100 μl) of this solution were stored at $-20^{\circ}C$. To obtain the dilution required for experiments (20 μg/ml), the following procedure was followed:-

stock solution of 6.0 mg/ml = (a)

0.1 ml (a) + 0.9 ml CMFH = 0.6 mg/ml = (b)

1.0 ml (b) + 4.0 ml CMFH = 0.12 mg/ml = (c)

1.0 ml (c) + 5.0 ml CMFH = 0.02 mg/ml = (d)

Dilution (d) was used in the procedure described above (section 6.2.1), giving a final con A-FITC concentration of 10 μg/ml during the incubation.

6.2.5 Preparation of phosphate-buffered saline:glycerol

The phosphate-buffered saline (PBS) of Dulbecco and Vogt (1954) was used for these experiments, and contained (per litre):-

8.0 g NaCl

0.2 g KCl

1.15 g disodium hydrogen phosphate

0.2 g potassium dihydrogen phosphate, to a pH of 7.3

The PBS:glycerine for mounting of coverslips
contained 1 volume of PBS to 1 volume of glycerol.

6.3   RESULTS

6.3.1   Post-incubation viability

As assessed by Eosin Y staining, the post-incubation
viability of con A-FITC labelled, drug-treated granulocytes was
$\geq$ 93%, whereas control (untreated) granulocytes were $\geq$ 95% viable.
The differences in viability between treated and untreated
granulocyte monolayers were not statistically significant.

6.3.2   The surface distribution of con A-FITC on drug-treated granulocytes

Untreated granulocytes displayed a predominantly
(> 80%) homogeneous or random distribution of fluorescence
(Figure 6.1). As shown in Tables 6.1 - 6.3, incubation of
granulocytes with any one of the three agents tested (colch, VBL
or VCR, respectively) caused con A receptor translocation as
indicated by the altered distribution of random, patched or
capped cells when compared with the control.

Very high percentages of granulocytes with a
patched distribution of bound con A-FITC were observed in the
drug-treated monolayers. For example, 65% of the cells were
patched in the colchicine-treated, 10 min incubated group (Table
6.1). In contrast, comparatively few patched granulocytes were
seen (5%) in the control (untreated) group (Figures 6.2, 6.3
and Tables 6.1 - 6.3). The percentage of capped cells (Fig 6.4) was
markedly increased in the drug-treated versus control granulocyte
preparations. By examining the data presented in Tables 6.1 -
6.3, it can be seen that the three agents showed only minor

differences in their ability to induce con A receptor

redistribution within the time periods studied.

6.3.3   Time course of con A receptor redistribution

Translocation of the con A receptors was very

rapid in the drug-treated (but not the untreated) monolayers,

being evident within 2 min of incubation with con A-FITC.

Redistribution was apparently complete within 5 min as very

little further change was observed at 10 min (Tables 6.1 - 6.3).

As the time course of redistribution was similar with all three

agents, the data from experiments with colchicine were used to

represent the sequential change in fluorescence distribution

(with time) as a bar graph (Figure 6.5).

6.4   DISCUSSION

In agreement with Oliver, Zurier and Berlin (1975),

Oliver (1976 a,b), and Oliver and Zurier (1976), it was observed

that concanavalin A receptors are found in a predominantly random

or homogeneous distribution on the granulocyte cell surface.   In

contrast, granulocytes incubated with any one of the three agents

tested (colch, VBL or VCR) displayed con A receptor translocation

as indicated by the altered distribution of random, patched or

capped cells. . As shown by Oliver and her coworkers, such

alterations in the surface distribution of the bound lectin signify

that cytoplasmic microtubules are disrupted (Oliver, Albertini

and Berlin, 1976).   The dose employed (1 µM) and brief incubation

periods used with these agents (2, 5 or 10 min) in these

experiments with con A were comparable to the experimental con-

ditions used in section 4.2 of this thesis.   In section 4.2, the

drugs in question (colch, VBL and VCR) produced significant

changes in granulocyte plasma membrane NBT reduction. It was

concluded that it was very likely that these changes were mediated

via a drug-induced disruption of microtubules (section 4.4). As

the correlative experiments on con A receptor redistribution pre-

sented herein indicate that microtubules were indeed disrupted

by these agents, this conclusion now seems valid.

In studying the redistribution of bound con A,

Oliver, Albertini and Berlin (1976) observed that the small

surface aggregates characteristic of patched granulocytes were

intracellular, presumably due to pinocytotic internalization of

the lectin during labelling. From their observations on mouse

and human granulocytes these authors suggested that development

of the three observed patterns seemed to follow the sequence:

random $\longrightarrow$ capped $\longrightarrow$ patched; or random $\longrightarrow$ patched directly

(endocytosis without the intermediate capped stage). In

lymphocytes the sequence is believed to proceed in the opposite

manner, that is: random $\longrightarrow$ patched[3] $\longrightarrow$ capped (reviewed by

Nicolson, 1976). [Once capped, the bound lectin is eventually

internalized through pinocytosis (reviewed by Schreiner and

Unanue, 1976)]. An explanation for the different sequence in

granulocytes and lymphocytes has not been offered by Oliver and

her colleagues, but the fact that (compared to granulocytes)

lymphocytes pinocytose con A very slowly (Loor, 1974; de Petris,

1975) may have some bearing on the observed discrepancy. That

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[3] It should also be pointed out that in lymphocytes the
patching phenomenon is thought to be the result of a non-
energy dependent cross-linking of multivalent ligands (such
as con A) on the surface of the cell (reviewed by Schreiner
and Unanue, 1976).

the redistribution of receptors on the cell surface varies with different cell types has also been discussed by other investigators. For example, although most cell types including lymphocytes (Edelman, Yahara and Wang, 1973) are able to redistribute bound lectin into caps (reviewed by Oliver, Zurier and Berlin, 1975; Nicolson, 1976), some cell types like SV40-transformed fibroblasts only do so in the presence of microtubule disruption (Rosenblith et al, 1973). These differences between cell types are not understood, but for the example cited, the fact that most fibroblasts have an extensive cytoplasmic microtubular system while lymphocytes do not may be relevant (reviewed by Nicolson, 1976).

Using electron microscopy to count the number of cytoplasmic microtubules within a defined cellular area, it has been shown that the binding of con A to the granulocyte cell surface induces the assembly of microtubules (Hoffstein et al, 1976; Oliver, Albertini and Berlin, 1976; Oliver, 1976 a,b). It is thought that the bound con A becomes firmly anchored in the membrane by virtue of transmembrane connections between the lectin's receptor and microtubules underlying the plasma membrane. In fact it is believed that Con A induces these transmembrane connections (Edelman, Yahara and Wang, 1973; Oliver, Zurier and Berlin, 1976; reviewed by Nicolson, 1976, 1977). It is thought that by disrupting microtubules, colchicine frees the bound con A from these transmembrane connections with the microtubules, and thus enhances the translocation of the lectin within the plane of the membrane. However, an alternative hypothesis to explain the colchicine-induced enhancement in receptor

redistribution has been put forward by Edidin and Weiss (1972)

and Unanue and Karnovsky (1974). These workers have suggested

that cell or membrane movements are required to produce aggregates

(such as caps) of ligand-receptor complexes. It is thought that

con A binding immobilizes the cell (indeed, this has been demon-

strated in granulocytes by Ryan, Borysenko and Karnovsky, 1974),

and that colchicine facilitates capping only by restoring cell

movement. In this regard it has been shown that normal and

leukaemic lymphocytes display enhanced cell movement (Jarvis,

Snyderman and Cohen, 1976) as well as enhanced con A

redistribution (reviewed by Schreiner and Unanue, 1976) after

colchicine treatment. Unfortunately, agents known to enhance

granulocyte cell movement do not concomitantly enhance con A

capping (Oliver, Zurier and Berlin, 1975).

Romeo and his coworkers have shown that the binding

of con A to the granulocyte cell surface activated the cell, in

a fashion akin to the post-phagocytic metabolic burst (reviewed

by Romeo *et al*, 1975). Furthermore, they found that removal

of the bound con A terminated or 'shut off' this activation.

These workers interpreted this to mean that a plasma membrane

switch or trigger for granulocyte activation is put in the 'off'

position when the clustered con A receptors on the cell

surface resume their normal homogeneous distribution upon

removal of the lectin.

With reference to their scheme, it seems there may

be some support *in vivo* from studies of humans and mice afflic-

ted with the Chediak-Higashi syndrome. The granulocytes of

affected mice or humans are able to ingest normally but then

they show a delay in degranulation and the release of lysosomal

enzymes into the phagocytic vacuoles (Oliver, 1976 a, b; Boxer

et al, 1976). Consequently the bactericidal ability of these

granulocytes is compromised. Furthermore, the azurophilic and

specific granules in these cells aggregate or fuse during

granulocyte maturation (Rausch, Pryzwansky and Spitznagel, 1978).

It is thought that the abnormal fusion of the granules and the

delay in degranulation both represent impaired microtubule

function in these cells (Oliver, 1976 a, b; Rausch, Pryzwansky

and Spitznagel, 1978). In addition, studies of the con A capping

phenomenon in these granulocytes have demonstrated that the

cells cap spontaneously with the binding of the lectin whereas

control granulocytes cap as readily only when treated with

colchicine (reviewed by Oliver, 1976 b). This finding further

emphasizes that the defect is at a microtubular level (Oliver,

Zurier and Berlin, 1975; Oliver, 1976 b). [Recently it was

shown that by elevating the levels of cGMP (a manoeuvre which

promotes microtubule assembly in Chediak-Higashi granulocytes),

the abnormal degranulation and capping phenomena were restored

to normal (reviewed by Oliver, 1976 b).]

The abnormal clustering or redistribution of con A

receptors on the cell surface may be related to the delay in

degranulation, as both are microtubule-mediated events (Oliver

1976 b; Boxer et al, 1977). That is, it seems reasonable to

assume that surface receptors redistribute before a signal is

conveyed (via the cytoskeleton) to the interior of the cell

(reviewed by Nicolson, 1977). In granulocytes such signals could

undoubtedly influence such events as the post-phagocytic

metabolic burst and degranulation[4]. Therefore if receptor

redistribution is aberrant as in the Chediak-Higashi syndrome, it

would not be unusual to find that degranulation is also impaired.

6.5   SUMMARY

In this section of the thesis the assay designed by

Oliver and her colleagues was used to determine if treatment of

granulocytes with μM doses of colchicine, VBL or VCR for brief

periods of time (< 10 min) resulted in the disruption of micro-

tubules. The assay involves examining the distribution of bound

fluorescein-labelled concanavalin A (con A-FITC) on the granulo-

cyte cell surface by fluorescence microscopy (Oliver, Albertini

and Berlin, 1976). Oliver and her coworkers have established that

alterations in the surface distribution of the bound lectin

signify that cytoplasmic microtubules are disrupted.

In the experiments presented herein, it was observed

that the fluorescence pattern of con A-FITC on the surface of

untreated granulocytes was predominantly random or homogeneous,

as other investigators have demonstrated (Oliver, Zurier and

Berlin, 1975; Oliver, 1976 a,b; Oliver and Zurier, 1976). In

contrast, granulocytes incubated with colchicine, VBL or VCR

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[4] It is likely that signalling of the cell interior would require
the participation of microfilaments as well as microtubules,
because cell surface phenomena including the redistribution of
bound ligands are known to be influenced by both cytoskeletal
elements (Nicolson, 1976; Nicolson, 1977). Such a dual influence
from both cytoskeletal elements appears to be established for
granulocytes, as a representative experiment demonstrated that
the colchicine-induced enhancement in con A capping was
inhibited when cytochalasin B was present during the incubation
(Ryan, Borysenko and Karnovsky, 1974).

displayed marked con A receptor translocation into patched

or capped surface distributions, indicating that cytoplasmic

microtubules were indeed disrupted by the drug treatments.

FIG. 6.1   Granulocytes (untreated) showing a random

(top)   (homogeneous) distribution of Con A-FITC on the

cell surface. These cells were stained with

methyl green as a fluorescent counterstain

(Schenk and Churukian, 1974) to demonstrate the

nuclear lobes. Magnification 1,400 x.

FIG. 6.2   Granulocytes (untreated) showing a patched distribution

(middle)   of con A-FITC. The patchy labelling is localized to

the perinuclear region in cells 'A' and 'B'.

Magnification 2,500 x.

FIG. 6.3   Granulocytes (untreated) displaying patched (A,C,D)

(bottom)   and capped (B) distributions of con A-FITC.

Magnification 3,200 x.
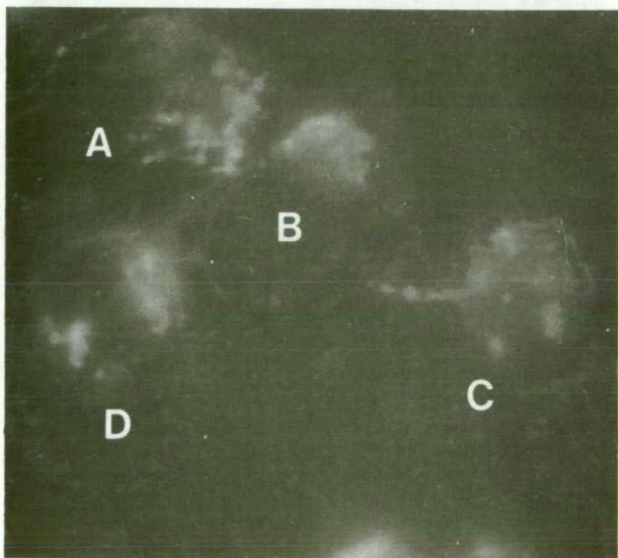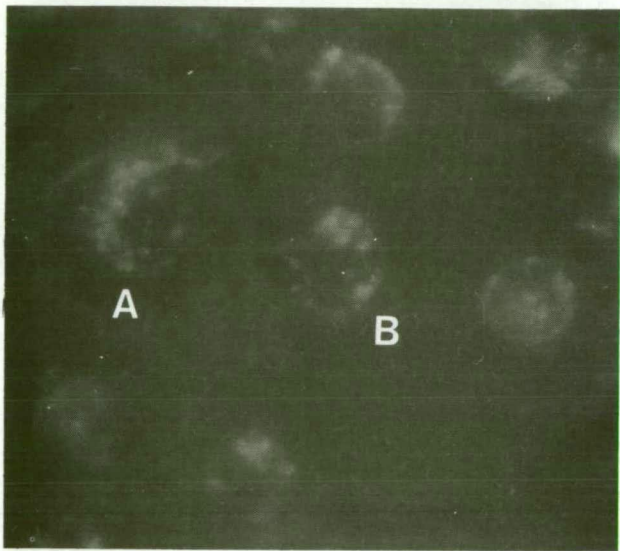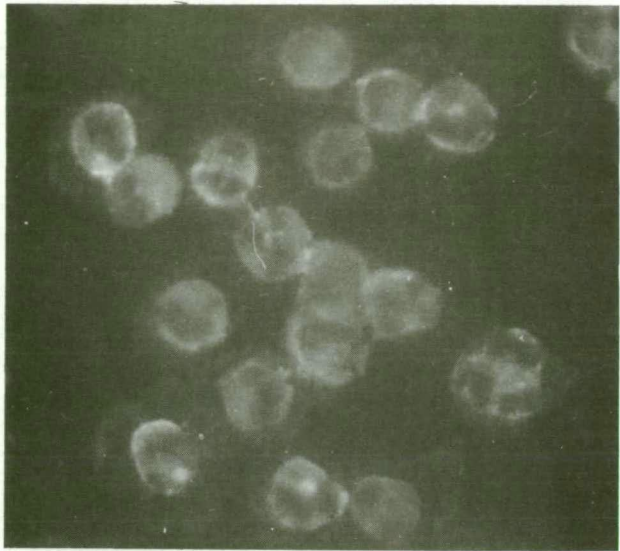
FIG. 6.4   Granulocytes which have been incubated 5 min

with colchicine (see text). Cells with a

capped distribution of con A-FITC are indicated

by the asterisks. A granulocyte with a random

fluorescence pattern is seen at 'A' (bottom).

Notice that the size of the cap is markedly

different in the two granulocytes shown in the

two photographs. Magnification (top and bottom)

4,400 x.
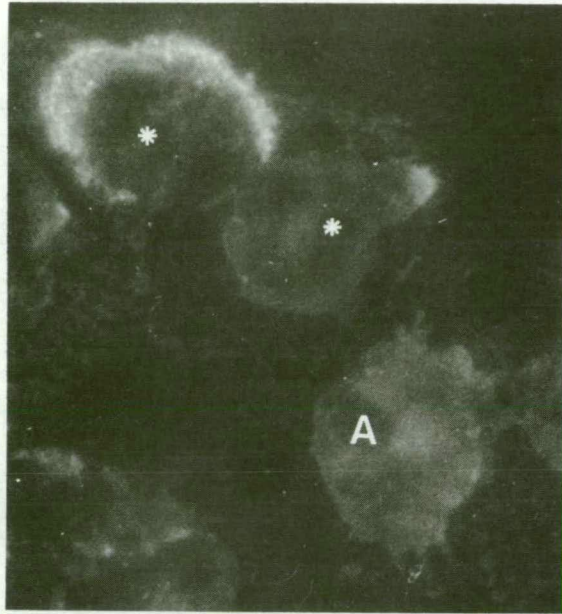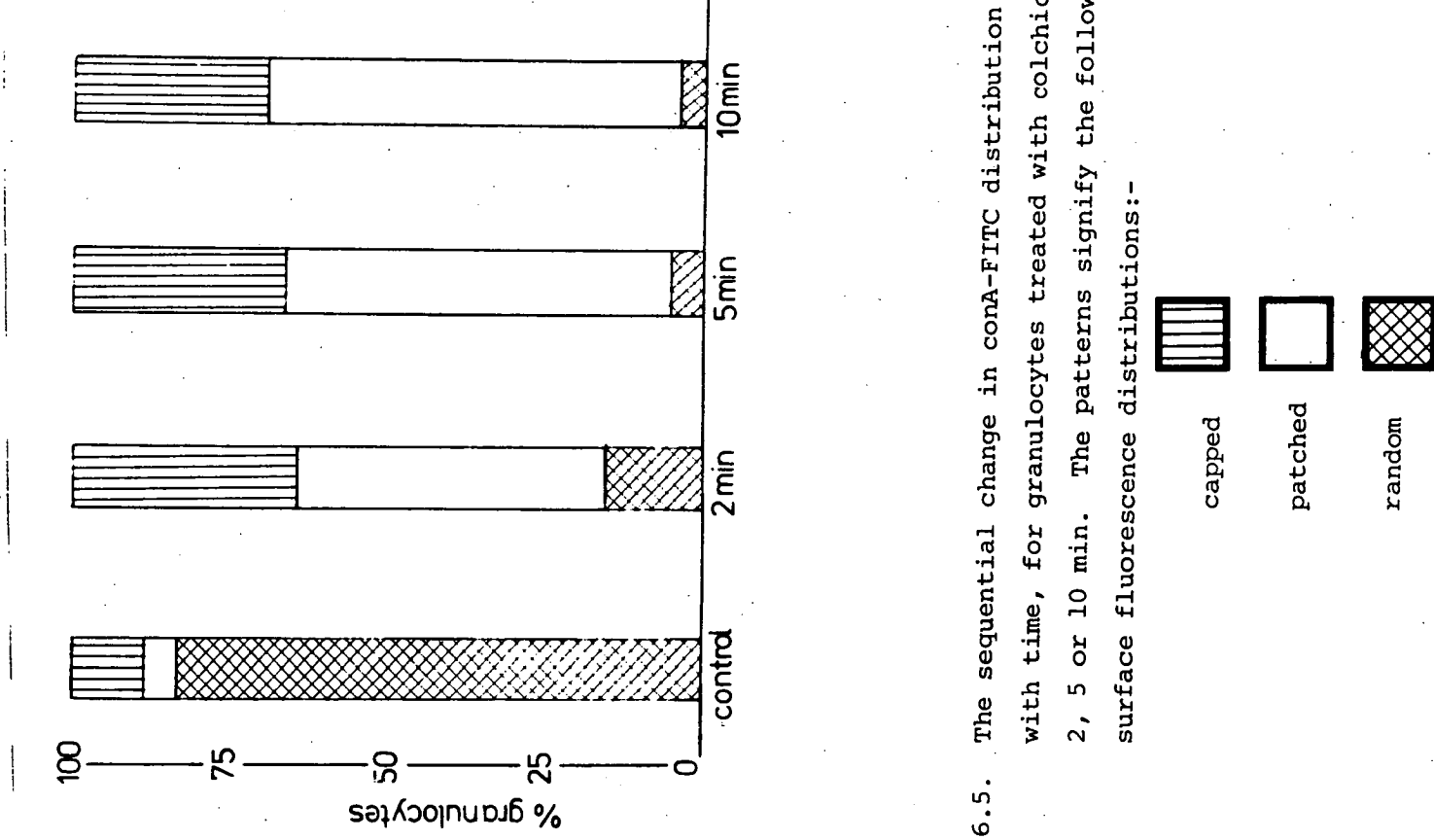
FIG. 6.5. The sequential change in conA-FITC distribution with time, for granulocytes treated with colchicine 2, 5 or 10 min. The patterns signify the following surface fluorescence distributions:-

capped

patched

random

TABLE 6.1   The effect of colchicine treatment on con A-FITC receptor translocation

Distribution	Control (untreated	Incubation time with con A-FITC in the presence of colchicine		
		2 min	5 min	10 min
capped	12	36	34	31
patched	5	49	62	65
random	83	15	5	4

Numbers represent the percentage of granulocytes showing a random, patched or capped distribution of con A-FITC. The cells were exposed to colchicine at 1 μM for 5 min and then incubated with con A-FITC (in the presence of the drug) for another 2, 5 or 10 min. At least 100 granulocytes were then counted (in each of 2 experiments) to determine the distributions given in this table.

TABLE 6.2   The effect of VBL treatment on con A-FITC receptor translocation

Distribution	Control (untreated)	Incubation time with con A-FITC in the presence of VBL		
		2 min	5 min	10 min
capped	12	39	40	34
patched	5	51	59	63
random	83	10	1	3

Numbers represent the percentage of granulocytes showing a random, patched or capped distribution of con A-FITC. The cells were exposed to VBL at 1 μM for 5 min and then incubated with con A-FITC (in the presence of the drug) for another 2, 5 or 10 min. At least 100 granulocytes were then counted (in each of 2 experiments) to determine the distributions given in this table.

TABLE 6.3   The effect of VCR treatment on con A-FITC receptor redistribution

Distribution	Control (untreated)	Incubation time with con A-FITC in the presence of VCR		
		2 min	5 min	10 min
capped	12	42	34	30
patched	5	50	62	65
random	83	8	4	5

Numbers represent the percentages of granulocytes showing a random, patched or capped distribution of con A-FITC. The cells were exposed to VCR at 1 µM for 5 min and then incubated with con A-FITC (in the presence of the drug) for another 2, 5 or 10 min. At least 100 granulocytes were then counted (in each of 2 experiments) to determine the distributions given in this table.

SECTION 7

EXPERIMENTS WITH THE FLUORESCENT MEMBRANE

PROBE, ANS

7.1   <u>INTRODUCTION</u>

l-anilino-8-naphthalene sulphonate (ANS) is a

chromophore which is not fluorescent in water (Stryer, 1965)

but becomes fluorescent in hydrophobic environments, for

example, when dissolved in organic solvents or associated with the

non-polar regions of proteins (Stryer, 1965; Dodd and Radda, 1967;

Edelman and McClure, 1968). ANS also interacts with biological

membranes and presumably binds at specific chemical sites

(possibly phospholipids) distributed throughout the thickness of

the membrane (Azzi et al, 1969; Vanderkooi and Martonosi, 1969).

The binding site is a relatively hydrophobic environment

compared to the water phase outside the cell membrane (Azzi

et al, 1969) and therefore the binding of ANS to the membrane

produces a fluorescent complex (Rubalcava et al, 1969; Harris,

1971; Vanderkooi and Martonosi, 1971). The changes in

fluorescence of membrane-bound ANS are a reflection of the

changes in the microenvironment of the chromophore (Stryer,

1965).

ANS has been used as a fluorescent probe to study

(i)   hydrophobic protein binding sites (Weber and Young,

1964; Stryer, 1965)

(ii)   conformational states of proteins (Stryer, 1965;

Dodd and Radda, 1967).

(iii) the rates of allosteric transitions (Dodd and Radda,

1968)

(iv)   the dynamic state of membranes, for example, skeletal

muscle membrane preparations (Vanderkooi and

Martonosi, 1969), mitochondrial membrane fractions

(Azzi *et al*, 1969), and erythrocyte membranes

(Freedman and Radda, 1969; Robak *et al*, 1975).

It is thought that changes in ANS fluorescence occur when the polarity of its environment is altered, for example, by spatial re-arrangements of proteins or changes in the lipid-protein interactions in the membrane (Azzi *et al*, 1969; Vanderkooi and Martonosi, 1969). Other changes in the micro-environment such as in the pH or viscosity of the medium are apparently of lesser quantitative significance (Vanderkooi and Martonosi, 1969). It has been said that ANS is a better probe for membrane structure than electron spin or nuclear magnetic resonance because it allows one to correlate the time course of the observed changes in membrane structure with the functional state of the membrane (Azzi *et al*, 1969). With mitochondrial membranes for example, a marked increase in fluorescence from the ANS-membrane complex was seen within seconds of the addition of substrates (succinate, ATP, NADH, O_2), and these changes in fluorescence were related to but not synchronous with electron transport processes in the mitochondrial membranes (Azzi *et al*, 1969).

The aim of the studies presented in this section of the thesis was to investigate (by using ANS-labelled granulocytes) whether the drug-induced changes in plasma membrane oxidase activity were associated with a structural change in the plasma membrane. The results of the experiments on plasma membrane oxidase activity (Section 4) suggested the possibility that the drugs in question influence oxidase activity by acting directly on the plasma membrane rather than on microtubules

and microfilaments.

7.2   <u>METHODS</u>

        The methods outlined below were taken from Romeo,

Cramer, and Rossi (1970) and Robak *et al* (1975).

7.2.1   <u>Labelling of the granulocyte plasma membrane with ANS</u>

        Granulocytes were isolated as described in section

2.2.13, except that they were resuspended (after the final wash)

at a concentration of 4.5 x 10^7 cells/ml in CMFH. The granulocytes

were divided into two aliquots: those to be labelled with ANS

and control (unlabelled) granulocytes. The viability of the

granulocytes as assessed by Eosin Y dye exclusion was 98%.

        To label the cells, excess ANS solutions (30 μM in

HSS) was added to the granulocytes in a plastic conical test

tube. The tube was then incubated for 5 min at 37°C in a water

bath. Unlabelled granulocytes were handled in parallel and

incubated in HSS without added ANS. After labelling, the

granulocytes were harvested by centrifugation, washed in CMFH

at 4°C, and resuspended in CMFH at 4.5 x 10^7/ml. Post-labelling

viability was 90%.

7.2.2   <u>Fluorescence intensity of ANS-labelled granulocytes</u>

        To compare the fluorescence intensity of labelled and

unlabelled granulocytes, fluorimeter cuvettes of 900 μl capacity

were filled with 70 μl CMFH at 37°C. To the cuvette was then

added 10 μl of labelled or unlabelled granulocytes (= 4.5 x 10^5

cells) in CMFH at 37°C. The contents of the cuvette were mixed

with the microlitre pipette and the fluorescence intensity was

measured with a Baird Atomic fluorimeter set at 380 nm

(excitation) and 472 nm (fluorescence). The fluorescence intensity

was recorded immediately after mixing and again after 5 and 10 minutes incubation in the cuvette.

The effect of varying the number of granulocytes added to the cuvette on the fluorescence intensity was also investigated. For these experiments 10 - 60 μl of granulocyte suspension (= 4.5 - 27.0 x 10^5 cells) were added to the cuvette and the procedure as outlined above was followed.

7.2.3   The fluorescence intensity of drug-treated granulocytes

The experimental procedures used were comparable to those presented in Section 4 with respect to the doses and incubation times with the microtubule or microfilament-disrupting agents.

The fluorimeter cuvettes were filled with 700 μl of CMFH at 37°C and to this was added 10 μl of labelled granulocytes. The baseline fluorescence intensity was recorded immediately after the addition of the cells. Then 80 μl of the drug or control in CMFH at 37°C was added to the cuvette and the contents mixed with the pipette. The final concentration of each particular drug was 1 μM at testing. The fluorescence intensity was recorded immediately after addition of the drug or control, and again after 5 or 10 min incubation. The drugs examined were cytochalasin B, VBL, VCR, colch and lumicolchicine (section 4.2, Appendix IV).

The control for experiments with cytochalasin B was DMSO in CMFH at a concentration of 0.11 % v/v at testing (section 7.2.5).

7.2.4   <u>Preparation of the drugs</u>

All drugs were dissolved in CMFH (except cyto B, which was dissolved in DMSO) at 1 mM. Successive dilutions of these 1 mM stock solutions were made to give a 11 µM drug dilution which was added to the fluorimeter cuvettes. As the drug was diluted 10-fold by the contents of the cuvette the concentration of each drug at testing was 1 µM. The dilution procedure was as follows:-

        stock solution of drug in CMFH   =   1 mM = (a)

        0.1 ml (a) + 2.9 ml CMFH   =   33 µM = (b)

        1.0 ml (b) + 2.0 ml CMFH   =   11 µM = (c)

Dilution (c) was added to the fluorimeter cuvettes for tests.

7.2.5   <u>Controls for the drug incubations</u>

As the stock solutions of colch, lumicolch, VBL, and VCR were prepared in CMFH the control for these drugs was CMFH without added drug. Cytochalasin B, in contrast, was prepared as a stock solution in DMSO and therefore successive dilutions of this solvent were made to obtain the appropriate control, as indicated below:-

        DMSO at 100 % v/v   =   control stock solution   =   (a)

        0.1 ml (a) + 2.9 ml CMFH = 3.3 % v/v DMSO   =   (b)

        1.0 ml (b) + 2.0 ml CMFH = 1.1 % v/v DMSO   =   (c)

Dilution (c) was used as the control for cyto B incubations, and therefore at testing the final concentration of DMSO in these experiments was 1.1 % x 1/10 = 0.11 % v/v DMSO in CMFH.

7.2.6   Experiments with phagocytic granulocytes

These experiments were a repeat of the studies performed

on guinea pig granulocytes by Romeo, Cramer, and Rossi (1970).

The only modification from their experimental procedure was in

the use of human rather than guinea pig granulocytes.

To 700 µl of CMFH at 37^OC in the fluorimeter cuvette

was added 10 µl of labelled granulocytes (= 4.5 x 10^5 cells).

The contents of the cuvette were mixed with the microlitre pipette

and the fluorescence intensity was recorded immediately. To the

cuvette was then added 10 µl of dialyzed polystyrene latex beads

(0.79 µm mean diameter, prepared as described in section 5.2).

The contents of the cuvette were again mixed and the fluorescence

intensity recorded immediately, and after 5 and 10 min incubation

with the beads.

For control experiments the beads were added to

unlabelled granulocytes, or to a solution of 33 µM ANS in CMFH,

or to CMFH at 37^OC. The procedure followed was otherwise exactly

as described above for labelled granulocytes.

7.3   RESULTS

7.3.1   Fluorescence intensity of ANS-labelled granulocytes

As shown in Fig. 7.1, unlabelled granulocytes dis-

played some intrinsic fluorescence but this was considerably

less than the fluorescence intensity of the ANS-labelled

granulocytes. In both cases the number of granulocytes in the

cuvette was 4.5 x 10^5.

The fluorescence intensity of the labelled granulocytes

was stable for at least 10 min (Fig. 7.1), and was a linear

function of the number of ANS-labelled granulocytes added to the

fluorimeter cuvette (Fig. 7.2).

7.3.2   The fluorescence intensity of drug-treated granulocytes

When the drugs (cyto B, colch, lumicolchicine, VBL, or VCR) were added to the cuvettes an immediate slight drop in the fluorescence intensity was observed (Fig. 7.3). No further change in the fluorescence intensity occurred with the passage of 5 or 10 min time.

The same immediate (slight) drop in fluorescence was seen when the drug-free controls (CMFH or DMSO, section 7.2.5) were added to the labelled granulocytes (Fig. 7.3). As with the drug-treated granulocytes, there was no further change in the fluorescence with the passage of time.

7.3.3   Experiments with phagocytic granulocytes

As shown in Fig. 7.4, polystyrene latex beads (in CMFH or in a solution of ANS in CMFH) displayed some intrinsic fluorescence. When the beads were added to unlabelled granulocytes a slight rise in fluorescence was observed, but this increase was not of the magnitude of the increase in fluorescence seen when beads were added to labelled granulocytes (Fig. 7.4). The increase in fluorescence occurred within a few seconds of the addition of beads to labelled or unlabelled granulocytes, and then showed no further rise with the passage of 5 or 10 min time.

7.4   DISCUSSION

1-anilino-8-naphthalene sulphonate (ANS) is a chromophore which when bound to biological membranes becomes fluorescent (Rubalcava et al, 1969; Harris, 1971; Vanderkooi and Martonosi, 1971). It is a very useful probe for studying the

dynamic state of membranes because a change in the fluorescence

intensity from the bound ANS indicates that an alteration in

plasma membrane structure has occurred, usually through bio-

chemical or physical activity (Stryer, 1965; Azzi et al, 1969;

Vanderkooi and Martonosi, 1969; Rossi, Romeo and Patriarca, 1972).

It has been shown for example, that membrane-active drugs can

increase or decrease the fluorescence intensity of ANS-labelled

human erythrocyte membranes (Wiethold et al, 1973; Robak et al,

1975). With this in mind experiments designed to monitor the

fluorescence intensity of drug-treated, ANS-labelled human

granulocytes were undertaken and are presented herein. As the

aim of this work was to investigate whether the drug-induced

changes in plasma membrane oxidase activity (see Section 4) were

associated with a structural change in the plasma membrane, the

design of these experiments was made comparable to that presented

in Section 4.

The linear relationship between the fluorescence

intensity and the number of granulocytes indicates that granulo-

cytes incubated with 30 μM ANS for 5 min at 37°C were successfully

labelled with ANS. The binding of ANS to the plasma membrane of

granulocytes or lysed erythrocytes has been shown to occur very

quickly, and is virtually complete by 5 min (Freedman and Radda,

1969; Romeo, Cramer and Rossi, 1970).

There was an immediate slight drop in the intensity of

fluorescence when labelled granulocytes were exposed to any of

the microtubule or microfilament-disrupting agents tested (colch,

VBL, VCR or cyto B). However the same response was seen when

the labelled granulocytes were incubated with the control

solutions (CMFH, DMSO or lumicolchicine). Therefore it was readily apparent that the immediate slight drop in fluorescence arose from adding the small volume of control or drug-containing solution to the existing volume in the fluorimeter cuvette. After this initial change no further change in the fluorescence intensity was seen even when the incubation with any one agent or the appropriate control was continued for as long as 10 min. This finding indicated that a true change in the plasma membrane structure of drug-treated granulocytes had not occurred.

As discussed in section 4.4, an incubation of 5 or 10 min with any one microtubule or microfilament-disrupting agent was sufficient to induce a marked change in plasma membrane oxidase activity. Therefore it may be concluded from these correlative experiments with ANS-labelled granulocytes that the microfilament and microtubule-disrupting agents were not acting directly on the plasma membrane to induce a change in plasma membrane structure, which thereby influenced plasma membrane oxidase activity. Employing complementary experimental techniques, Schlessinger et al (1976), Alstiel and Landsberger (1977), and Edidin (1977) have also concluded that microfilament and microtubule-disrupting agents do not act directly upon the plasma membrane to bring about a response.

In addition to the experiments with drug-treated granulocytes, the experiments of Romeo, Cramer and Rossi (1970), and Rossi, Romeo and Patriarca (1972) on ANS-labelled phagocytic guinea pig granulocytes were repeated, using human granulocytes. These authors hypothesized that the metabolic burst of phagocytos- ing granulocytes (and in particular the enhanced activity of a

granule-associated NADPH oxidase) was regulated by an alteration

of the granulocyte plasma membrane. This theory was based on

their own experimental observations and the studies of several

other groups of investigators (reviewed in Rossi, Romeo and

Patriarca, 1972) which showed that:-

(i)   the increases in respiration and HMPS activity seen

        in phagocytosing granulocytes occur within a few

        seconds of contact with particles

(ii)   these increases in metabolic activity are accompanied

        by a precocious activation of a granule-associated

        oxidase

(iii) the increases can only be seen when intact phagocytes

        are used, and

(iv)   phagocytosis is not necessary for these increased

        metabolic events, in that the increased metabolic

        events can be reproduced by agents (such as

        surfactants and antibodies) which act on the

        granulocyte cell membrane.

        To study whether an alteration in the cell membrane

had occurred with phagocytosis Romeo, Cramer and Rossi (1970)

conducted experiments with the fluorescent membrane probe, ANS.

For their studies the plasma membrane of intact guinea pig

granulocytes was labelled with ANS. Upon observing a dramatic

and instantaneous increase in the fluorescence intensity

of the ANS-granulocyte complex when granulocytes were presented

with polystyrene latex beads, the authors suggested that a

conformational change in the plasma membrane had taken place

once the granulocytes had contacted the beads.

As shown in Fig. 7.4, their experimental observations with guinea pig granulocytes were confirmed in this laboratory, using human granulocytes. A dramatic increase in the fluorescence intensity of ANS-labelled human granulocytes occurred within seconds of contact with polystyrene latex beads. The magnitude of this increase in fluorescence was greater than that seen when the beads (which displayed some intrinsic fluorescence) were added to unlabelled granulocytes, a solution of ANS in CMFH, or CMFH alone. There was no further increase in the fluorescence intensity with the passage of time. A similar finding by Romeo, Cramer and Rossi (1970) led these workers to conclude that the observed change in fluorescence was due to the initial collision between granulocytes and beads and not phagocytosis *per se*. From such experimental observations these workers postulated a scheme wherein (i) contact between beads and the granulocyte plasma membrane produced a change in the membrane, and (ii) this change in the plasma membrane was followed by either release of an unidentified chemical mediator or direct communication between the plasma membrane and cytoplasmic granules, so that (iii) a signal was transmitted to the granule-bound NADPH oxidase, resulting in (iv) enhanced oxidase activity (H_2O_2 and $NADP^+$ production) and thence HMPS activation (see Appendix I).

It seems reasonable to re-evaluate their hypothetical scheme, taking into account the more recent papers showing that the cyanide-insensitive oxidase is not restricted to a granule location, but is found in the plasma membrane of granulocytes as well (Appendix I). With this in mind, the instantaneous enhancement in plasma membrane ANS fluorescence and oxidase

activity upon contact between beads and granulocytes (Romeo,

Cramer and Rossi, 1970) may indicate that contact-induced changes

in the structure of the plasma membrane directly stimulate plasma

membrane oxidase rather than granule-associated oxidase activity.

With respect to this point, it is interesting to note that these

authors later emphasize that their granule fractions are probably

contaminated with plasma membrane (Rossi, Romeo and Patriarca,

1976). Hence it is not possible for these workers to ascribe

their observed contact-induced increase in oxidase activity to a

granule fraction-associated oxidase only. A similar problem

arises when working with intact phagocytic granulocytes, in that it

is very difficult to partition an observed change in oxidase

activity into that which has come from either the plasma membrane

or granule-associated oxidase (see section 5.4).

7.5   SUMMARY

In this section of the thesis experiments with ANS-

labelled granulocytes are described. ANS is a chromophore

which, in binding to the plasma membrane of intact granulocytes

becomes fluorescent. A change in the fluorescence intensity

of this membrane-bound probe signifies that a change in the

probe's microenvironment (presumably the structure of the membrane)

has occurred. Therefore this chemical has been used by several

groups of investigators to study the dynamic state of biological

membranes after treatment with membrane-active drugs.

In section 4.3 of this thesis it was found that

treatment of granulocytes with microtubule or microfilament-

disrupting agents led to changes in the activity of the plasma

membrane oxidase. The aim of the correlative experiments

presented herein was to determine if the microtuble or microfilament-disrupting drugs were acting directly on the plasma membrane to induce a change in the structure of the membrane (as measured by the fluorescence of ANS), which might then be responsible for the observed changes in oxidase activity. As no such drug-induced changes in plasma membrane structure (ANS fluorescence) were observed, it was concluded (in agreement with Schlessinger *et al*, 1976; Alstiel and Landsberger, 1977; and Edidin, 1977) that the microfilament and microtubule-disrupting agents do not act directly upon the plasma membrane to bring about a response.
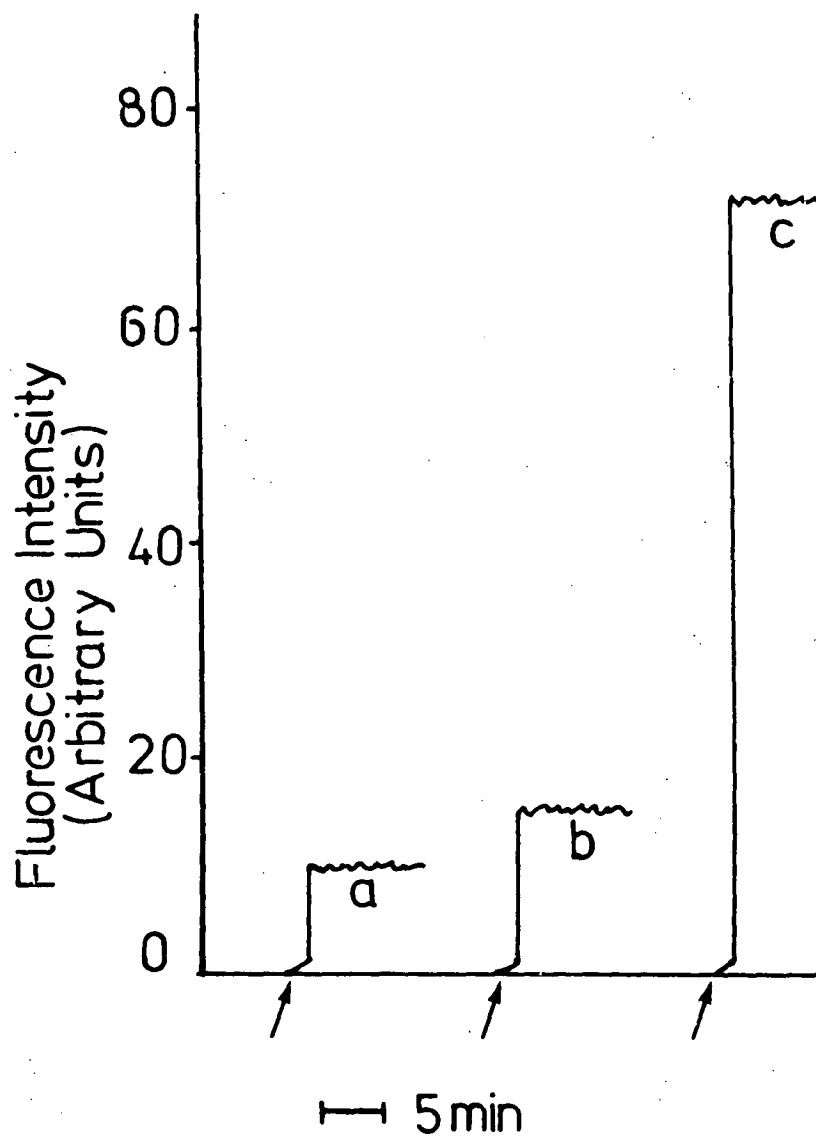
FIG. 7.1. The fluorescence intensity of CMFH (baseline, at 'a');
unlabelled granulocytes (b) and ANS-labelled
granulocytes (c). The pen recorder was allowed to
run for 10 min, as indicated.
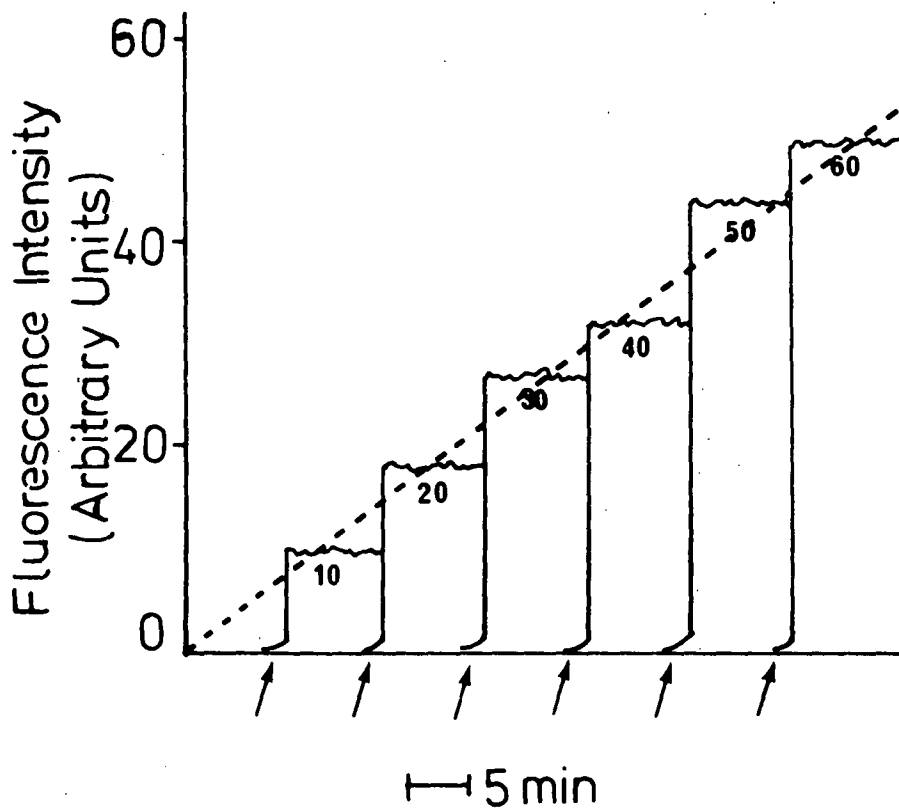
FIG. 7.2. The fluorescence intensity as a function of the
number of ANS-labelled granulocytes present in the
cuvette. For this experiment 10-60 µl of cells were
present (as indicated). The fluorescence intensity of
10 µl (= 4.5 x 10^5 granulocytes) has arbitrarily been
set at 10 units.
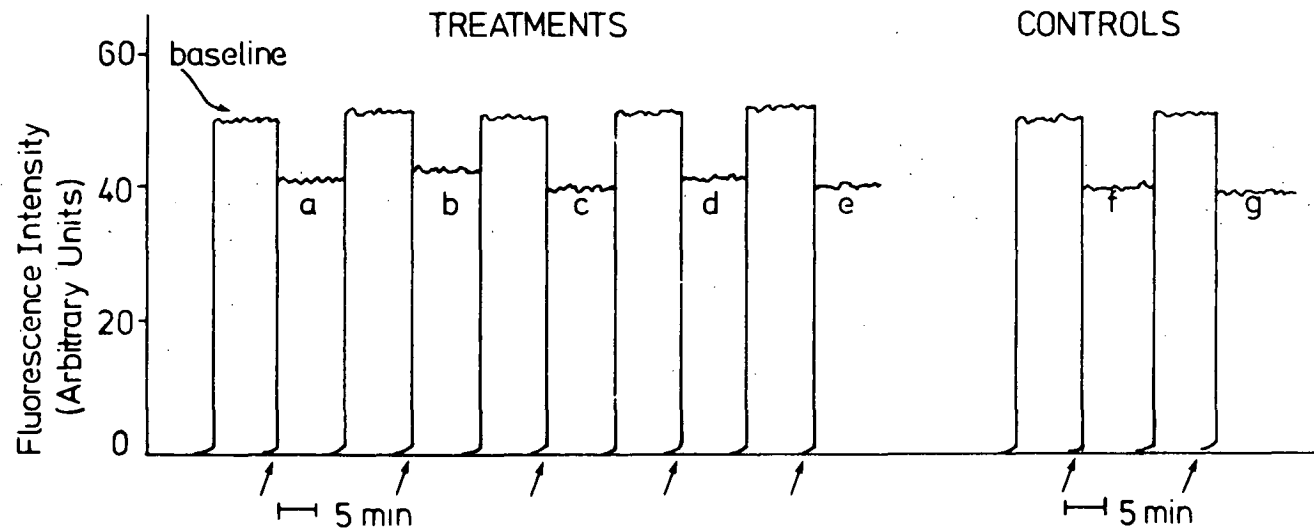
FIG. 7.3. The fluorescence intensity of drug-treated, ANS-labelled granulocytes.

Where indicated by the small arrows, 80 µl of cyto B (a); colch (b);

lumicolchicine (c); VBL (d) or VCR (e) was added to a fresh (control)

cuvette. At (f) and (g), 80 µl of CMFH or DMSO in CMFH (respectively)

was added to the cuvette.
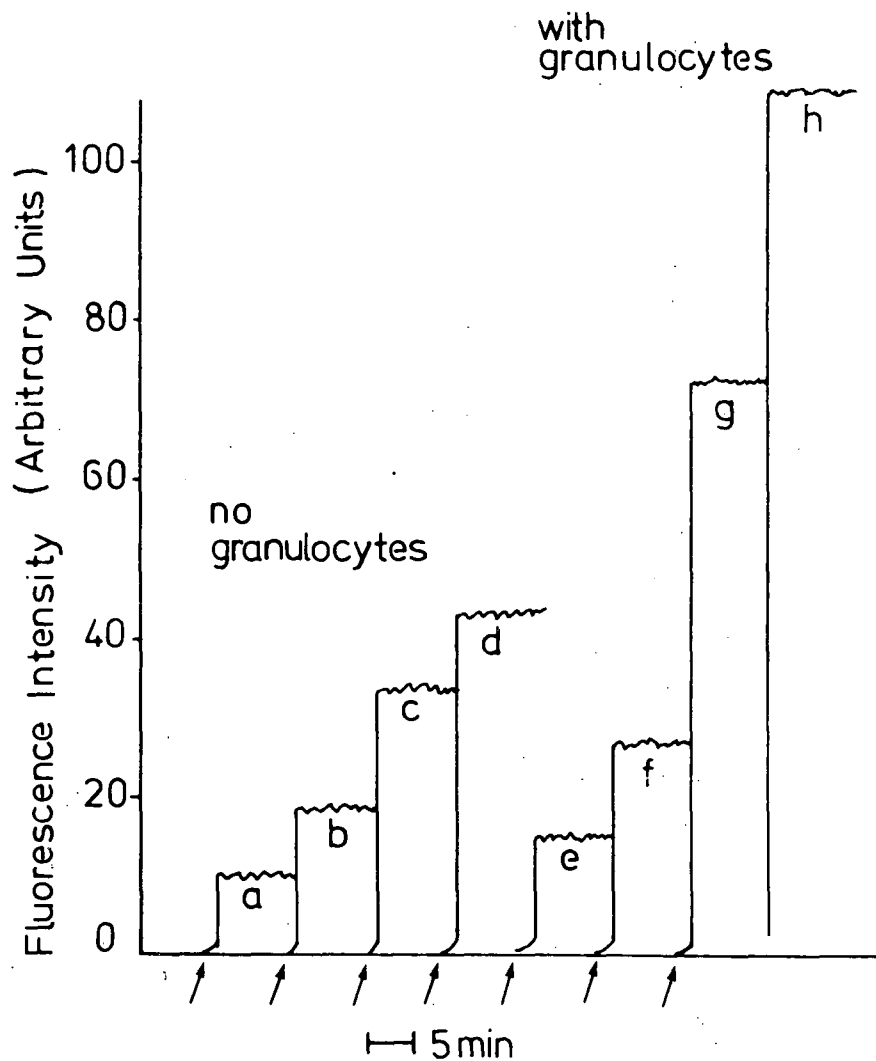
FIG. 7.4. The fluorescence intensity of drug-treated, un-
labelled and ANS-labelled granulocytes. The
additions (arrows) to the fresh (control) cuvettes were CMFH (a);
CMFH + polystyrene latex beads (b); ANS (c); ANS + beads (d).
On the right-hand side of the tracing the additions to fresh
cuvettes were unlabelled granulocytes (e); unlabelled
granulocytes + beads (f); labelled granulocytes (g) and labelled
granulocytes + beads (h). By subtraction the changes in
fluorescence intensity were:-

    (b) - (a) = 8   (f) - (e) = 12
    (d) - (c) = 9   (h) - (g) = 38

SECTION 8

MICROFILAMENT AND MICROTUBULE DISRUPTION

AND HMPS ACTIVITY

8.1   INTRODUCTION

The hexose monophosphate shunt (HMPS) is a metabolic pathway whereby glucose is oxidized to a five-carbon sugar (ribulose-5-phosphate) and CO_2 while $NADP^+$ acts as an electron acceptor (Babior, 1978). The pathway is shown in Figure 8.1.

In erythrocytes the HMPS serves to generate reducing equivalents (in the form of NADPH) which are used by the cell to protect both haemoglobin and the red cell membrane from oxidant stress (Lionetti, 1974; Desforges, 1976). It has not been established whether the HMPS in granulocytes has a similar role, that is, to generate 'protective' reducing power. However it has been suggested that the formation of reducing equivalents (NADPH) coupled to the glutathione peroxidase – glutathione reductase system might protect the granulocyte's enzymes against inactivation by H_2O_2 (Little and O'Brien, 1968). This H_2O_2 could come from the granulocyte itself (as excess H_2O_2 generated above anti-microbial requirements) or from ingested micro-organisms (Klebanoff, 1968).

As discussed in Section 1, phagocytic granulocytes display a metabolic burst characterized by a cyanide-insensitive increase in O_2 uptake, the production of O_2^- and H_2O_2, and a cyanide-insensitive stimulation of HMPS activity, events which are crucial for oxygen-dependent killing by granulocytes. Phagocytosis activates a cyanide-insensitive NADH oxidase and/or a cyanide-insensitive NADPH oxidase, either of which is capable of the O_2^- production. The activation of this O_2^- producing enzyme accounts for the observed increase in O_2 uptake and the

stimulation of the HMPS[1]. Normally only 10% of the granulocyte's glucose enters the HMPS (Beck, 1958), but with phagocytosis and the resultant $NADP^+$ and GSSG production (Eggleston and Krebs, 1974, Appendix I), a 7-fold increase in the glucose oxidized via the HMPS occurs (Sbarra and Karnovsky, 1959).

The aim of the experiments described in this section was to examine HMPS activity in non-phagocytic granulocytes which had been exposed to cytochalasin B, colchicine, vinblastine (VBL) or vincristine (VCR). These drugs had been found to affect O_2^- production in non-phagocytic granulocytes (Section 4), therefore it was desirable to investigate whether or not this change in O_2^- production was associated with any change in glucose oxidation via the HMPS. The effects of some of these drugs on non-phagocytic granulocytes have been examined by other investigators, and their findings are discussed in section 8.4 along with the results of these experiments.

8.2 METHODS

8.2.1 Pooled human serum

Venous blood (60 ml) was collected from six healthy volunteers and centrifuged for 15 min at room temperature at a force of 1500 x g. The plasma was then removed and allowed to clot. The resultant serum was pooled and stored in 2 ml aliquots at $-70^\circ C$.

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[1] This is the view held by the majority of workers, for example Baehner et al, (1970) and DeChatelet et al (1972b). However, some investigators believe that the increase in HMPS activity precedes oxidase activity, and hence superoxide production (Strauss et al, 1969). In this alternative scheme, phagocytosis activates glutathione reductase and thence the HMPS, and these events are followed by the increase in oxygen uptake and oxidase activity (see also Appendix I).

8.2.2   Phosphate buffer

The buffer used in these experiments contained
(in g/ml):-

NaCl 8.0, $MgCl_2$ x $6H_2O$ 0.2, KCl 0.2, $CaCl_2$
(fused) 0.11, Na_2HPO_4 1.15, and KH_2PO_4 0.2,
with a final pH of 7.4.

8.2.3   Colchicine, VBL, VCR, cytochalasin B, DMSO

8.2.3.1   Drug dilutions

All drugs were prepared as a stock solution of 1 mM
in water and then dilutions of this stock solution were used in
the preparation of HSS. The only exception was cytochalasin B
which was prepared as a stock solution in DMSO. However all
subsequent dilutions of the drugs (including cytochalasin B)
were made with water. Successive dilutions as outlined below
were made to obtain concentrations of the drugs which, when
used to prepare HSS, would result in a final drug concentration
at testing of 1, 0.1, or 0.01 µM in HSS:-

<div align="center">

stock solution = 1   mM (a)

0.1 ml (a) + 2.9 ml water = 33   µM (b)

0.1 ml (b) + 0.9 ml water = 3   µM (c)

0.1 ml (c) + 0.9 ml water = 0.3 µM (d)

</div>

To obtain HSS with 1 µM drug, 1 volume of (b) was
used to replace 1 volume of the water used in preparing HSS
(Appendix II). HSS containing drug at 0.1 µM and 0.01 µM were
prepared in the same way, using drug dilutions (c) and (d)
respectively.

8.2.3.2 Pre-incubation of granulocytes with the drugs

Isolated granulocytes (2×10^6 cells in 100 µl CMFH) were incubated in 200 µl HSS with or without the drugs, for 5 or 15 min at 37°C. These incubations were carried out in plastic tubes. The drug concentrations used for these incubations were 1, 0.1 and 0.01 µM. The control for cytochalasin B-treated granulocytes was HSS with the appropriate DMSO content (section 8.2.3.3.). The control for colchicine, VBL or VCR-treated cells was HSS.

During the pre-incubations with the agents or controls, KCN was present at 1.5 mM. Experiments with granulocytes pre-incubated in 3.0 mM KCN were also performed.

After the 5 or 15 min pre-incubations, the granulocytes were washed and then resuspended in 100 µl CMFH for HMPS activity measurements (section 8.2.4).

8.2.3.3 DMSO control for cytochalasin B-treated granulocytes

Cytochalasin B was dissolved in DMSO at 1 mM to give stock solution (a) (section 8.2.3.1). Therefore in those experiments where the effects of cytochalasin B were being studied, control HSS containing DMSO at the appropriate concentration had to be prepared. DMSO was used as the control stock solution, and using the dilution format outlined in section 8.2.3.1, the DMSO concentrations were:-

			100%	v/v	=	(a)
(a) x 1/30	=	3.3%	v/v	=	(b)
(b) x 1/10	=	0.33%	v/v	=	(c)
(c) x 1/10	=	0.03%	v/v	=	(d)

Dilutions (b), (c) and (d) were then used in the preparation of control HSS for the cytochalasin B experiments. Following the method discussed in section 8.2.3.1, the final DMSO content (% v/v) during pre-incubation of the granulocytes was therefore:-

for 1 µM cytochalasin B or its control ... 0.25% v/v DMSO

for 0.1 µM " " " ... 0.03% v/v DMSO

for 0.01 µM " " " ... 0.003% v/v DMSO

8.2.4 HMPS activity assay

The HMPS assay used in these experiments was that of Hosking, Fitzgerald and Shelton (1978). It is based on the principle of quantitating ^{14}C-1-glucose oxidation to $^{14}CO_2$, as discussed by Beck (1958), Sbarra and Karnovsky (1959), and Reed (1969). As indicated in the reaction below, for each molecule of $^{14}CO_2$ evolved, one molecule of ^{14}C-1-glucose is oxidized via the HMPS:-

$$2 \text{ NADP}^+ + {}^{14}C\text{-1-glucose} \xrightarrow{\text{HMPS}} 2 \text{ NADPH} + 2 \text{ H}^+ + {}^{14}CO_2$$

A universal container with two compartments and a screw-top with rubber plug, through which injections could be made, was used for the assay (Fig. 8.2). The smaller compartment contained the pre-incubated granulocytes (2 x 10^6 cells in 100 µl CMFH), 700 µl of buffer (8.2.2) containing 0.8 µCi of ^{14}C-1-glucose, 25 µl of pooled human serum (8.2.1), and an additional 100 µl of buffer to replace the phagocytosable stimulus normally used by Hosking *et al.*

The larger compartment contained a mini-scintillation

vial containing 0.4 ml Hyamine Hydroxide 10X.

Once the granulocytes had been added, the universal container was capped and then incubated for 1 hour at $37^{O}C$ to allow glucose oxidation to take place. After this time 1 ml of 2M-HCl was injected through the rubber plug into the smaller compartment to arrest the glucose oxidation. The vials were left capped and allowed to stand at room temperature for one hour to ensure complete absorption of the liberated $^{14}CO_2$ by the Hyamine Hydroxide. After this time the mini-vial was removed and 5 ml of Packard liquid scintillation fluid was added. The vials were sealed with caps and counted in a Packard 2450 Tri-Carb liquid scintillation spectrometer to quantitate HMPS activity.

8.2.5   <u>Quantitation of HMPS activity</u>

Activity is expressed as nmol glucose oxidized with the liberation of $^{14}CO_2$ from the formula:

$$\text{glucose oxidized (nmol)} = \frac{\text{cpm (drug treated)} - \text{cpm (control)}}{0.87^{(a)} \times 2220^{(b)} \times 3^{(c)}}$$

where (a) = the counting efficiency of the beta

scintillation counter (87%)

(b) = cpm in one nanoCurie (Braestrup and Vikterlöf, 1974).

(c) = activity of the purchased ^{14}C-1-glucose, i.e.

3 nanoCurie/nanomole.

and cpm = counts per minute.

8.3   RESULTS

8.3.1   Pre-incubation with cytochalasin B

Pre-incubation of non-phagocytic granulocytes for 5 or 15 min with cyto B in DMSO had no effect on HMPS activity (Table 8.1). Similarly the concentrations of DMSO present during these experiments had no significant effect on HMPS activity (Table 8.1).

8.3.2   Pre-incubations with VCR, VBL, or colchicine

Some interesting results were seen when non-phagocytic granulocytes were pre-incubated with these agents. However, only one of these agents (VCR) produced statistically significant effects. After 5 min pre-incubation, VCR at 0.1 and 1.0 μM showed a significant stimulation in HMPS activity compared with the control (Table 8.2). By examining the results for both 5 and 15 min pre-incubations with VCR it is apparent that the stimulation observed was dose-dependent. Although this trend was seen with VCR, it was not observed with the other two microtubule-disrupting agents, VBL and colch. In fact, VBL and colch pre-treatment for 15 min resulted in a trend which was opposite that seen with VCR. That is, with increasing doses of VBL or colch, the HMPS activity diminished compared with the appropriate control. With 5 min pre-incubations, VBL and colch showed no such dose-response trend (Table 8.2).

8.3.3   The effect of incubation time with the agents or controls

The number of nanomoles of glucose oxidized after 5 or 15 min pre-incubations with the control buffer were virtually identical (65±5 versus 67±8, respectively, Tables 8.1 and 8.2).

Similarly there was very little difference in the values for 5

min or 15 min pre-incubated granulocytes treated with DMSO or

cyto B (Table 8.1). Granulocytes pre-incubated for 5 min or

15 min with the microtubule-disrupting drugs, VBL, VCR, or colch,

showed almost identical values also (Table 8.2). The exception

was once again with VCR, where the nanomoles glucose oxidized

after 5 min pre-incubation were slightly higher, for each of the

three doses tested, than after 15 min pre-incubation.

8.3.4   The effect of potassium cyanide

Non-phagocytic granulocytes exposed to 3.0 mM KCN

for 5 min or 15 min showed a pronounced stimulation of HMPS

activity when compared with the appropriate 5 or 15 min KCN-free

controls (Table 8.3). Exposure to 1.5 mM KCN resulted in a

statistically significant stimulation as well, but only with the 5

min pre-incubation. The stimulations with KCN at 5 and 15 min

were dose-dependent.

8.4   DISCUSSION

These experiments were conducted to determine if

the observed enhancement or depression in superoxide production

from non-phagocytic granulocytes (Section 4) was associated with

a similar change in the activity of the HMPS. It has been

established that phagocytic granulocytes display a stimulation

of the HMPS along with enhanced superoxide production (see

section 1.3). It is generally believed that the production of

superoxide occurs before HMPS activation (Baehner *et al*, 1970;

DeChatelet *et al*, 1972b, but some investigators hold the opposite

viewpoint (Strauss *et al*, 1969). With this latter possibility

in mind, it was necessary to investigate whether the HMPS was

affected by the drug treatments. The experiments described in this section were not designed to clarify if superoxide production follows or precedes HMPS stimulation, however.

In studying the activity of the plasma membrane oxidase in Section 4, potassium cyanide was present to ensure that the appropriate 'cyanide-insensitive' metabolic burst (section 1.3) was being measured. Therefore, KCN was also present in these experiments so that it could be determined whether the drug-induced changes in oxidase activity were associated with a parallel change in HMPS activity. Only a few studies of the effects of cyto B, VBL, VCR or colch on HMPS activity in non-phagocytic granulocytes have been reported, and none of the studies involved KCN-treated non-phagocytic granulocytes. Nevertheless, although they cannot be considered comparable to the experiments presented herein, those studies must be discussed.

The findings from one group of investigators are very difficult to evaluate because they mention that the HMPS activity of non-phagocytic granulocytes was both inhibited by cyto B (Roos et al, 1976), and unaffected (Roos, Homan-Müller and Weening, 1976). In agreement with the latter report, Hawkins (1973) also found that cyto B treatment of non-phagocytic granulocytes does not affect HMPS activity. Although an inhibition of HMPS activity by cyto B was observed in this thesis (in agreement with the reports from Malawista, Gee and Bensch, 1971; Zigmond and Hirsch, 1972; Okuda et al, 1976; and Roos et al, 1976), this inhibition was not statistically significant (Table 8.1). Furthermore, parallel results were seen with DMSO at the appropriate control concentrations (Roos et al, 1976, and

this thesis), and therefore the inhibition of HMPS activity by cyto B can be attributed to the DMSO used as a solvent for the cyto B. As none of the above investigators reported an enhancement in HMPS activity, it seems certain that the stimulated oxidase activity of cyto B-treated granulocytes is not due to or associated with HMPS activity.

Several laboratories have studied the effects of VBL, VCR or colch on glucose oxidation via the HMPS. Lehrer (1973), using colch, and more recently Pickering *et al* (1978) with VBL and VCR, have observed an inhibition of non-phagocytic HMPS activity by these agents. In contrast to these findings, and therefore in agreement with the results presented herein, Malawista and Bodel (1967), Malawista (1971), Tsan *et al* (1976a) and Zabucchi *et al* (1978) have found that VBL and/or colch have no effect on HMPS activity of non-phagocytic granulocytes. These latter studies were conducted under experimental conditions which were comparable to the methods used in this thesis (except for the omission of KCN), whereas the experiments described by Pickering *et al*, and Lehrer cannot be considered comparable. For example, Lehrer observed an inhibition of HMPS activity with doses of colchicine (6000 μM) far in excess of those used in this thesis (and by others) to specifically cause the disruption of microtubules (Wilson *et al*, 1974; Wilson, 1975; Owellen *et al*, 1976). Similarly the experiments of Pickering *et al* (1978) are not comparable to those presented herein because they measured HMPS activity of drug-treated whole blood, rather than isolated granulocytes. It would appear therefore, from the results presented in this section, and from those of the most comparable

studies that VBL or colch treatment does not affect the HMPS activity of non-phagocytic granulocytes.

It is interesting to note that a significant stimulation in HMPS activity with VCR treatment was observed, and this stimulation was dose-dependent. It was most unusual to find this stimulation when colch, and in particular VBL, did not give the same result[2]. In most reports describing *in vitro* work with VCR and VBL, the same response is observed with these two vinca alkaloids, presumably because both drugs possess the entire dimeric alkaloid structure necessary for biological activity mediated by binding to tubulin and disruption of microtubules (Wilson, 1975; Wilson *et al*, 1975; Owellen *et al*, 1976). However, VCR differs slightly from VBL in that VCR has a CHO (rather than CH_3) group attached to nitrogen in the lower indole portion of the molecule (Appendix IV). It is certain that this slight structural alteration accounts for the different therapeutic response observed *in vivo* and therefore the clinical applications of these two alkaloids (Spiers, 1974). It is likely that this slight structural difference is also responsible for the unexpected stimulation of HMPS activity observed herein with VCR.

It should be mentioned that Pickering *et al* (1978) also observed an enhancement of resting HMPS activity with VCR.

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[2] A similar discrepancy was found by Malawista (1971), who showed that colchicine had no effect whereas VBL caused an inhibition in the HMPS activity of non-phagocytic granulocytes.

However, because of their unusual methodology (as described above) and the presence of prednisone in the incubation with VCR, these results do not necessarily confirm those presented herein.

In conclusion, the results of these experiments on HMPS activity do not parallel the findings of the experiments on the oxidase activity of drug-treated granulocytes (see section 4.3). Therefore it is not reasonable to assume that the observed changes in oxidase activity were associated with or due to alterations in HMPS activity brought about by these agents. The apparent separation between oxidase activity and the HMPS in these experiments was at first viewed as an unexpected finding. However, chronic granulomatous disease granulocytes have abnormal oxidase yet normal HMPS activity (Baehner and Nathan, 1967; Holmes, Page and Good, 1967; Tsan et al, 1976a; Oliver et al, 1976). Therefore the results presented in this section of the thesis cannot be considered unusual.

As mentioned above, potassium cyanide was present during the drug incubations to ensure that the appropriate 'cyanide-insensitive' metabolic burst was being measured. It has long been maintained that the post-phagocytic HMPS activation is cyanide-insensitive (section 1.3). However, recent reports show that cyanide can in fact stimulate post-phagocytic oxygen consumption (Reed and Tepperman, 1969; Klebanoff and Hamon, 1972, DeChatelet et al, 1977), as well as HMPS activity (Klebanoff and Pincus, 1971; DeChatelet et al, 1977). An enhancement in the HMPS activity of non-phagocytic granulocytes was observed in this thesis (Table 8.3). This enhanced HMPS activity in resting or phagocytosing granulocytes exposed to cyanide occurs because cyanide inhibits the heme-

containing proteins, catalase and myeloperoxidase. These two

enzymes are used by the granulocyte to detoxify the peroxide

produced by oxidase activity (Reed, 1969). In the presence of

cyanide this peroxide is not detoxified, and it can therefore

be consumed by the glutathione reductase-glutathione peroxidase

system. The utilization of peroxide by this system will result

in the formation of NADP$^+$, which stimulates the HMPS (Reed, 1969,

as illustrated in Appendix I, Fig. I.1).

8.5   <u>SUMMARY</u>

In this section of the thesis, the HMPS activity of

drug-treated (cyto B, VBL, VCR, colch) non-phagocytic granulocytes

was examined. Overall, these agents had no effect on HMPS activity

from non-phagocytic granulocytes, although one of the drugs (VCR)

produced an unexpected stimulation compared with the control.

As the results from these and similar experiments

on HMPS activity by Malawista and Bodel (1967), Malawista (1971),

Tsan *et al* (1976), Roos *et al* (1976) and Zabucchi *et al* (1978)

did not parallel the results of experiments on the oxidase

activity of drug-treated granulocytes, it cannot be concluded that

the drug-induced changes in oxidase activity (see section 4.3) were

associated with or due to a change in HMPS activity. The apparent

separation between oxidase activity and the HMPS in these experiments

was at first viewed as an unexpected finding. However, chronic

granulomatous disease granulocytes have abnormal oxidase yet normal

HMPS activity, therefore the results presented herein are not

unusual.

glucose-6-phosphate

$NADP^+$

H^+ + NADPH

1

6-phosphogluconolactone

H_2O

2

6-phosphogluconate

$NADP^+$

H^+ + NADPH

3

CO_2

ribulose-5-phosphate

4

5

xylulose-5-phosphate

ribose-5-phosphate

further metabolism
via the pentose
phosphate pathway

further metabolism
to form nucleic
acids, for example
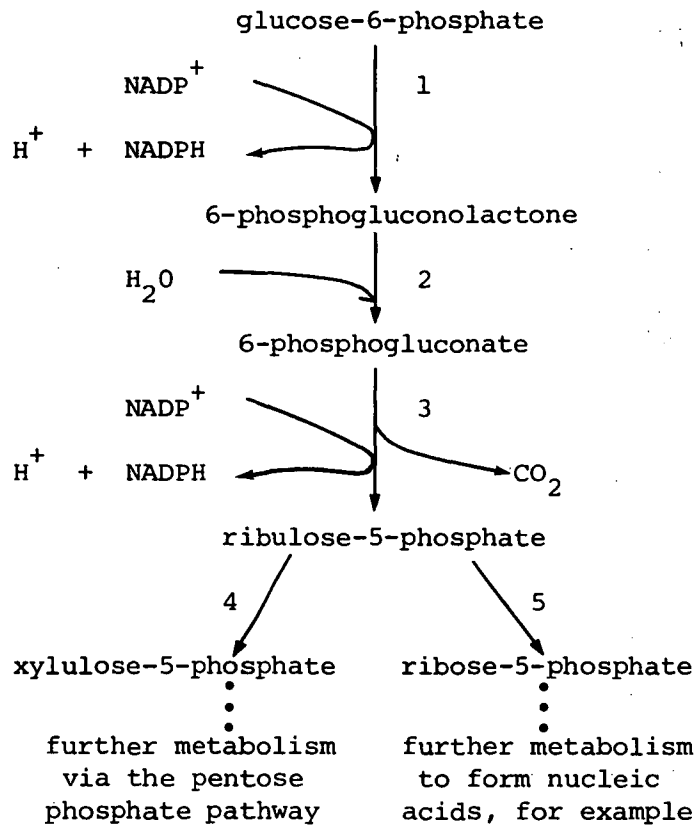
FIG. 8.1   The first steps of the hexose monophosphate
shunt, as modified from Chayen *et al* (1973).
Enzymes involved are:-

1. G-6-P dehydrogenase
2. hydrolase
3. 6-PG dehydrogenase
4. epimerase
5. isomerase

FIG. 8.2.   The universal container with two compartments and a
            screw-top with rubber plug.   The smaller compartment
            contained the granulocytes, buffer, ^{14}C-1-glucose and
            pooled human serum.   The larger compartment contained
            (as shown in the photograph) a plastic mini-
            scintillation vial.
            Magnification approx. = 1.1X

TABLE 8.1

Values given are the $\bar{X}$ ± SEM of 4 determinations (2 experiments, each conducted in duplicate), and indicate the number of nmoles glucose oxidized. The values are not statistically significant when compared with the appropriate (5 or 15 min) control .

a)   DMSO at 0.25% v/v

b)   DMSO at 0.03% v/v

c)   DMSO at 0.003% v/v

TABLE 8.1   5 or 15 min pre-incubation with cyto B or DMSO

Agent and dose		Incubation time	
		5 min	15 min
Cyto B	1 µM	57 ± 6	62 ± 2
	0.1 µM	73 ± 2	71 ± 2
	0.01 µM	55 ± 3	60 ± 7
Control for cyto B	1 µM[a]	56 ± 1	61 ± 3
	0.1 µM[b]	75 ± 1	68 ± 3
	0.01 µM[c]	54 ± 1	62 ± 7
Control	0 µM	65 ± 5	67 ± 8

TABLE 8.2

Values given are the $\bar{X} \pm$ SEM of 4 determinations (2 experiments,

each conducted in duplicate), and indicate the number of nmoles

glucose oxidized. The values [except for a) and b)] are not

statistically significant when compared with the appropriate

control.

For a) p < .005 compared with the control (5 min)

and b) p < .02 compared with the control (5 min)

TABLE 8.2   5 or 15 min pre-incubation with VBL, VCR or colch

Agent and dose			Incubation time	
			5 min	15 min
VBL	1	μM	60 ± 11	60 ± 3
	0.1		64 ± 8	65 ± 4
	0.01		55 ± 8	72 ± 3
VCR	1	μM	90 ± 1[a)]	90 ± 6
	0.1		83 ± 2[b)]	74 ± 5
	0.01		79 ± 13	67 ± 4
colch	1	μM	73 ± 2	61 ± 5
	0.1		63 ± 13	69 ± 3
	0.01		76 ± 1	74 ± 3
control	0		65 ± 5	67 ± 8

TABLE 8.3   The effects of cyanide on HMPS activity

Incubation Period (min)	Dose (mM)		
	(Control) 0	1.5	3.0
5	54 ± 1	65 ± 5[a]	91 ± 5[b]
15	51 ± 5	67 ± 8[c]	127 ± 4[d]

Values given are the $\bar{X}$ ± SEM of 4 determinations (2 experiments, each conducted in duplicate), and indicate the number of nmoles glucose oxidized. Except for c), the values are significantly greater than the appropriate control, that is:-

a)   p < .025   and b), d)   p < 0.01

SECTION   9

ULTRASTRUCTURAL STUDIES OF NBT REDUCTION

BY GRANULOCYTES

9.1   INTRODUCTION

Using two complementary techniques, Briggs et al (1975)

examined the ultrastructural location of cyanide-insensitive

oxidase activity in granulocytes. The first technique involved

studying the sites of oxidase activity through the formation of

H_2O_2. As discussed in section 1.4, the cyanide-insensitive oxidase

catalyzes the formation of superoxide, and is therefore ultimately

responsible for the production of H_2O_2 in the following reactions:-

$$2O_2 + NAD(P)H \xrightarrow[\text{oxidase}]{} 2O_2^- + NAD(P)^+ + H^+$$

$$2O_2^- + 2H^+ \xrightarrow[\substack{\text{superoxide}\\ \text{dismutase}}]{} O_2 + H_2O_2$$
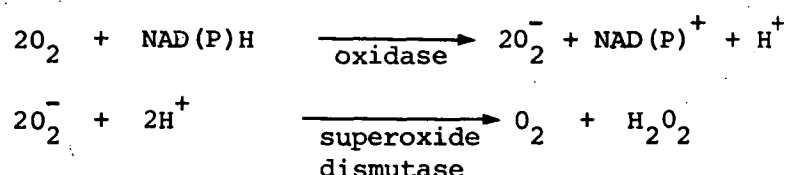
The authors detected the cellular locations of H_2O_2

production with exogenous $CeCl_3$. The cerium ions formed a

precipitate with the H_2O_2 (presumably cerium perhydroxide), and

this precipitate was deposited at the site of its formation.

The second correlative study by Briggs et al

involved examining the deposition of reduced NBT as the result

of oxidase activity. As discussed in section 3.1, NBT is

reduced to formazan via O_2^- generated as a result of oxidase

activity, and the formazan remains in situ at the cellular site

of its production.

With these complementary studies the authors showed

that the cyanide-insensitive oxidase was located on the surface

of granulocytes, and that the oxidase was NADH-dependent (see

Appendix I). Moreover they showed that the plasma membrane

enzyme was internalized during phagocytosis and retained its

enzymic capacity (as detected by H_2O_2-dependent cerium deposition)

within the phagocytic vacuole.

In this section, electron microscopical studies of the
cellular sites of oxidase activity, as monitored by formazan
deposition, are described. The aim of these experiments was to
examine the surface distribution of reduced NBT dye, and to study
the effects of prior drug treatment of phagocytic or resting
granulocytes, on the distribution of these sites of enzyme activity.

9.2   METHODS

9.2.1   Preparation of drugs in HSS

Colchicine and cytochalasin B were prepared in HSS
as described in section 4.2. The final concentration of these
agents in HSS was 1 μM.

9.2.2   Granulocyte pre-incubations with the drugs

Isolated granulocytes (section 2.2.13) were centri-
fuged in plastic conical test tubes and the supernatant CMFH
removed. The cells were then resuspended in HSS containing
colchicine or cyto B, and incubated with the agents for 10 min
at 37°C. If the granulocytes were to be used for phagocytic
studies, the procedure outlined in section 9.2.3 was followed,
otherwise the drug-treated granulocytes were washed in CMFH
at 4°C. Control (untreated) granulocytes were handled in
parallel but not exposed to the drugs.

9.2.3   Granulocyte incubations for phagocytic stimulation

Drug-treated or untreated granulocytes to be used for
phagocytic studies were presented with polystyrene latex beads
in HSS (as detailed in section 5.2), and incubated with the
beads for 10 min at 37°C. Ingestion was stopped by washing the
cells in excess CMFH at 4°C to minimize the particle-cell contact.
Control granulocytes were handled in parallel but not exposed

to the beads.

9.2.4   Incubation of granulocytes for NBT reduction studies

Resting or phagocytic, drug-treated (or control) granulocytes were used in the incubations for NBT reduction (section 9.2.4.1). The solutions and procedure followed were taken from Briggs *et al* (1975).

9.2.4.1   Incubation for NBT reduction

The granulocytes were centrifuged and resuspended in Tris/sucrose solution (pH 7.0, section 9.2.4.2) for a brief wash of 5 min at 4°C. They were then resuspended in the NBT incubating solution (section 9.2.4.3) and incubated for 30 min at 37°C. After this time the cells were washed for 5 min in cold Tris/ sucrose washing solution (9.2.4.2), and then fixed in glutaraldehyde (section 9.2.5.1). Control granulocytes were handled in the same way but not exposed to NBT dye. After fixation in glutaralde-hyde, the cells were prepared for electron microscopy, as detailed in section 9.2.7.

9.2.4.2   Washing solution for NBT incubations

This solution consisted of 0.1 M Tris maleate with 7% sucrose, pH 7.0 (adjusted with 5 M- NaOH). The washing solution was prepared on the day of use.

9.2.4.3   NBT incubating solution

This solution consisted of 0.1 M Tris maleate, pH 7.0 with 0.71 mM NADH and 0.6 mM NBT. The solution was prepared just before use and Millipore-filtered (0.45 µm) to remove any undissolved NBT.

9.2.5   Electron microscopy solutions

9.2.5.1   Glutaraldehyde fixative

      Electron microscopy grade glutaraldehyde was used to make a 2% glutaraldehyde in CMFH solution. The solution was prepared by mixing 1.1 ml glutaraldehyde (25%), 1 ml Ca^{+2} and Mg^{+2} free concentrated salt solution, 1 ml glucose, 155 mM, 2 ml HEPES buffer, 58.5 mM, pH 7.4 (Appendix II), and 7.9 ml water. The final composition of the fixative was:-

    2% glutaraldehyde, 142.9 mM Na^{+}, 6.0 mM K^{+}, 11.9 mM glucose, 9.0 mM HEPES, with a pH of 7.4. The fixative was prepared on the day of use.

9.2.5.2   Washing solution for electron microscopy

      The solution described by Sabatini *et al* (1963) was used for washes, and consisted of 0.2 M sucrose in 0.1 M sodium cacodylate, pH 7.4.

9.2.5.3   Osmium tetroxide fixative

      The osmium tetroxide fixative contained 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.4. The fixative was prepared every few months, as required, and stored at $4^{o}C$ in a glass container with a tight-fitting (glass) stopper.

9.2.5.4   Spurr's resin for electron microscopy

      The embedding material used for electron microscopy was a low viscosity epoxy resin described by Spurr (1969). The resin was prepared by mixing (in order):-

        10.0 g vinyl cyclohexene dioxide (resin)

        6.0 g diglycidyl ether of polypropylene glycol (resin)

26.0 g nonenyl succinic anhydride (hardener) and

0.4 g dimethylaminoethanol (accelerator)

Mixing was accomplished by gently swirling the Erlenmeyer flask after the addition of each component. The resin was prepared every few weeks and was stored covered (to protect from moisture) at -20°C.

9.2.6   Thin section stains for electron microscopy

9.2.6.1   Uranyl acetate

This heavy metal uranium stain was adapted from Watson (1958). It was prepared as a saturated solution in 50% ethanol by adding 8 g UO_2 $(C_2H_3O_2)_2$ x 2 H_2O to 100 ml of 50% ethanol in water. After mixing, the solution was stored in an amber bottle and allowed to settle for 2 days before use. The stain was made up monthly, and it was centrifuged for clarification before use.

9.2.6.2   Lead citrate

The lead citrate solution was prepared following the suggestions of Reynolds (1963). 1.76 g sodium citrate $(Na_3C_6H_5O_7$ x $2H_2O)$ was dissolved in 40 ml water and to this was added 1.33 g lead nitrate. The mixture was tightly stoppered and stirred for 30 min. After this time 10 ml of 1 M - NaOH was added and the solution stirred for a further 5 min. This solution was prepared monthly and stored in a clear glass bottle.

9.2.7   Processing of granulocytes for electron microscopy

Treated or untreated granulocytes to be examined by electron microscopy were fixed in glutaraldehyde, post-fixed in osmium tetroxide, embedded in resin (using a modification of

Spurr's technique (Spurr, 1969), thin-sectioned, and finally

stained on grids with uranyl acetate and lead citrate.

Fixation in 2% glutaraldehyde (section 9.2.5.1) was

started at room temperature ($^\sim$25°C) for 5-10 min and was then

completed at 4°C for a total fixation of 3 hours. The unreacted

glutaraldehyde was removed by centrifuging the cells in two

successive washes at 4°C (solution 9.2.5.2). The cells were then

post-fixed in osmium tetroxide for 30 min at room temperature

(section 9.2.5.3). After osmium the granulocytes were again

washed twice, and then dehydrated in a graded series of ethanols.

The ethanol in water solutions (in order of use) were 50, 70, and

100% v/v. The cells were exposed to each ethanol solution for

10 min, and to the absolute ethanol twice. To further ensure

dehydration and to provide a solvent which would be compatible

with the embedding resin, the cells were then exposed to

propylene oxide twice (10 min and 30 min, respectively). The

granulocytes were then resuspended in 300 µl of a mixture of

Spurr's resin (9.2.5.4) and propylene oxide (1:1) for 60 min at

room temperature. Then 300 µl of fresh Spurr's resin was added

to increase the ratio of resin:propylene oxide to 3:1. The cells

were left in this mixture at room temperature for one hour to

promote infiltration of resin into the cells. After this time

the granulocytes were centrifuged and the resin/propylene oxide

mixture removed. They were then resuspended in 300 µl of fresh

resin and transferred with a Pasteur pipette to BEEM capsules.

At this point the granulocytes were centrifuged (60 min x 500 g x

25°C) to the bottom cavity of the capsules. These capsules were

then incubated at 70°C for 24 hours to polymerize the resin.

The polymerized blocks were removed from the capsules and sectioned on an LKB III ultramicrotome with glass knives. Thin sections were collected on 400-mesh Formvar-coated (Pease, 1964) copper grids . These grids were placed section-side down on drops of uranyl acetate stain (one grid per drop of stain), and stained for 15 min at room temperature. They were then rinsed of stain by immersion 20 times in two successive beakers of water. The grids were not allowed to dry before being placed onto drops of lead citrate, and stained 8 min at room temperature. The stained grids were thoroughly rinsed once more, allowed to dry, and then examined in an Hitachi HS-7S electron microscope.

9.3   RESULTS

9.3.1   Untreated granulocytes

The ultrastructure of the mature granulocyte has been described by Bainton *et al* (1971). The most prominent feature of the granulocyte is the segmented or multi-lobed nucleus. Sometimes a connecting strand of chromatin can be seen between two lobes. The cytoplasm is replete with large azurophilic granules which can vary in shape from round to oval. The smaller specifics are interspersed with the larger azurophilic granules in the cytoplasm. Very few if any mitochondria are present in the mature granulocyte. Occasional glycogen deposits may be seen, and sometimes the cell has been sectioned through the cell centre, revealing the centriole. Vacuoles may be found in the cytoplasm and occasionally they contain post-phagocytic, partially digested debris. The surface outline of the cell is usually irregular, in contrast to the rounded shape of lymphocytes. Sometimes microtubules

are visible in the cytoplasm, and in the region underneath

the plasma membrane. Many of these features can be seen in Figures

9.1.A and B.

9.3.2   Phagocytic granulocytes

Granulocytes which have ingested polystyrene latex

beads can be distinguished from those which have not by virtue

of the presence of numberous beads within the cell, and the

absence of many cytoplasmic granules. The granule numbers are

depleted through degranulation into the phagocytic vacuoles, as

described in section 1.2. The phagocytic vacuoles are

bordered by membrane derived from the cell surface during the

process of ingestion (section 1.2). Sometimes the polystyrene

latex beads are partially extracted during processing for electron

microscopy and as a result, appear as a sphere with variations in

electron density. The cell surface of post-phagocytic granulocytes

is usually fairly smooth, although sometimes a long projection or

pseudopod is seen. The typical features of granulocytes which

have ingested polystyrene latex beads are seen in Fig. 9.2.

9.3.3   Non-phagocytic granulocytes exposed to NBT dye

Reduced NBT (formazan) deposition at the cell surface

was of low electron density and evenly distributed over the cell

surface. The reduced dye was visible mainly where two cells

came in contact (Fig. 9.3).

9.3.4   Colchicine-treated non-phagocytic granulocytes

Granulocytes exposed to colchicine often showed a

rounded cell profile with numberous small projections off the

cell surface. Occasionally small vacuoles were seen at

the cell surface as well. The granules were present and appeared
to be intact (Figs. 9.4.A and B). When these colchicine-treated
cells were incubated with NBT dye, patchy deposits of reduced dye
were seen on the cell surface. Occasionally a very large patch
of formazan was seen at the cell surface, and the surface depos-
ition of dye was always more visible where two cells touched
(Figs. 9.5.A and B).

9.3.5   Cyto B-treated non-phagocytic granulocytes

Almost all cyto B-treated cells showed some ultra-
structural change at the cell surface. Blebbing of the membrane
or unusual cellular projections were prominent findings. Vacuole
formation at the cell surface (especially within the blebs) was
often seen. Sometimes the granules, although not depleted in
numbers, were localized to a particular region or end of the
cell after cyto B treatment. These features are shown in Figs.
9.6.A,B,C, and D .

When cyto B-treated granulocytes were incubated with
NBT dye, deposits of formazan were localized to the peripheral
vacuoles found at regions of cell surface blebbing. Not all
vacuoles contained formazan, however (Figs. 9.7.A and B).

9.3.6   Phagocytic granulocytes incubated with NBT

Phagocytic granulocytes incubated with NBT showed heavy
formazan deposits within the phagocytic vacuoles, and only a
faint deposition at the cell surface. Cytoplasmic granules were
often depleted. Most phagocytic granulocytes showed a large
number of beads per cell section examined (mean number from 20
cells counted = 40 beads). These cells are shown in Fig. 9.8.

9.3.7   Colchicine-treated phagocytic granulocytes

Colchicine-treated phagocytic granulocytes presented fewer beads per cell section examined (mean value from counts performed on 20 cells = 20 beads). Otherwise the appearance of these cells was similar to the control phagocytic granulocytes [cf. Figs. 9.9 (colchicine) and 9.2 (control)]. Colchicine-treated phagocytic granulocytes incubated with NBT dye showed light formazan deposition within the phagocytic vacuoles (Figs. 9.10.A and B), but little or no dye on the cell surface.

9.3.8   Cyto B-treated phagocytic granulocytes

Cytochalasin B-treated phagocytic granulocytes showed fewer beads eaten per cell section examined, than control granulocytes (mean value for 20 cells counted = 18). Granules were often depleted in numbers. The most striking finding was the presence of partially ingested beads attached firmly to the cell surface. For example, in Figure 9.11 at least 12 such beads can be seen.

When cyto B-treated phagocytic granulocytes were incubated with NBT dye, a very heavy deposit of formazan within the phagocytic vacuoles was observed. Where beads were partially ingested, formazan was found outside the cell, close to the sites of attachment of these beads (Fig. 9.12). In the granulocyte illustrated, an incompletely closed phagocytic vacuole is seen, and formazan appears to be deposited outside the cell surface in this region (Fig. 9.12). There are few granules present in the section of the cell shown.

9.4   DISCUSSION

Treatment of granulocytes with colchicine produced ultrastructural changes at the cell surface. Colchicine-treated cells were generally rounded, whereas control granulocytes had an irregular cell profile. In addition, small projections off the cell surface were commonly observed after colchicine treatment. The 'rounding-up' of colchicine-treated cells has been documented by others (Brown and Bruck, 1973; reviewed by Burnside, 1975). The formation of projections and ruffles at the cell surface of colchicine-treated fibroblasts has also been observed (Vasiliev et al 1970; Gail and Boone, 1971). It has been suggested by Vasiliev et al that the ruffling is a form of enhanced cell surface activity due to colchicine-induced microtubule disruption (see Appendix IV). Therefore, Vasiliev et al proposed that microtubules provide a way of stabilizing the resting cell surface.

Colchicine-treated granulocytes showed a patchy, rather than the usual faint, homogeneous deposition of reduced NBT dye at the cell surface. (In both colchicine-treated and control granulocytes, the formazan deposits were best seen where two cells came in contact.) The patchy distribution of NBT dye at the cell surface indicates that the location of the plasma membrane oxidase within the plasma membrane has been altered by colchicine treatment. In this regard, it has been shown that the topographical organization of transport sites (Ukena and Berlin, 1972) and lectin-binding sites (Oliver et al, 1974) within the plasma membrane of granulocytes is altered after colchicine treatment. It was concluded that microtubules, which were disrupted by colchicine, were required for the topographical organization of the

granulocyte plasma membrane (Oliver *et al*, 1974; Berlin, 1975).

It is likely therefore, that the observed inhibition of plasma

membrane NBT reduction by colchicine (see section 4.3) arose

from a detrimental alteration of plasma membrane organization

after microtubule disruption. A recent review by Coleman has

outlined the evidence with respect to how the overall organiz-

ation of the membrane is very often crucial for the activity of

a membrane-bound enzyme (Coleman, 1973).

Cytochalasin B-treated granulocytes displayed blebbing

and vacuole formation at the cell surface. These features of

cytochalasin B treatment have been observed with human, horse,

and rabbit granulocytes (Zigmond and Hirsch, 1972) as well as

other cell types (Butcher and Perdue, 1973; Holtzer *et al*,

1973; Mayhew and Maslow, 1974; Godman *et al*, 1975; Bhisey

and Freed, 1975). Based on such morphological observations

with mouse peritoneal macrophages, Bhisey and Freed (1975)

proposed that the blebs may have been due to cytochalasin B-

induced changes in the composition of the cell surface and per-

haps the plasma membrane itself. This view is supported by

the more recent finding that cytochalasin B, by acting to dis-

rupt a microfilament system located just underneath the cell

membrane (see Appendix IV) can bring about marked changes in the

movements of cell surface components (see also section 6.4).

When cytochalasin B-treated granulocytes were

incubated with NBT dye, it was found that the deposits of formazan

were localized to those vacuoles contained within surface blebs.

It was initially thought that the dye in those vacuoles might

have been reduced by granule rather than plasma membrane-produced

superoxide, and hence that these vacuoles were very similar to
phagocytic vacuoles. However the vacuoles inside surface blebs
were morphologically unlike phagocytic vacuoles (cf. Figs. 9.2 and
9.6.A,B, and C), and cytoplasmic granules were not observed fusing
with the membrane of the vacuole. Other investigators have shown
that the granules do not fuse with the plasma membrane of cyto B-
treated, non-phagocytic granulocytes (Henson, 1971 a,b; Henson, 1972;
Zurier, Hoffstein and Weissmann, 1973; Henson and Oades, 1975).
In addition, Goldstein *et al* (1975, 1977) and Roos *et al* (1976)
showed that cytochalasin B treatment of non-phagocytic granulocytes
resulted in a release of superoxide which was quite separate from
the release of granule enzymes from the cell, and suggested there-
fore, that the superoxide was released from the cell surface.
Thus it seems certain that the reduced dye in these surface vacuoles
emanated from plasma membrane oxidase activity, and that the vacuoles
contain the oxidase in their limiting membrane. There is morphologi-
cal evidence of their formation from the plasma membrane in Fig.
9.6.C. The reduction of NBT dye at the cell surface and the form-
ation of these vacuoles occurred too quickly to determine if vacuole
formation had to occur before dye reduction in cytochalasin B-treated
granulocytes. It can be concluded, however, that the reduction of
dye is not required before vacuole formation occurs, because other
workers have observed vacuolization after cytochalasin B treatment
of granulocytes in the absence of NBT (Zigmond and Hirsch, 1972).
As detailed in section 4.3, cytochalasin B treatment of non-phagocytic
granulocytes resulted in a time and dose-dependent stimulation of
NBT reduction by the plasma membrane oxidase. That finding, in
combination with the increased cell surface activity (blebbing and
vacuole formation) reported herein, suggests that cytochalasin

B-sensitive microfilaments underlying the membrane may provide

a constraint against plasma membrane oxidase activity.

Ultrastructural studies with drug-treated phagocytic

granulocytes provided some interesting and unexpected results.

Formazan deposition in the phagocytic vacuoles of the colchicine-

treated phagocytic granulocytes was considerably less than in

control cells. This finding agrees with the observed inhibition

of superoxide production (NBT reduction) by colchicine-treated

phagocytic granulocytes (section 5.3), and the experimental

results of others (Curnutte and Babior, 1975). Cytochalasin

B-treated granulocytes, in contrast, showed very heavy formazan

deposition within the phagocytic vacuoles, and some deposition

outside the cell near sites of bead attachment to the membrane.

Some of the formazan within the vacuoles may have been formed

first at the plasma membrane and then internalized with phago-

cytosis, because formazan was seen outside the cell at an

incompletely fused phagocytic vacuole (Fig. 9.12). An alternative

explanation provided by the studies of Weissmann *et al* (1975a,b)

is that the NBT dye was reduced by superoxide from a granule

oxidase. These authors found that cytochalasin B inhibited

the ingestion of zymosan particles[1] but did not affect the

binding of zymosan to the granulocyte membrane. Consequently,

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[1] Although Weissman *et al* (1975a,b) found that cytochalasin B
completely inhibited the ingestion of zymosan, it is clear
from the experiments presented in this thesis (section 5.3
and Figs. 9.11 and 9.12) and by others (Table 5.9) that
cytochalasin B does not completely inhibit phagocytosis of
all types of particles by the granulocyte.

this situation resulted in the release of granule enzymes to the exterior of the cell through a process the authors termed 'reverse endocytosis'. In effect, these granulocytes were degranulating to the exterior because the granules were merging with the plasma membrane underlying the attached bead, rather than the proper (interior) phagosome membrane (see also section 5.4).

In agreement with Briggs *et al* (1975) very little formazan was found on the surface of phagocytic granulocytes even though heavy deposits were evident within the phagocytic vacuoles. The absence of formazan at the surface of these cells would suggest that during phagocytosis, the plasma membrane oxidase is very rapidly and selectively incorporated[2] into the phagosome membranes at the expense of the cell surface.

9.5   SUMMARY

Colchicine and cytochalasin B both produced ultra-structural changes at the granulocyte cell membrane. The effects of cyto B treatment were the more dramatic and included plasma membrane blebbing and vacuole formation. Colchicine-treated granulocytes showed a patchy distribution of reduced NBT dye (indicative of the production of superoxide by the cyanide-insensitive oxidase) at the cell membrane. Untreated cells in contrast showed a homogeneous distribution of reduced dye at the

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[2] The granulocyte plasma membrane proteins which bind the lectins, concanavalin A and Ricinus communis agglutinin have been shown to be rapidly and selectively incorporated into the phagosome membrane during phagocytosis (Berlin, 1975).

cell surface. The patchiness probably represents a colchicine-induced alteration in the location of the oxidase within the plasma membrane.

Cytochalasin B-treated granulocytes exposed to NBT dye displayed formazan deposits in cell surface vacuoles located at areas of plasma membrane blebbing. Based on the ultrastructural observations presented herein and the experiments of Goldstein *et al*, Henson *et al*, and Zurier, Hoffstein and Weissmann (see section 9.4) with cytochalasin B, it seems certain that the dye within these vacuoles was reduced via plasma membrane, rather than the granule-produced superoxide.

Phagocytic granulocytes incubated with NBT dye showed very heavy deposits of formazan within the phagocytic vacuoles but little dye at the cell surface. A likely explanation for this finding is that the plasma membrane oxidase is rapidly and selectively incorporated into the phagosome membrane at the expense of the cell surface location.

FIG. 9.1.A.   Untreated, non-phagocytic granulocytes. Features

   (top)   evident are the multilobed nucleus and the

                azurophilic (large) and specific (small) granules.

                A few cytoplasmic vacuoles are seen.

                Magnification 15,300X.

FIG. 9.1.B.   A higher magnification of the granulocyte cytoplasm.

  (bottom)   Microtubules in cross section can be seen at A and

                B; and underneath the plasma membrane (arrows)

                several longitudinal profiles of microtubules are

                evident. Magnification 52,300X.
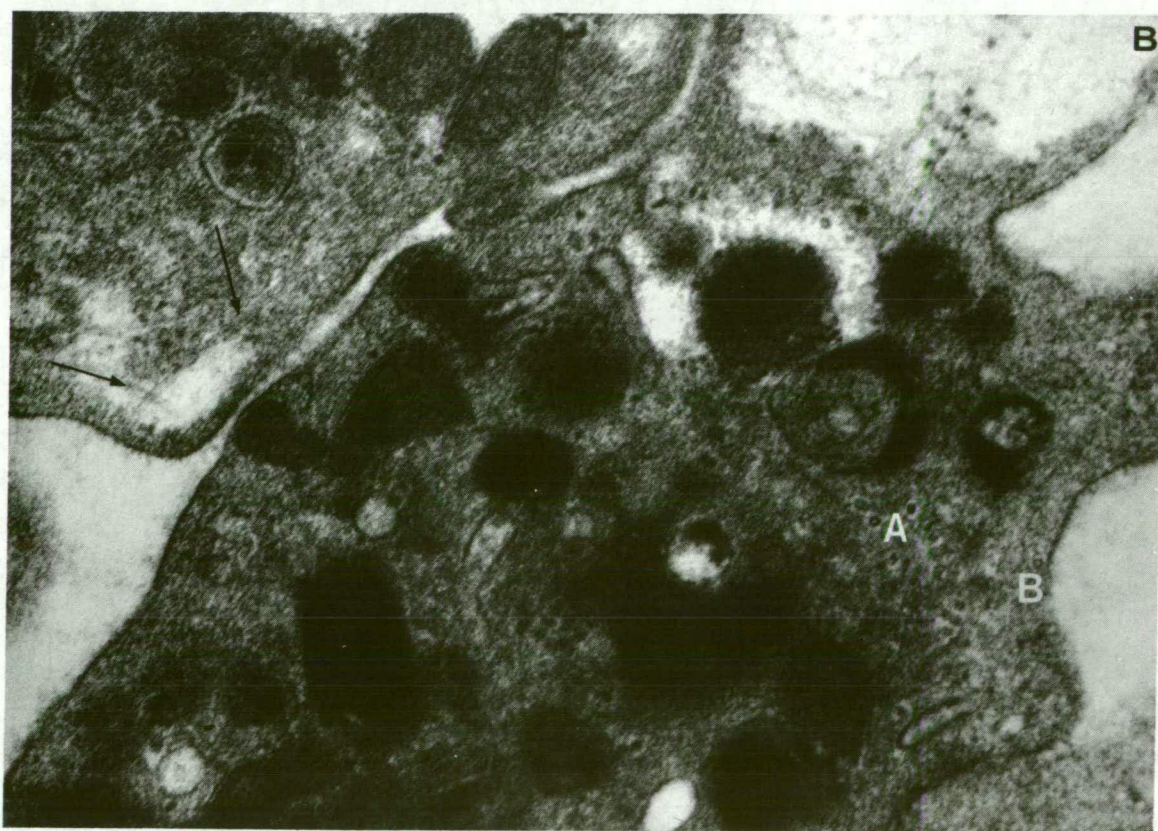
FIG. 9.2. A granulocyte which has ingested polystyrene latex
beads. Fewer granules are seen in such cells
(cf. FIG. 9.1.A). Magnification 19,500X.
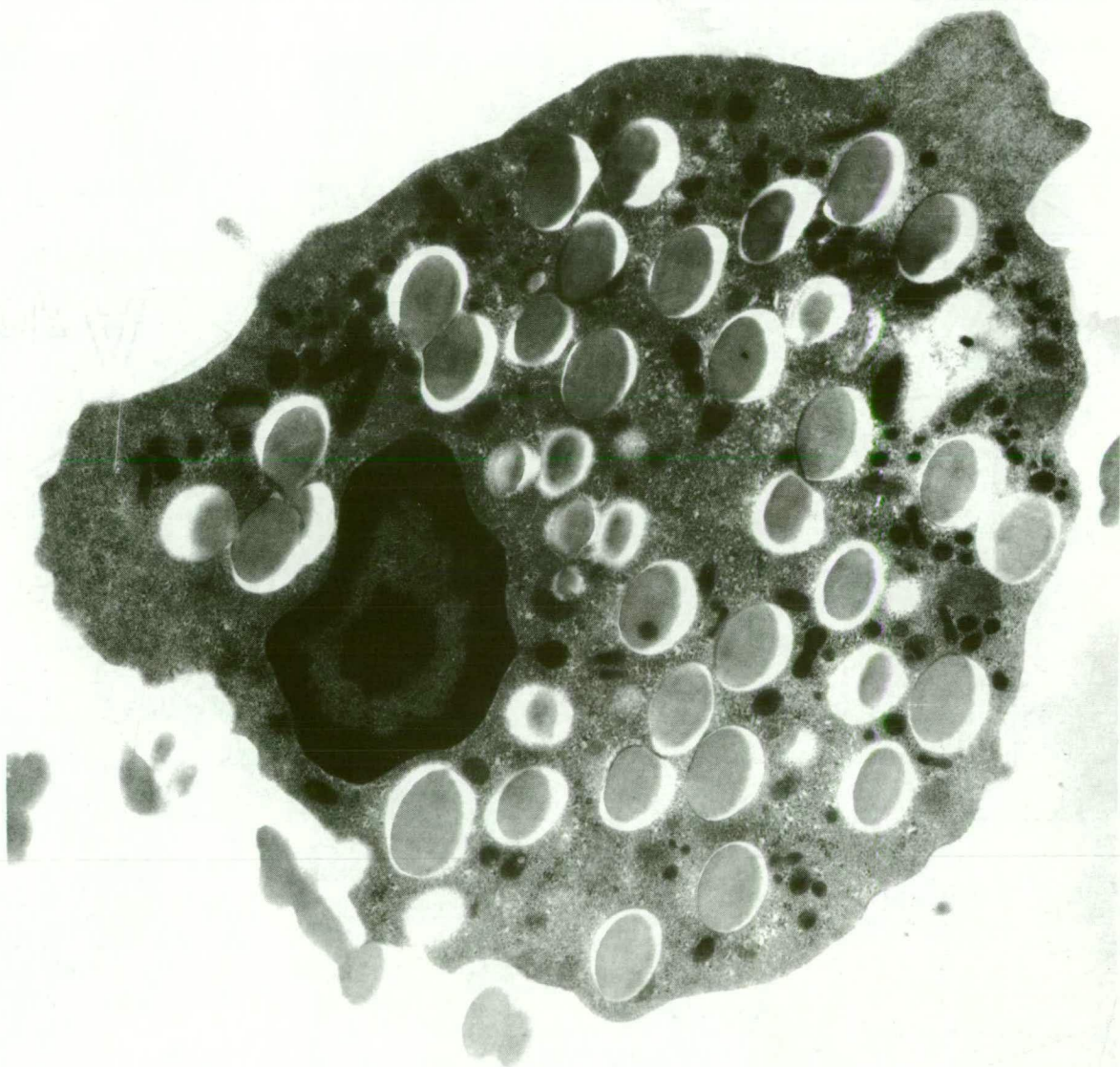
FIG. 9.3.   Two non-phagocytic granulocytes which have been ex-
            posed to NBT dye.   The deposition of reduced dye at
            the cell surface is visible where the two cells touch
            (arrows).   Magnification 15,100X.

FIGS. 9.4 A and B. Non-phagocytic granulocytes which have been
        exposed to colchicine. Small projections off the
        cell surface are often seen (arrows). Cytoplasmic
        and surface vacuoles are also frequently observed.
        The azurophilic and specific granules are present
        and appear to be intact. The granulocyte in the
        bottom photograph has the rounded appearance of
        colchicine-treated granulocytes (see section 9.4).
        Very few microtubules were seen in these cells.
        Magnification   (top) 16,000X
                    (bottom) 14,800X
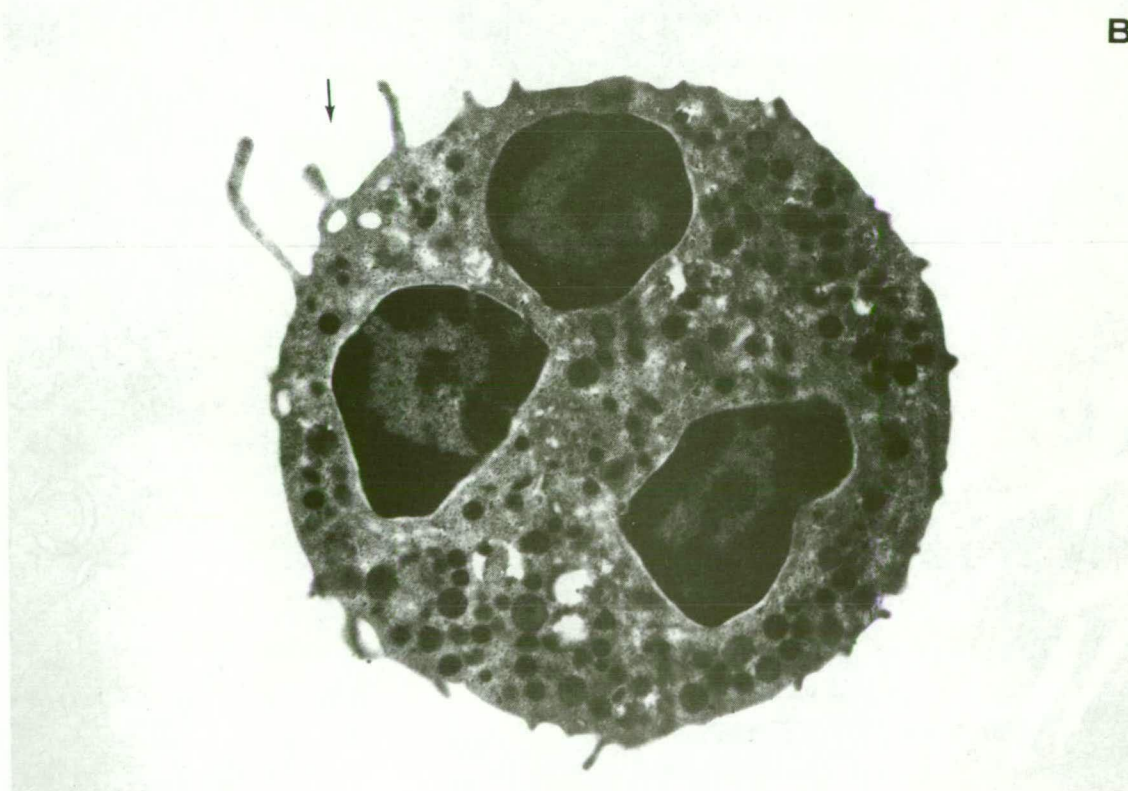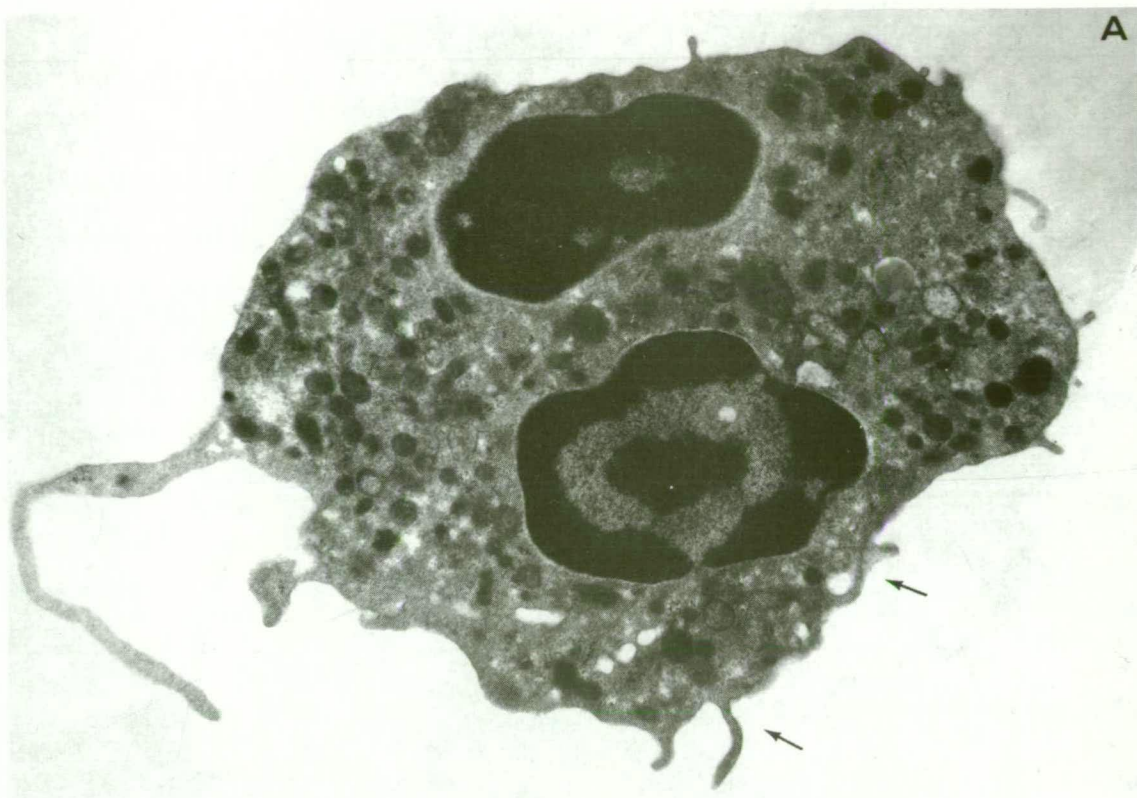
FIGS. 9.5 A and B. Non-phagocytic granulocytes exposed to
colchicine and incubated with NBT dye. Reduced
dye is evident in a patchy distribution on the
cell surface (top, arrows). As indicated in
FIG. 9.3, the reduced dye was readily seen where
two cells touched (bottom photograph). Occasionally
a very heavy deposit of formazan was present on
the plasma membrane (asterisk, bottom photograph).
Magnification   (top) . 18,400X
                   (bottom)   42,100X
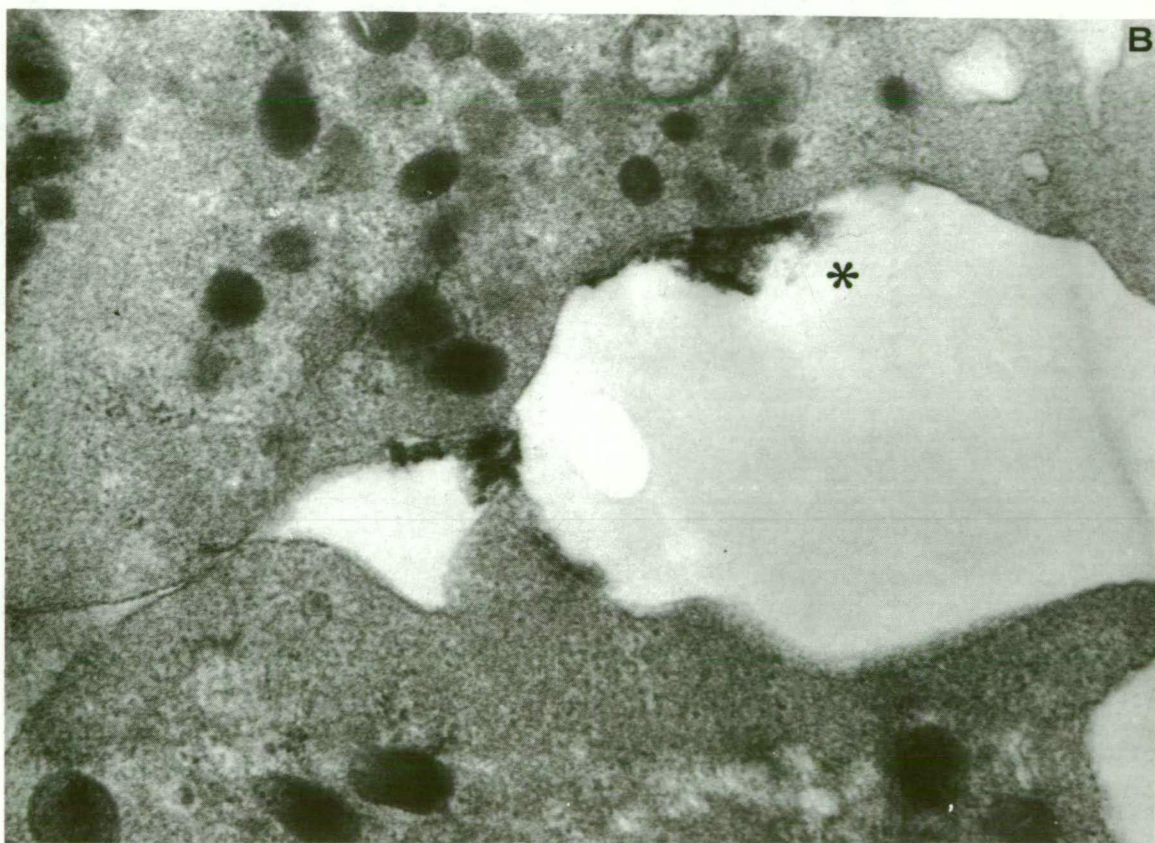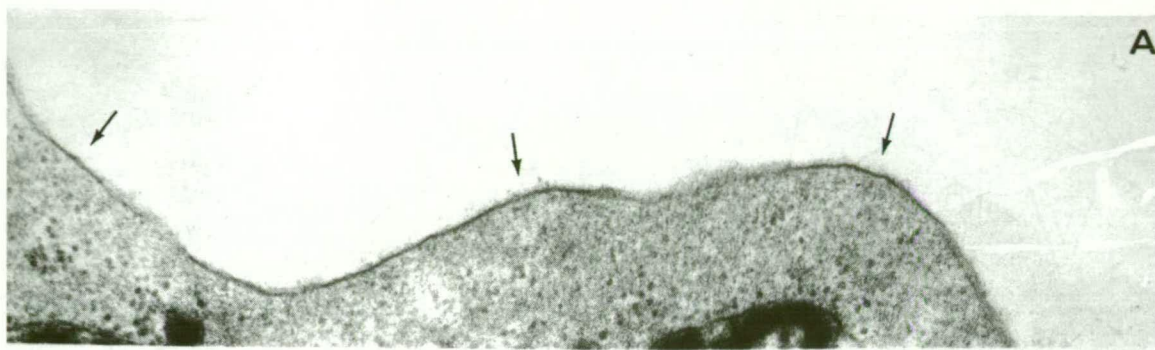
FIGS. 9.6 A and B. Cytochalasin B-treated non-phagocytic
        granulocytes. Ultrastructural changes at the
        cell surface are evident in the form of blebs
        (arrows) and projections (asterisks). Vacuole
        formation in the region of these blebs and pro-
        jections is seen in the lower photograph, at
        1, 2, 3 and 4. The granules are present and
        appear to be intact, although they seem to be
        localized to one half of the cell in the top
        photograph.

        Magnification   (top)   12,000X
                          (bottom)   48,400X
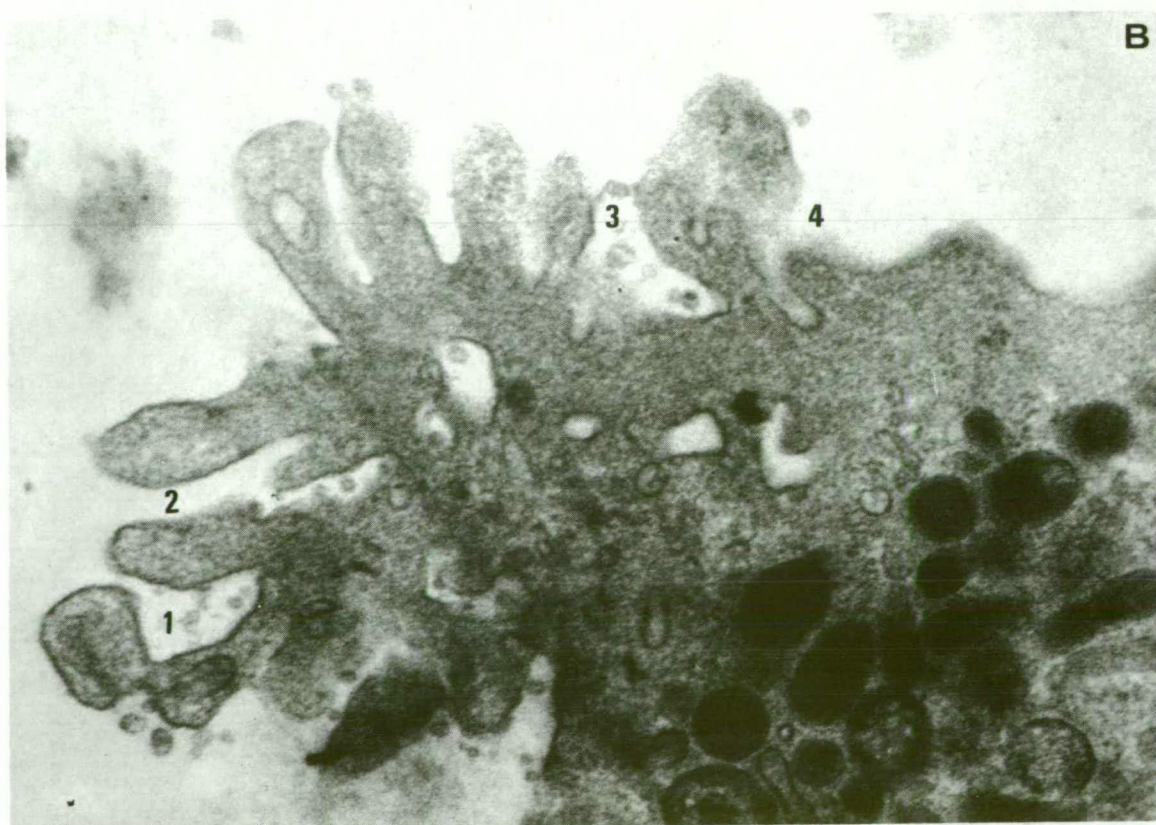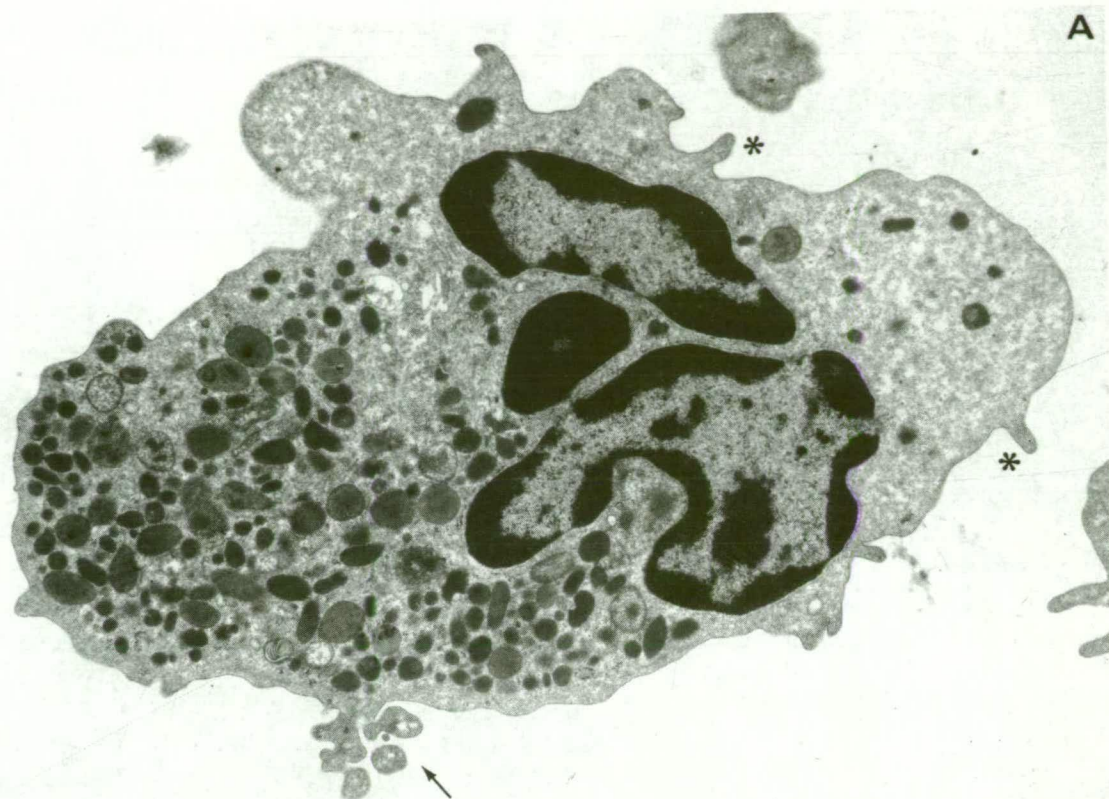
FIGS. 9.6 C and D. Cytochalasin B-treated, non-phagocytic

granulocytes. Alterations to the cell surface

are present in the form of projections (arrows)

and vacuole formation (asterisks). As indicated

for FIGS. 9.6 A and B, the granules are present

and appear to be intact in these cells.

Magnification   (top)   15,200X

                          (bottom)   35,700X
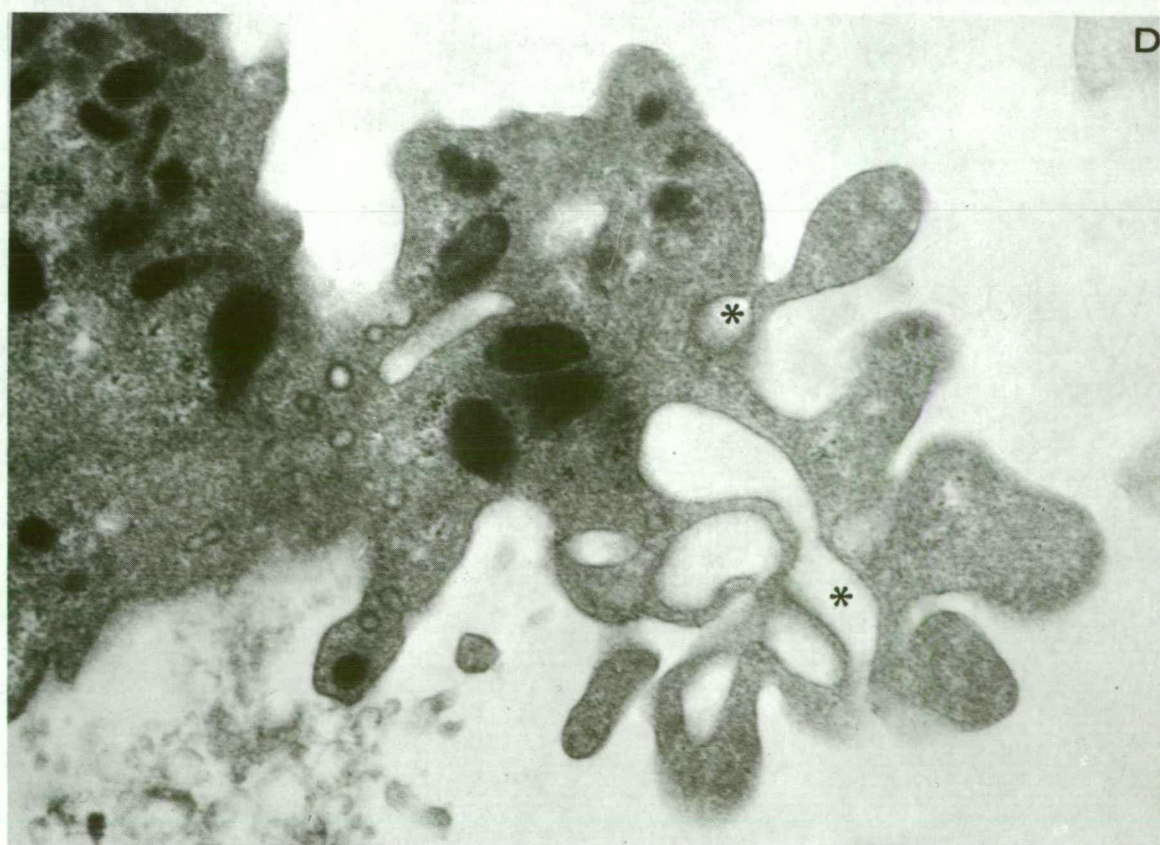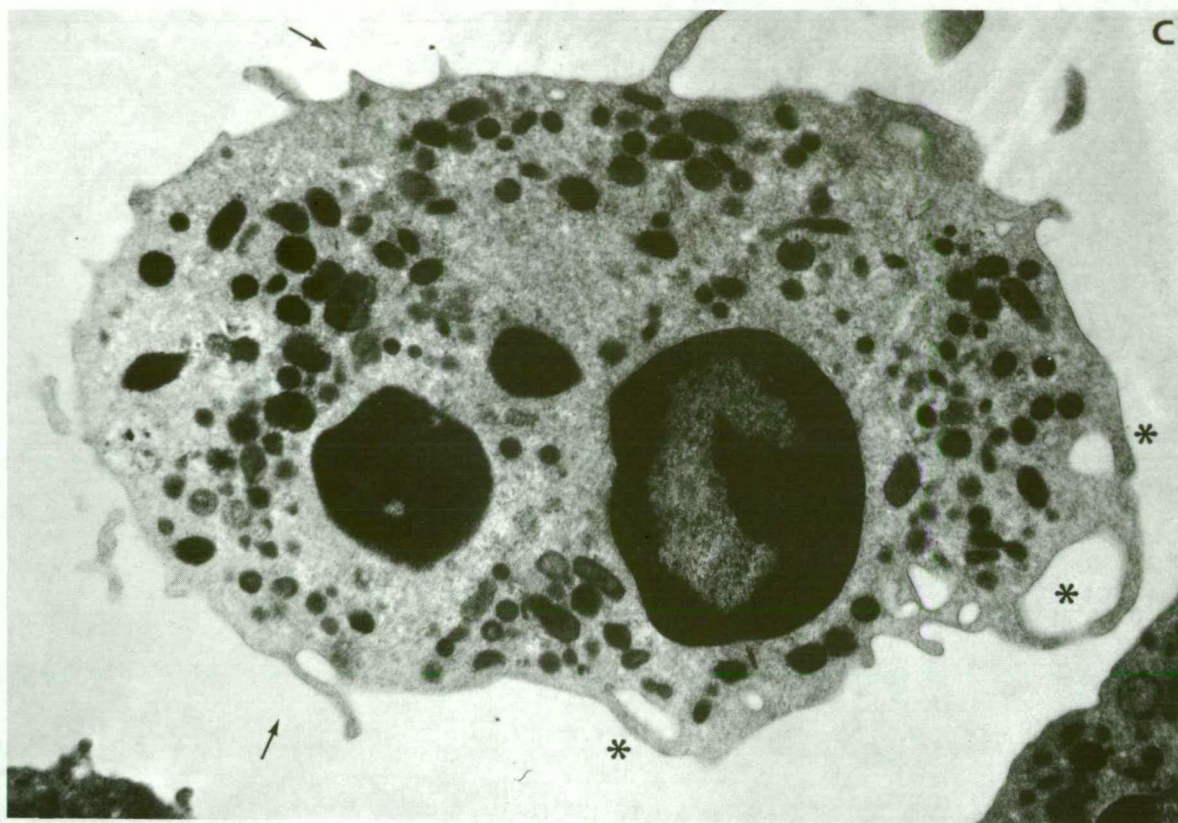
FIGS. 9.7 A and B. Cytochalasin B-treated, non-phagocytic
        granulocytes which have been incubated with NBT
        dye. Deposits of formazan are present in the
        peripheral vacuoles found at regions of cell
        surface blebbing (arrows).
        Magnification   (top)   20,100X
                            (bottom)   20,700X

A
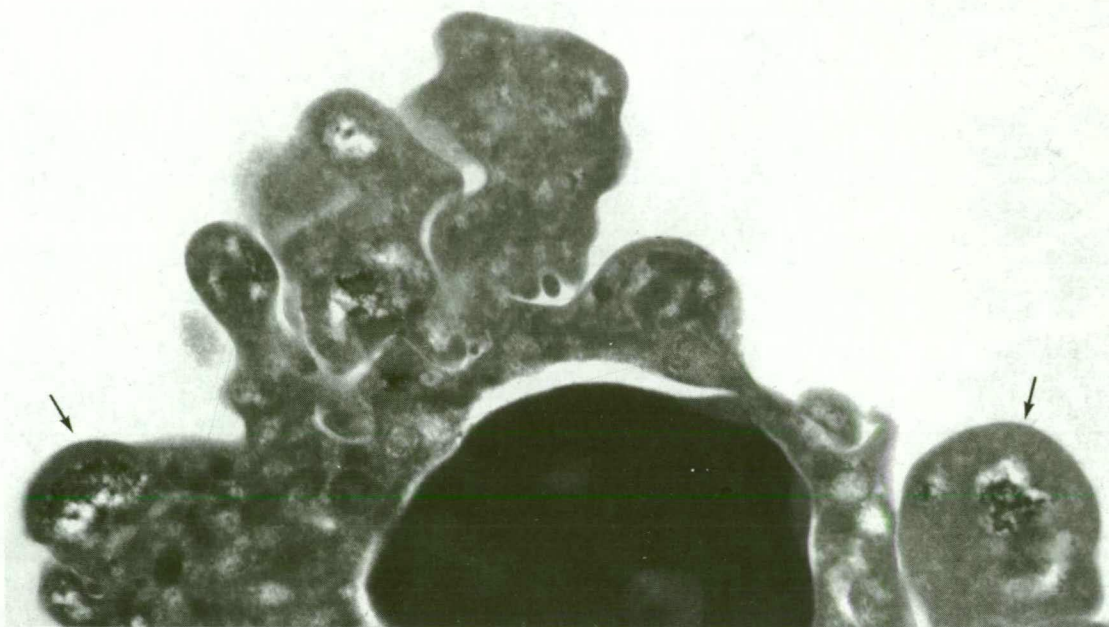
B
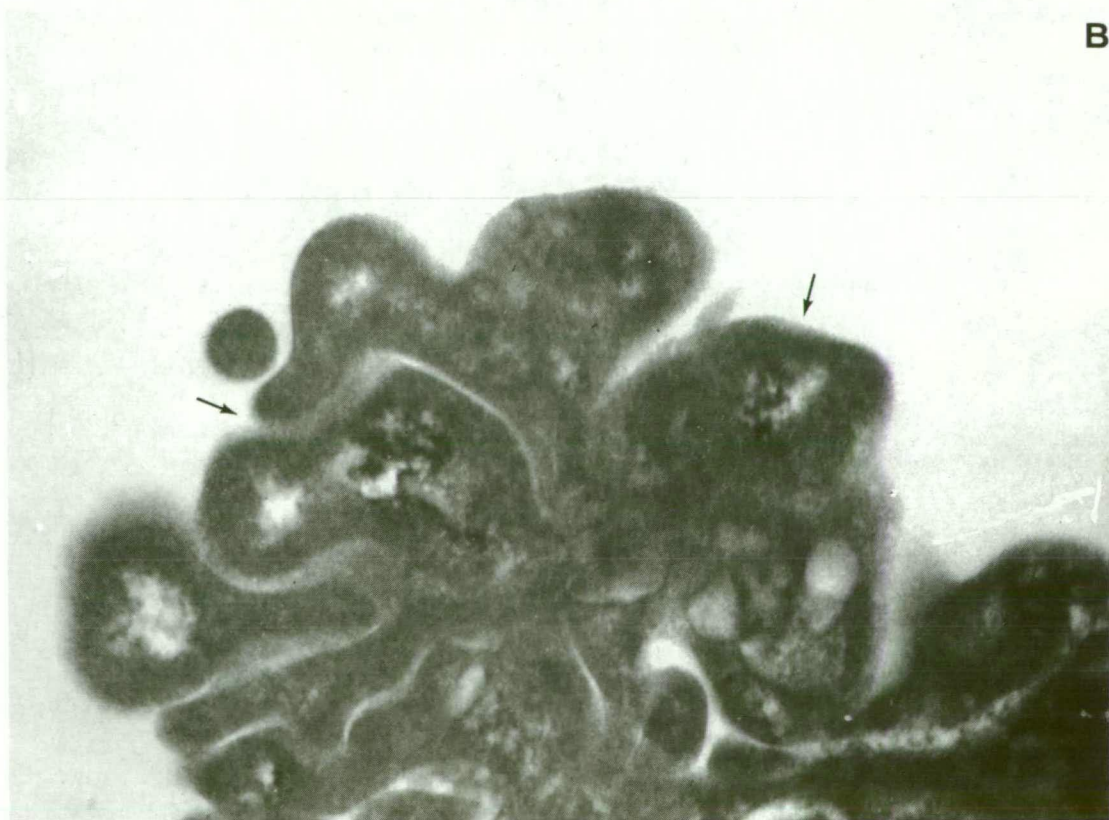
FIG. 9.8. Phagocytic granulocytes incubated with NBT dye.
         Heavy formazan deposits are seen within the
         phagocytic vacuoles (arrows). Cytoplasmic granules
         are depleted in numbers.
         Magnification 20,800X

FIG. 9.9. Colchicine-treated phagocytic granulocytes. These

        cells contained fewer beads per cell (on average)

        than control granulocytes. (cf. FIG. 9.2).

        Magnification 19,200X

FIG. 9.10.A and B. Colchicine-treated phagocytic granulocytes
        incubated with NBT dye. In contrast to untreated
        cells (see FIG. 9.8), these granulocytes displayed
        a light deposition of formazan with the phagocytic
        vacuoles (arrows).

        Magnification   (top)   32,800X
                            (bottom)   15,800X
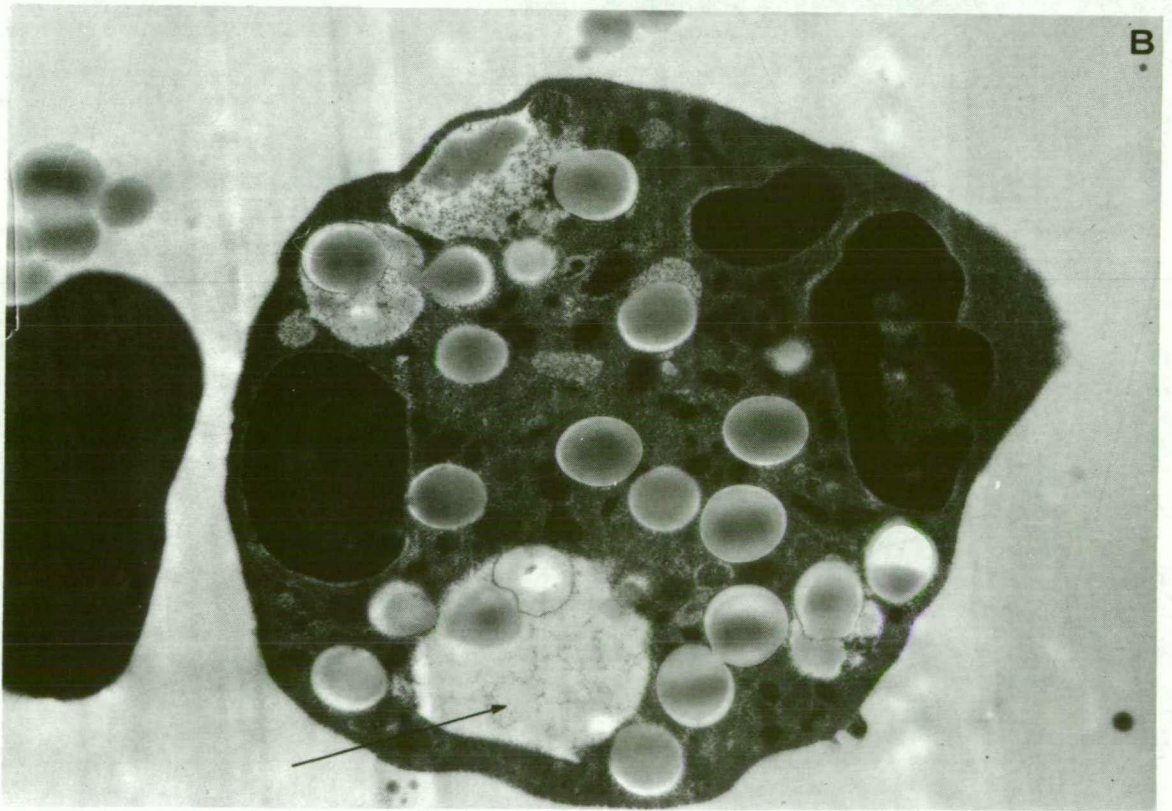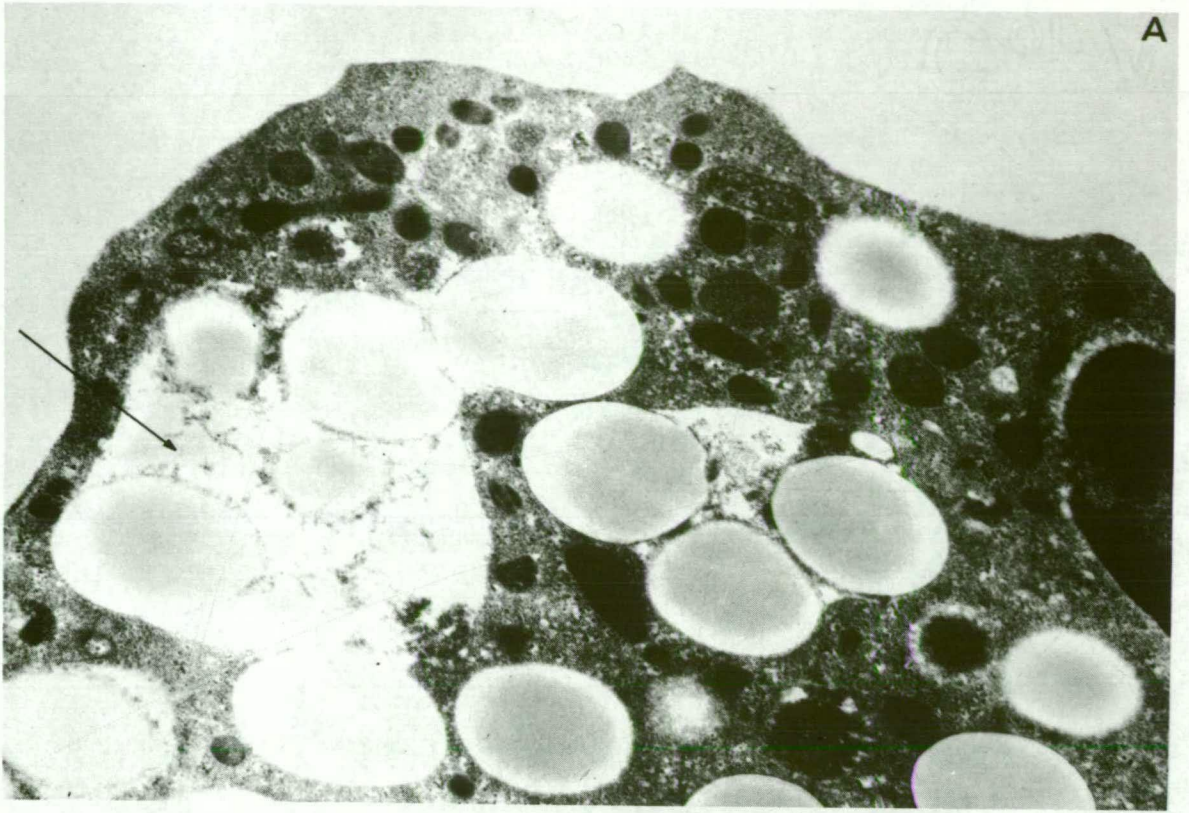
FIG. 9.11.   Cytochalasin B-treated phagocytic granulocytes. These
   (top)   cells contained fewer beads per cell (on average) than

             control phagocytic granulocytes. Cytoplasmic granules

             appear to be depleted in numbers. Many adherent or

             partially internalized beads were seen at the cell

             surface of these granulocytes (arrows).

             Magnification   14,600X.

FIG. 9.12.   Cytochalasin B-treated, phagocytic granulocytes
  (bottom)   which have been incubated with NBT dye. A very

             heavy deposit of formazan is present in the

             phagocytic vacuoles. On the right-hand side, an

             incompletely closed phagocytic vacuole appears to

             be releasing dye to the outside of the cell

             (arrow). Faint deposits of reduced dye are seen

             at points of bead attachment to the cell surface

             (at 1, 2 and 3). Few cytoplasmic granules are

             evident in this cell section.

             Magnification   16,000X.
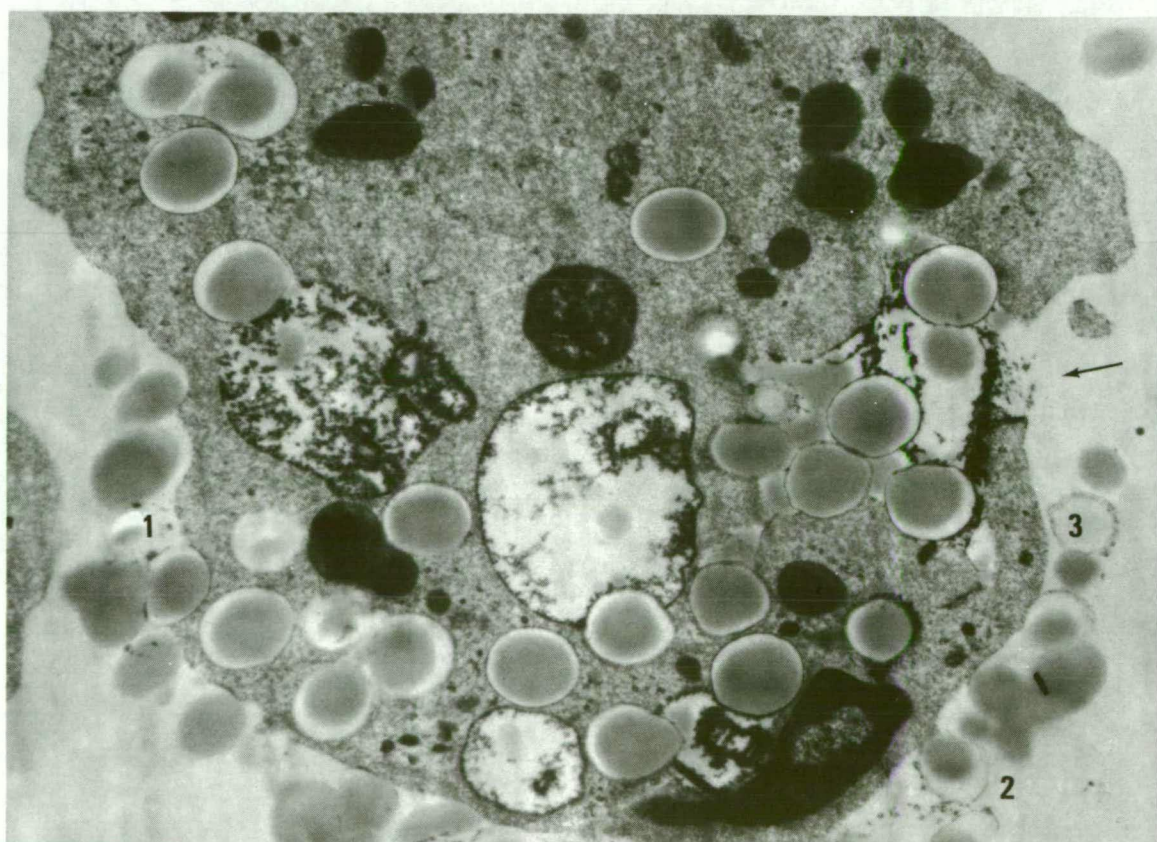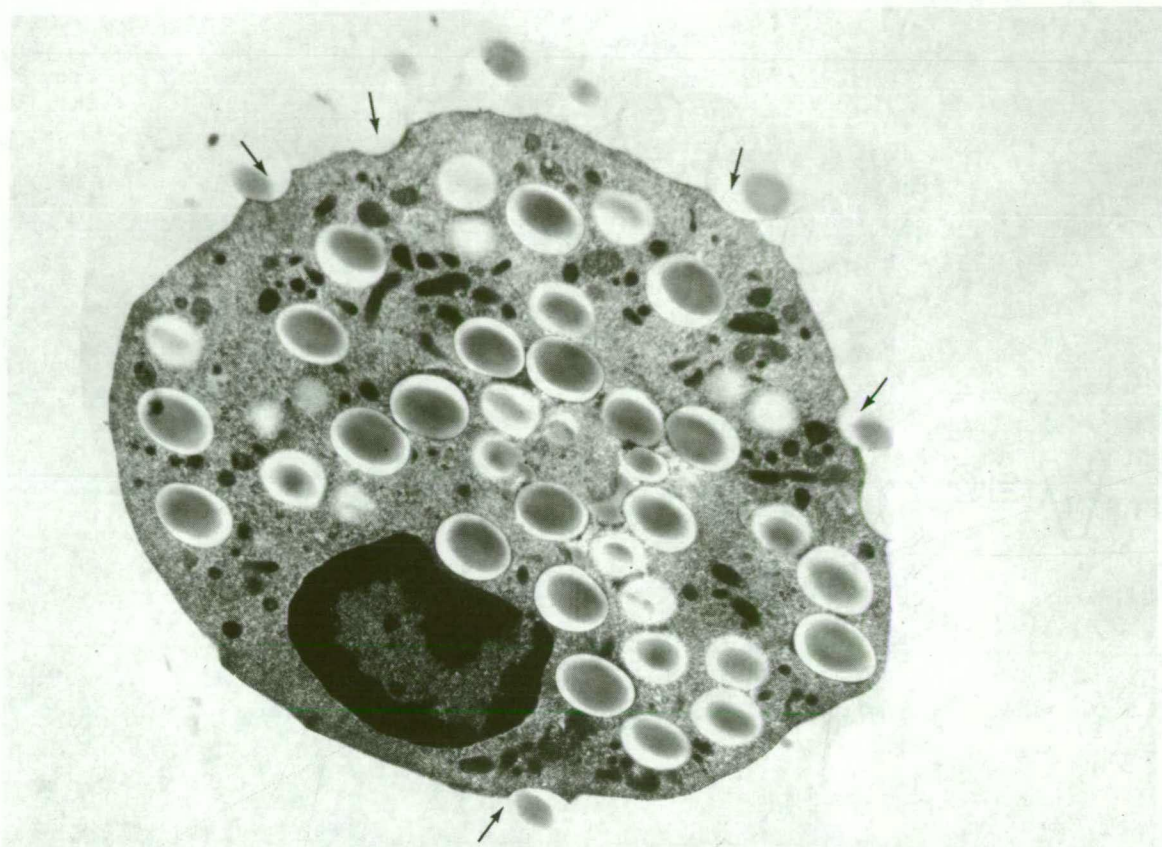
SECTION   10

DISCUSSION

10.1   <u>Introductory Remarks</u>

The studies described in this thesis examined the possible role of the cytoskeletal elements (microfilaments and microtubules) in controlling the activity of the granulocyte plasma membrane oxidase. There have been no detailed investigations of this topic published to date. These studies were prompted by the recent reports of

(i)   a plasma membrane location for this enzyme (Appendix I)

and

(ii)   plasma membrane phenomena (such as capping and the control of the topographical organization of the granulocyte plasma membrane during phagocytosis) which are under the control of the cytoskeletal elements (see section 1.7).

This thesis examined the conflicting literature on the nucleotide specificity and location of the oxidase within the granulocyte (Appendix I). *In vitro* techniques for isolating granulocytes from human peripheral blood, and for quantitating the activity of the plasma membrane oxidase were developed (Sections 2, 3 and Appendix III) and used for these investigations of the role of the cytoskeleton in controlling oxidase activity (Sections 4, 5, 9). The involvement of microfilaments and microtubules in oxidase activity was studied by using pharmacological agents known to disrupt these structures *in vitro* (cytochalasin B, and colchicine, vinblastine, vincristine, respectively - see Appendix IV). Control experiments were also performed to ensure that microfilament and microtubule disruption by these agents provided the best explanation for the results presented herein (see Sections, 5, 6, 7 and

Appendix IV). Correlative experiments were conducted to determine

if a change in HMPS activity was associated with the observed

changes in plasma membrane oxidase activity brought about by the

drug treatments (Section 8). In this final section of the thesis

the evidence supporting the involvement of microfilaments and

microtubules in controlling plasma membrane oxidase activity is

discussed and presented in conjunction with the relevant findings

from other laboratories.

10.2   The problems of studying the granulocyte plasma membrane oxidase

For these studies it was necessary to devise an

in vitro technique to monitor plasma membrane oxidase activity

without interference from the granule oxidase (see section 3.1

and Appendix I). It was reasoned that it would be desirable

to work with intact granulocytes because methods for isolating

plasma membrane fragments which retain their full biological

activity and are not physically altered (for example, turned

'inside-out' during preparation) have not been perfected

(See Bennett and Cuatrecasas, 1973 and Crumpton *et al*, 1977).

Furthermore, these plasma membrane fragments do not maintain

functional or physical relationships with the cytoskeletal

elements, and are therefore not suitable for studies on the

role of microfilaments and microtubules in controlling plasma

membrane oxidase activity.

It was also reasoned that the best method for examining

the plasma membrane oxidase would be to use non-phagocytic

granulocytes and to quantitate oxidase activity via NBT reduction.

In the absence of phagocytosis NBT is unable to cross the

granulocyte plasma membrane and therefore is reduced at the cell surface

and remains *in situ* at the site of reduction (see section 3.4).

In phagocytic granulocytes however, NBT enters the cell along with

the ingested particle and once reduced intracellularly it cannot

be determined if the dye reduction was the result of granular

or plasma membrane oxidase activity (see section 5.4). Although

it seemed that granulocytes exposed to colchicine or cytochalasin

B (to arrest phagocytosis) would be useful for studying the plasma

membrane oxidase, this was not so because it was observed that

the inhibition of phagocytosis was only partial with these agents

(see sections 5.4 and 9.4). Cytochalasin B-treated granulocytes

were also unsuitable because many particles adhered to the surface

of these cells. This situation resulted in the fusion of cyto-

plasmic granules with the plasma membrane subtending the particle,

and the inability therefore to ascribe oxidase activity to a

granule or plasma membrane oxidase only (see section 5.4). For

these reasons it was decided to conduct experiments on non-

phagocytic granulocytes. In support of this decision, it has

been shown by many investigators that granulocyte oxidase activity

is not dependent upon phagocytosis in any case (Lehrer, 1973;

Goldstein *et al*, 1975, 1977; Roos *et al*, 1976; Johnston and

Lehmeyer, 1976; DeChatelet *et al*, 1976; Stendahl *et al*, 1977).

10.3   The relevant experimental findings

Experiments on non-phagocytic granulocytes showed

that microfilament disruption by cytochalasin B resulted in

enhanced oxidase activity, while microtubule disruption by

colchicine, vinblastine or vincristine produced a dual effect :

a paradoxical enhancement (with low doses or brief exposures to

the agents) or an impairment in oxidase activity. A few other

investigators have also reported either the stimulation with micro-
filament or inhibition with microtubule disruption (Malawista and
Bodel, 1967;   Curnutte and Babior, 1975;   Nakagawara et al, 1975,
1976 a; Okuda et al, 1976;   Zabucchi et al, 1978).   Such results
suggest that the cytoskeletal elements play opposite roles in con-
trolling plasma membrane oxidase activity, with microtubules being
necessary for, and microfilaments acting as a constraint against
oxidase activity.   The requirement for microtubules was highlighted
in the experiments described herein, where the usual enhancement in
oxidase activity with cytochalasin B was ablated when the granulo-
cytes were concomitantly incubated with a microtubule-disrupting
agent (see section 4.3).   Qualitatively similar results with these
agents were also observed in experiments with phagocytic granulo-
cytes, but for the reasons outlined above (section 10.2), these
experiments were deemed unsuitable for studying plasma membrane
oxidase activity after drug treatments.

The activity of the granulocyte HMPS was not similarly
affected by the drug treatments and thus oxidase activity and HMPS
activity seem to be independently regulated in the granulocyte.
This situation is reminiscent of the separation between

(i)   oxidase activity and phagocytosis (section 10.2)

(ii)   phagocytosis and lysosomal enzyme release (Zurier,

       Hoffstein and Weissman, 1973 a,b;   Goldstein et al, 1975;

       Roos et al, 1976;   Roos, Homan-Müller and Weening, 1976)

(iii) the abnormal oxidase yet normal HMPS activity of chronic

       granulomatous disease granulocytes (Baehner and Nathan,

       1967;   Holmes, Page and Good, 1967;   Tsan et al, 1976a;

       Oliver et al, 1977).

Electron microscopy revealed that colchicine-treated granulocytes had a patchy distribution while untreated granulocytes showed an homogeneous pattern of NBT-dye reduction at the cell surface. This suggests that the decrease in oxidase activity observed with colchicine was associated with or due to a loss in the topographical organization of the plasma membrane. It is very likely that a colchicine-induced disruption of microtubules produced the loss of plasma membrane organization by facilitating the movement of proteins (perhaps including the oxidase) within the membrane (see section 1.7). It was shown by fluorescence microscopy that colchicine caused a translocation of concanavalin A-binding proteins in the membrane. Therefore it seems reasonable to assume that a similar movement of the oxidase occurred because the concanavalin A-binding proteins and the oxidase share two important properties, namely

(i) both appear to be granulocyte plasma membrane ecto-
proteins [Nicolson and Singer, 1974; Oliver, Ukena and Berlin, 1974 (con A-binding proteins); Briggs *et al*, 1975; Goldstein *et al*, 1975, 1977; Root and Metcalf, 1977 (oxidase)], and

(ii) both appear to be selectively removed from the plasma
membrane and incorporated into the phagosome membrane during phagocytosis [Oliver, Ukena and Berlin, 1974 (con A-binding proteins); Briggs *et al*, 1975; Segal *et al*, 1978; Segal and Jones, 1978, 1979 (oxidase - see Appendix I)].

As mentioned above, cytochalasin B treatment of granulocytes resulted in enhanced plasma membrane oxidase activity spectrophotometrically. In addition, cytochalasin B

treatment appeared to induce increased cell surface activity in general, as electron micrographs showed surface undulations and blebs as well as peripheral vacuole formation in these granulocytes (section 9.3). These ultrastructural features of cytochalasin B-treated cells have also been described by others (Zigmond and Hirsch, 1972; Butcher and Perdue, 1973; Holtzer et al, 1973; Mayhew and Maslow, 1974; Godman et al, 1975; Bhisey and Freed, 1975; Sundqvist and Otteskog, 1978).

An examination of these peripheral vacuoles in cytochalasin B-treated non-phagocytic granulocytes showed them to be a location for NBT dye reduction. The reduced dye in these vacuoles was most likely emanating from a plasma membrane rather than granule oxidase activity because granules were not seen fusing with these non-phagocytic vacuoles (section 9.3). This conclusion is strengthened by the reports from other laboratories, showing that

(i)   the granules do not fuse with the plasma membrane of
        cytochalasin B-treated, non-phagocytic granulocytes
(Henson, 1971 a,b; Henson, 1972; Zurier, Hoffstein and Weissmann, 1973 a; Henson and Oades, 1975) and that

(ii)   oxidase activity and lysosomal enzyme release from the
        granules occur independently in cytochalasin B-treated
granulocytes (Goldstein et al, 1975; Roos et al, 1976). Assuming the dye reduction in these vacuoles to be from plasma membrane oxidase activity, the oxidase was probably incorporated into the vacuole membrane at the time of vacuole formation in the cytochalasin-B treated cells. The experiments presented in this thesis did not attempt to determine if this dye was

reduced before or after vacuole formation. However, it can be reasoned that dye reduction from the cell surface oxidase does not have to occur before these peripheral vacuoles form, because Zigmond and Hirsch (1972) have observed vacuole formation in cytochalasin B-treated granulocytes not reducing NBT dye.

The observations presented herein, and the suggestions from the other workers that cytochalasin B treatment:-

(i)   disrupts a microfilamentous barrier under the plasma
      membrane, thereby facilitating the fusion of granules
with the plasma membrane subtending attached particles (Zurier, Hoffstein and Weissmann, 1973a; Goldstein *et al*, 1975; Hoffstein, Goldstein and Weissmann, 1977)

(ii) prevents the granulocyte respiratory burst from being
      terminated (Jandl *et al*, 1978) and

(iii) enhances immunoglobulin receptor redistribution on
      the granulocyte cell surface (Sajnani, Ranadive
and Movat, 1976) indicate that microfilaments generally play a negative role in granulocyte function. Therefore, the usual consequence of microfilament disruption by cytochalasin B is enhanced granulocyte activity.

10.4   Conclusions to be drawn from these studies

Although the evidence presented herein suggests that the cytoskeletal elements play a role in controlling the activity of the plasma membrane oxidase, it is difficult to devise a scheme incorporating the results. It is a particularly hard task when so many divergent results have been reported from studies with the traditional microfilament and microtubule-disrupting agents (see sections 4.4 and 5.4), and especially

when dual effects are observed with the same agent. It is

interesting to note that comparable studies of granulocyte

functions other than oxidase activity are also difficult to

interpret for the same reasons. [For example, Becker and Showell

(1974) observed that colchicine and vinblastine inhibit chemo-

taxis at high doses yet enhance it at low doses. Similarly, the

dose of cytochalasin B employed will determine whether random

migration (Becker et al, 1972) or phagocytosis is enhanced or

inhibited by drug treatment (Cannarozzi and Malawista, 1973).

From these reports it is also evident that the different

granulocyte functions which have been shown to be mediated via

the cytoskeletal elements (see Becker, 1976 for a brief review)

are not all interdependent or inseparable events. Thus, micro-

filament disruption is known to inhibit chemotaxis and

phagocytosis (Borel and Stäh/ein, 1972; Zigmond and Hirsch, 1972)

but enhance lysosomal enzyme release (Zurier, Hoffstein and

Weissmann, 1973a; Becker and Showell, 1974; Goldstein et al,

1975; Hoffstein, Goldstein and Weissmann, 1977) and oxidase

activity (Nakagawara et al, 1976a; Okuda et al, 1976a and

Zabucchi et al, 1978).] Nevertheless, a scheme concerning the

participation of the microfilaments and microtubules in

granulocyte plasma membrane oxidase activity has been devised,

and is shown in Fig. 10.1. By virtue of their attachments to

the enzyme or to adjacent proteins, the microfilaments are

envisaged to physically constrain the oxidase within the plasma

membrane. In this way the microfilaments hold the activity of

the oxidase in check, and thus the enzyme is freed and active once

the microfilaments are removed. However, the oxidase is optimally

active only as long as the microtubules are present to ensure

that the necessary plasma membrane topography for the

enzyme's activity is maintained. As shown in Fig 10.1, the

microtubules may also be attached to the enzyme and to the micro-

filaments. With such a scheme it is reasonable to assume that

the binding of particles to the surface of the granulocyte would

result in the recruitment of microtubules to that area to aid

ingestion and promote oxidase activity. However, some recent

work by Berlin and Oliver (1978) would suggest that the opposite

situation occurs. These workers found that microtubules were not

located in the pseudopod region of phagocytic granulocytes,

or the region underneath concanavalin A caps. Conversely,

microfilaments were found in those regions. These findings imply

that microfilaments are needed but microtubules are not required

for ingestion, and perhaps the metabolic burst accompanying

phagocytosis. Similar findings have not been reported by others,

and in fact Berlin and Oliver (1978) admit that it is not easy to

explain some aspects of their work. For example, it is difficult

to understand how concanavalin A receptors can move from a region

containing microtubules[1] to one free of them and yet rich in

microfilaments (Berlin and Oliver, 1978; Oliver, 1978). To

explain their unique findings they suggest that the microtubules

control the distribution and function of the microfilaments, and

through the microfilaments,are able to alter the topography of the

cell surface. This is somewhat different from the scheme

---

[1] The binding of concanavalin A to the cell surface induces
microtubule assembly in that region and therefore micro-
tubules subtend the regions of concanavalin A binding (see
Oliver (1978),and section 6.4).

presented above and the one in which microtubules (or a complex of microfilaments and microtubules) modulate the cell surface topography, and where microfilaments control the movement of cell surface receptors once the microtubules are disrupted (see Nicolson, 1976 and Edelman, 1976). It must be emphasized that all of these schemes suffer from the weakness of inferring positive roles for the cytoskeletal elements based upon the results of experiments where these structures are disrupted by drug treatments. Nevertheless, there appears to be some justification for devising roles for the microfilaments and microtubules in granulocyte plasma membrane oxidase activity, as outlined in the next section (10.5).

10.5   The relevance of these findings to *in vivo* studies

Studies of the granulocytes from patients with chronic granulomatous disease (CGD) have provided evidence that there is some justification (*in vivo*) for suggesting that the cytoskeletal elements play a role in granulocyte plasma membrane oxidase activity. As detailed in Appendix I, CGD granulocytes are unable to respond to phagocytosis with an increase in oxidase activity. There are a few recent reports indicating that the plasma membrane rather than the granule oxidase activity is defective in these cells (Segal and Peters, 1976, 1978; Gabig, Kipnes and Babior, 1978). Furthermore, lectins such as phytohaemagglutinin (PHA) and concanavalin A (con A) bind to the CGD granulocyte cell surface, but the cells do not then respond with the usual increase in HMPS and oxidase activity, even though the HMPS is intact (Romeo and Rossi, 1973; DeChatelet, 1975; Tsan *et al* 1976a; Tsan *et al*, 1977; Oliver *et al*, 1977). In contrast,

the binding of either lectin by healthy granulocytes is followed

by an increase in the number of microtubules in the cells

(see Hoffstein, Soberman *et al* 1976; Weissmann *et al*, 1975 a,b;

Oliver, 1976b; Oliver, Albertini and Berlin, 1976; Hoffstein

et al, 1976; Oliver *et al,* 1977) as well as an increase in

oxidase and HMPS activity (Repine *et al*, 1974; DeChatelet,

Shirley and Johnston, 1976; Oliver *et al*, 1977). This enhancement

in HMPS activity could be ablated if the healthy granulocytes

were pretreated with colchicine prior to binding PHA to the cell

surface. Thus it seems very likely that the defective oxidase

activity of CGD granulocytes is tied in to this colchicine-

sensitive (microtubule-dependent?) mechanism for transducing

the signal from the cell surface (PHA binding) to the interior

(HMPS activation). [Edelman (1976) and DeChatelet (1978) have

presented schemes where the cytoplasmic microtubules are involved

in transducing such signals to the interior of the cell.]

However, the defect in CGD granulocytes could arise from a more

complex situation because:-

(i)   in contrast to the results with PHA, pretreatment

        of healthy granulocytes with colchicine prior to bind-

ing con A to the cell surface did not ablate the con A-

induced signal to increase HMPS activity (Tsan *et al*, 1976a)

(ii)   the amount and distribution of con A binding to the

        CGD granulocyte cell surface is normal (Tsan *et al*, 1976a;

Oliver *et al*, 1977 respectively). Furthermore, the bound con A

becomes redistributed into a cap in both healthy and CGD granulo-

cytes after colchicine treatment (Oliver *et al* , 1977) suggesting

that CGD granulocytes have functionally normal microtubules

(see section 6.1).

(iii)   the studies of Nakagawara *et al* (1976a, 1977) showed that

        CGD granulocytes did not respond to incubation with the

cytochalasins by exhibiting the usual enhancement in oxidase

activity. These studies are difficult to interpret but suggest

that the increase in oxidase activity which occurs after micro-

filament disruption is missing in CGD granulocytes.

(iv)   the cell surface of CGD granulocytes is abnormal (Lehrer,

        Olofsson and Ferrari, 1977;   Segal and Peters, 1978)

and could be responsible along with abnormal plasma membrane-

cytoskeletal interactions for the features of CGD granulocytes

(Oliver, 1978).

One other disease involving the granulocytes has been

investigated thoroughly. Chediak-Higashi granulocytes show

impaired degranulation and chemotaxis which appear to be

secondary to impaired microtubule function[2]. In spite of these

two microtubule-mediated defects in function, no impairment in

oxidase activity exists (Wolff *et al*, 1972). Therefore it must

be concluded that this disease does not provide any evidence in

favour of a role for the cytoskeleton in oxidase activity.

It is relevant to mention that the microtubules themselves

can be 'regulated' by oxidase activity, in that the products of

oxidase activity (especially H_2O_2) can oxidize and thereby impair

the function of the microtubules if the enzyme systems for

detoxifying H_2O_2 are not functioning normally. Thus in patients

---

[2] These defects can apparently be corrected by altering the
     levels of cyclic nucleotides in the granulocytes, and thereby
     affecting the dynamic state of the microtubules (see Oliver,
     1978).

with a deficiency in glutathione reductase or glutathione synthetase there is a generalized loss of granulocyte function because of the microtubular defect (see Oliver, 1978).

In conclusion, although there are only a few diseases which provide *in vivo* support, the information from these diseases in combination with the evidence from the *in vitro* work described herein is strongly suggestive of a role for the cytoskeletal elements in controlling granulocyte plasma membrane oxidase activity.

10.6   Future Work

During the course of these studies, several lines of investigation which would be suitable for future work were uncovered, and will be mentioned at this point.

(1)   It is clear that a careful re-examination of the

oxidase activity associated with the cytoplasmic granules is required.   The granule oxidase activity should be investigated by using plasma membrane and myeloperoxidase-free granule fractions (see Appendix I).

(2)   Further studies of the plasma membrane oxidase of

CGD granulocytes are required (see Appendix I).   In addition, the granulocyte functions which have been shown to be mediated or influenced by the cytoskeletal elements need to be examined by independent laboratories.

(3)   Studies of the temporal sequence of events during the post-

phagocytic metabolic burst are needed.   Recently Tsan, Newman and McIntyre (1976 b) provided some evidence indicating that superoxide production is the initial event after phagocytosis, but this finding needs to be confirmed by other workers.

(4)   For several reasons, an investigation of the influence of

plasma membrane compositional changes on cell surface oxi-

dase activity is warranted.   Firstly, there is evidence that the

phospholipid composition of the granulocyte plasma membrane

changes with phagocytosis (Elsbach, 1968;   Elsbach *et al*, 1972).

Secondly, studies of an NADH-dependent NBT-reductase in human

erythrocyte membranes (Zamudio, Cellino and Canessa-Fischer, 1969)

and an NADH-dependent, O_2^--generating ubiquinone reductase in beef

heart mitochondria (Cadenas *et al*, 1977;   Heron, Corina and Ragan,

1977) suggest that only the latter enzyme has a phospholipid

requirement for optimal activity.   Briggs *et al* (1975) and Segal

and Peters (1976, 1977, 1978) have provided some evidence that

the plasma membrane oxidase of the granulocyte utilizes NADH

as the electron acceptor.   Therefore, it seems reasonable to

suggest that studies are needed to determine if the changes in

phospholipid composition of the membrane during phagocytosis

can influence the activity of the plasma membrane oxidase.
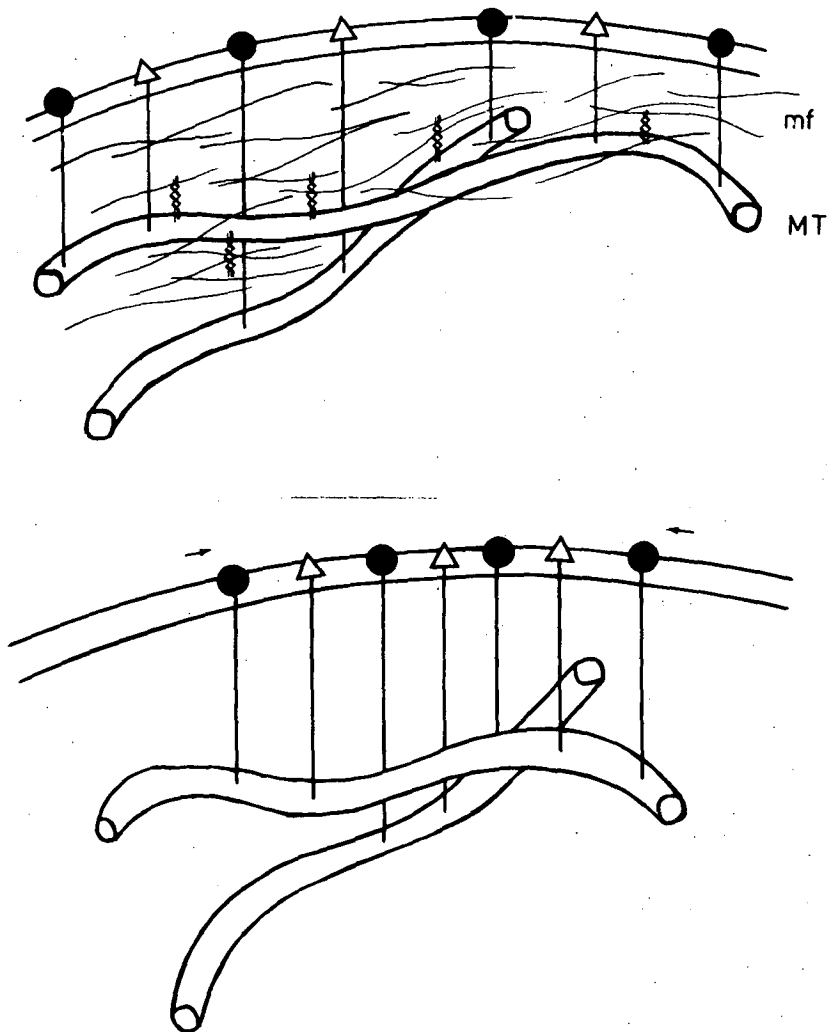
FIG. 10.1. A scheme to represent the role of microfilaments (mf)
           and microtubules (MT) in controlling plasma membrane
oxidase activity. The oxidase (triangles) and other plasma membrane
components (circles) are shown localized to particular regions
within the membrane by virtue of attachments to the underlying
mesh of microfilaments. The plasma membrane components are
envisaged to be connected to the subtending microtubules as well.
Hypothetical connections between the cytoskeletal elements
are indicated by the wavy lines (top).

          As shown in the lower diagram, a suitable chemical or
physical perturbation of the membrane disrupts the microfilaments
in that region, leaving the microtubules free to control (and
alter) the topography of the cell surface. With the change in
cell surface organization the enzyme is shown located in a milieu
favourable for its activity (see also section 10.4).

APPENDIX I

THE OXIDASE RESPONSIBLE FOR THE

METABOLIC BURST

APPENDIX   I   THE OXIDASE RESPONSIBLE FOR THE METABOLIC BURST

I.1   INTRODUCTION

With phagocytosis, granulocytes display a metabolic
burst characterised by a cyanide-insensitive increase in O_2 uptake,
O_2^- and H_2O_2 production, and an increase in the oxidation of
glucose via the HMPS (section 1.3 and 1.4 of this thesis).
Several granulocyte enzymes have been suggested as the source of
the O_2^- and H_2O_2, including myeloperoxidase (Roberts and Quastel,
1964), D-amino acid oxidase (Cline and Lehrer, 1969; Eckstein,
Baehner and Nathan, 1971; DeChatelet, McCall and Cooper, 1972a),
and an NADH or NADPH-dependent oxidase (see discussion to follow).
As outlined by Baehner and Karnovsky (1968), the enzyme of the
metabolic burst must display the following features:-

(i)   an insensitivity to cyanide

(ii) H_2O_2 must be a product of the enzyme's activity[1]

(iii) the enzyme would have to be present in a large

       enough quantity in granulocytes to account for

       the oxidative stimulations seen during

       phagocytosis

(iv) a link or relationship between the enzyme and

     the HMPS should exist.

---

[1] More recent work has shown that superoxide (O_2^-) is the actual
     product of the enzyme's activity and that H_2O_2 is formed from
     the O_2^- as shown in equation I.1 (Babior, Curnutte and McMurrich,
     1976; Babior, 1978).

For several reasons, D-amino acid oxidase (DAAO) is no longer considered to be the oxidase of the metabolic burst. The studies of Eckstein, Baehner and Nathan (1971) showed no difference in the DAAO activity of healthy or chronic granulomatous disease (CGD) granulocytes (a disease characterised by a failure of the granulocytes to produce O_2^- and H_2O_2 after phagocytosis, as discussed in section I.2), indicating that DAAO was not responsible for the O_2^- or H_2O_2 production. Similarly, no increase in HMPS activity was observed when resting or phagocytosing granulocytes were incubated with D-amino acids as substrates for DAAO (DeChatelet, McCall and Cooper, 1972a). It has therefore been concluded that DAAO is unlikely to be the oxidase of the metabolic burst even though H_2O_2 is a product of this enzyme's activity (Eckstein, Baehner and Nathan, 1971; DeChatelet, McCall and Cooper, 1972a).

As mentioned above, myeloperoxidase (MPO) has also been considered responsible for the production of O_2^- and H_2O_2 during phagocytosis. However, for three reasons this enzyme has been discounted as being involved in the oxidative events of phagocytosis:-

(i)   MPO-deficient patients have normal oxidase activities (reviewed by Patriarca et al, 1976; McPhail, DeChatelet and Shirley, 1977a).

(ii) the MPO activity of resting and phagocytosing granulocytes is similar, while the activity of the crucial oxidase rises dramatically with phagocytosis (McPhail, DeChatelet and Shirley, 1977a).

(iii) the oxidase is cyanide-insensitive while

peroxidases, such as MPO, are known to be

inhibited by KCN (Roberts and Quastel, 1964;

Patriarca *et al*, 1976).

Recent evidence indicates that while MPO is very
unlikely to be the enzyme in question, it does possess NAD(P)H
oxidase ability which, unlike its peroxidase activity, is not
inhibited by cyanide. This and other studies which necessitate
a re-evaluation of the role of MPO in generating O_2^- and H_2O_2 are
discussed in section I.3.6.2.

I.2   Studies with CGD and normal granulocytes

It is widely accepted that the crucial enzyme is a
reduced pyridine nucleotide oxidase, but there has been consider-
able debate for many years over which nucleotide (NADH or NADPH)
is employed by the oxidase as the electron acceptor (see for
example Karnovsky, 1962; Karnovsky, 1973; Babior, Curnutte and
McMurrich, 1976; Babior, 1978). To study this question many
investigators have examined the granulocytes from CGD patients.
CGD was first recognized as a distinct clinical entity by
Berendes, Bridges and Good (1957). It is classically an X-linked
recessive disorder of males but can also arise in females from
an autosomal recessive pattern of inheritance (reviewed by
Babior, 1978). CGD granulocytes appear to ingest micro-
organisms normally (Quie *et al*, 1967; Stossel, Root and Vaughan,
1972) and possess normal glycolytic pathways (Holmes, Page and
Good, 1967), but they lack the usual post-phagocytic metabolic
burst. They are missing the increase in O_2 uptake, HMPS activity
and O_2^-/H_2O_2 production (Baehner and Nathan, 1967; Curnutte,

Whitten and Babior, 1974; Johnston *et al*, 1975), and they may or may not be able to degranulate normally (Quie *et al*, 1967; Baehner, Karnovsky and Karnovsky, 1968; Gold *et al*, 1974). As a result CGD granulocytes are unable to kill certain species of bacteria and the patients suffer from severe infections with granuloma formation (reviewed by Babior, 1978; Hosking, Fitzgerald and Shelton, 1978). Studies of CGD granulocytes have not ended the controversy over which pyridine nucleotide oxidase is involved, as investigators have demonstrated defects in NADPH-dependent oxidase activity (Hohn and Lehrer, 1975; McPhail *et al*, 1977b; DeChatelet *et al*, 1978; Gabig, Kipnes and Babior, 1978); NADH-dependent oxidase activity (Baehner and Nathan, 1968; Baehner and Karnovsky, 1968; Karnovsky, 1973; Segal and Peters, 1976, 1978); both oxidases (Hohn and Lehrer, 1975; Babior, Curnutte and McMurrich, 1976; Iverson *et al*, 1977); or neither oxidase (Nathan, Baehner and Weaver, 1969).

Similarly, investigations of the oxidase activity of healthy (non-CGD) granulocytes have proved just as contradictory. A survey of the literature reveals that most authors favour an NADPH oxidase, and for that reason the remainder of this Appendix will be primarily concerned with examining the arguments for and against the NADPH oxidase. It is clear that the debate over which oxidase is responsible for the metabolic burst is still unresolved, although Babior (1978) recently stated that he believed the evidence to overwhelmingly favour NADPH.

I.3   Arguments for and against an NADPH oxidase

I.3.1   The link between the oxidase and the HMPS

It has been pointed out by several workers that a direct link between the activity of the oxidase and the stimulation of the HMPS exists when the oxidase employs NADPH, but not NADH (Cagan and Karnovsky, 1964; Baehner and Karnovsky, 1968; Baehner, Gilman and Karnovsky, 1970; Patriarca et al, 1971b). A reduction of O_2 to O_2^- (and ultimately H_2O_2) by an NADPH oxidase would result in the production of $NADP^+$, as shown by Babior (1978):-

$$NADPH \quad + \quad 2O_2 \quad \xrightarrow{1} \quad NADP^+ \quad + \quad 2O_2^- \quad + \quad H^+$$

$$2H^+ \quad + \quad 2O_2^- \quad \xrightarrow{2} \quad H_2O_2 \quad + \quad O_2 \qquad (I.1)$$
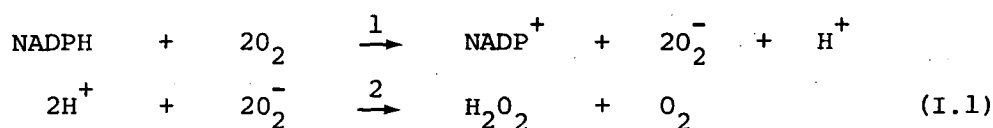
where 1 = NADPH oxidase

2 = superoxide dismutase

The $NADP^+$ thus produced would directly stimulate the HMPS, as the level of $NADP^+$ controls the rate of glucose oxidation by this pathway (Beck, 1958). HMPS activity then regenerates NADPH and completes the cycle. As shown in Figure I.1, H_2O_2 which leaks from the phagosome would be destroyed by catalase, or would react with reduced glutathione (reviewed by Babior, 1978). The coupling of the glutathione peroxidase and glutathione reductase systems would result in the regeneration of reduced glutathione, and the production of another source of $NADP^+$ to stimulate the HMPS (Reed, 1969; Stossel, 1974; Baehner et al, 1975).

If the oxidase utilized NADH however, there would

no longer be a direct link between the production of O_2^-/H_2O_2 and

the stimulation of the HMPS (Cagan and Karnovsky, 1964; Baehner and

Karnovsky, 1968; Baehner, Gilman and Karnovsky, 1970; Patriarca

et al, 1971b). As shown in Figure I.2, NADH is formed in the

Embden-Meyerhof pathway where triose phosphate is converted to

1,3-diphosphoglycerate[2]. An indirect link between the oxidation

of NADH and the stimulation of the HMPS could be achieved in

three ways:-

I.3.1.1   The glutathione peroxidase/glutathione reductase scheme

The NADH oxidase activity would produce O_2^- and

H_2O_2 as shown below in equation I.2. The H_2O_2 thus produced would

be detoxified via glutathione peroxidase, followed by the reduction

of GSSG coupled to $NADP^+$ formation via glutathione reductase.

The formation of $NADP^+$ in this way would then drive the HMPS (Reed,

1969; Baehner, Gilman and Karnovsky, 1970).

Jacobs and Jandl (1966) and Reed (1969) have shown

that this pathway is quantitatively very important for H_2O_2

utilization in granulocytes from species other than man. Further-

more, it has been shown that the key enzymes glutathione

peroxidase and reductase exist in human granulocytes (Holmes

et al, 1970; Baehner, Gilman and Karnovsky, 1970; Bass *et al*,

1977), and that enough glutathione reductase activity is present

to account for the required re-oxidation of NADPH during

phagocytosis (Baehner, Gilman and Karnovsky, 1970). In addition,

---

2

As discussed in sections I.3.1.3 and I.3.4, the NADH thus
formed would be the substrate for both NADH oxidase and
lactate dehydrogenase.

reduced glutathione levels fall dramatically (Mendelson,

Metz and Sagone, 1977) and glutathione reductase activity rises

within seconds of phagocytosis (Strauss *et al*, 1969) suggesting

the existence of this scheme. The pathway as shown by Wilkinson,

Powars and Hochstein (1975) is:-

$$NADH \ + \ H^+ \ \text{\huge 1} \ O_2 \qquad 2H_2O \ \text{\huge 3} \ GSSG \quad NADPH \ + \ H^+ \tag{I.2}$$

$$NAD^+ \ \text{\huge 1} \ O_2^- \xrightarrow{\ 2\ } H_2O_2 \ \text{\huge 3} \ 2GSH \ \text{\huge 4} \ NADP^+ \qquad ....HMPS$$

stimulation
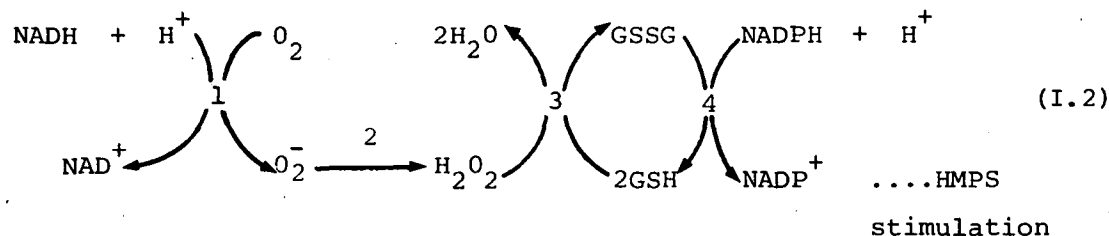
where 1 = NADH oxidase

      2 = superoxide dismutase

      3 = glutathione peroxidase

      4 = glutathione reductase

  GSSG = oxidized glutathione

   GSH = reduced glutathione

I.3.1.2   A transhydrogenase

A transhydrogenase (TH) may link the oxidized and

reduced forms of the two pyridine nucleotides, thereby producing

the $NADP^+$ required to drive the HMPS (Evans and Karnovsky, 1961;

Evans and Kaplan, 1966):-

$$NAD^+ \ + \ NADPH \ \xrightarrow{\ TH\ } NADH \ + \ NADP^+ \tag{I.3}$$

This pathway does operate in the guinea pig, and

transhydrogenase levels in human granulocytes are greatly in

excess of those required to produce enough $NADP^+$ to drive the

HMPS (Evans and Kaplan, 1966; Baehner, Johnston and Nathan, 1972).

I.3.1.3   A lactate dehydrogenase

Evans and Karnovsky (1961) have described a guinea pig lactate dehydrogenase (LDH) which uses NADPH and therefore provides the $NADP^+$ required to stimulate the HMPS. The scheme can be shown as:-

$$\text{pyruvate} \quad + \quad \text{NADPH} \quad \xrightarrow{\text{LDH}} \quad \text{lactate} \quad + \quad NADP^+ \qquad (I.4)$$
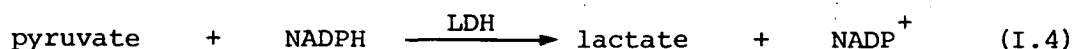
This pathway also overcomes the problem of NADH (the usual substrate for LDH) becoming depleted by the NADH oxidase and therefore un-available for the LDH-catalyzed reaction. An NADPH-dependent LDH has not yet been found in the human granulocyte, however (Baehner, Gilman and Karnovsky, 1970).

In conclusion, any one of these three pathways could provide a link, albeit indirect, to couple the activity of an NADH oxidase to the stimulation of the HMPS. It is apparent that further studies are required to establish the importance of these three pathways in human granulocytes.

I.3.2   Evidence supporting the NADPH oxidase from studies with G6PD-deficient patients

Another argument put forward to support an NADPH oxidase is that a deficiency in G6PD can lead to a CGD-like syndrome, with failure in H_2O_2 production and antimicrobial activity after ingestion (Cooper et al, 1970; Baehner, Johnston and Nathan, 1971; Cooper et al, 1972; Gray et al, 1973; Babior, 1978). As shown in Figure I.2 the activity of the enzyme G6PD and the reduction/oxidation of $NADP^+$/NADPH are linked as the first step of the HMPS, providing the explan-ation for the lowered NADPH oxidase activity of G6PD-deficient

granulocytes (reviewed by Babior, 1978). However, it has been pointed out that both NADH and NADPH oxidase activities are low in this disease, because of transhydrogenase reactions (Baehner, Johnston and Nathan, 1972). Therefore the existence of a diminished production of H_2O_2 in patients with G6PD deficiency cannot be construed as evidence in favour of an NADPH oxidase as the enzyme of the metabolic burst.

I.3.3   Studies of the changes in NAD(P)$^+$/NAD(P)H levels after phagocytosis

Selvaraj and Sbarra (1967) have suggested that the changes in the ratios NAD$^+$/NADH and NADP$^+$/NADPH reliably reflect the events of the post-phagocytic metabolic burst. They found, as did Rossi, Romeo and Patriarca (1972) that in guinea pig granulocytes, the quotient NADP$^+$/NADPH (but not NAD$^+$/NADH) rose after phagocytosis, and therefore concluded that the oxidase of the post-phagocytic metabolic burst utilized NADPH. Recently Aellig *et al* (1977) also examined the ratios, and found that for guinea pigs these workers were correct, but for human granulocytes both ratios were affected similarly. Such studies indicate that neither oxidase is favoured, and that both NADH and NADPH oxidases could be involved in the oxidative changes in human granulocytes.

I.3.4   The problem of competition for NADH as substrate

Another argument put forward to support the case for NADPH and against NADH is the fact that the NADH oxidase would have to compete against lactate dehydrogenase (LDH) for glycolytic NADH (Patriarca *et al*, 1971a,b). In guinea pig granulocytes, the K_m for LDH with respect to NADH has been

calculated to be 4×10^{-6} M while for NADH oxidase it is

4×10^{-3} M (Rossi, Romeo and Patriarca, 1972). Thus it has been

suggested that the NADH oxidase would not be able to compete

successfully against LDH for NADH as substrate, and therefore

an NADH oxidase is not likely to be the critical enzyme of the

metabolic burst (Rossi, Romeo and Patriarca, 1972). Other

investigators had pointed out earlier that this might be a

problem (Cagan and Karnovsky, 1964). Nevertheless, this argument

in favour of the NADPH oxidase may be criticized for several

reasons:-

(i) the experiments of Rossi, Romeo and Patriarca (1972)
were not conducted on human granulocytes, and, as
indicated by Aellig *et al* above (section I.3.3),
guinea pigs cannot be considered to be comparable to
humans with respect to the events of the metabolic
burst. That the species are not identical can also
be seen by examining the levels of granulocyte
glutathione peroxidase (Baehner, Gilman and Karnovsky,
1970; Bass *et al*, 1977)

(ii) the K_m values for the NADH oxidase towards NADH have
been reported by others to be much lower. For
example, when a plasma membrane NADH oxidase of
human granulocytes was studied the K_m was 1.7 µM
(Segal and Peters, 1976). As the cyanide-insenstive
NADH oxidase may be located in the plasma membrane
rather than the granules (the fraction studied by
Patriarca's group), the K_m value of 1.7 µM may be
more accurate (see section I.4).

I.3.5   The K_m of the NAD(P)H oxidase towards its substrate

It has been pointed out that the K_m of the NADPH

oxidase with respect to NADPH is much lower than the K_m of

the NADH oxidase towards NADH, and therefore an NADPH oxidase

is more likely to be the enzyme of the metabolic burst (Rossi,

Romeo and Patriarca, 1972; Babior, Curnutte and Kipnes, 1975;

Babior, Curnutte and McMurrich, 1976). A major criticism can be

levelled at these studies, namely that all of the workers examined

the K_m values for oxidases found in granule fractions from

disrupted cells, while recent evidence indicates that the KCN-

insensitive NADH oxidase at least is localized in the plasma

membrane rather than the granule fraction (see section I.4).

I.3.6   The Mn^{+2} requirement of the NADPH oxidase

I.3.6.1   The oxidase of the granule-containing fractions

In 1971 and 1972, Rossi and Patriarca's group

suggested that the granulocyte NADPH oxidase located in the

granule fraction of disrupted cells required Mn^{+2} for

full activity *in vitro* (Patriarca *et al*, 1971b; Rossi, Romeo

and Patriarca, 1972). Since those reports most investigators

have added Mn^{+2} to their fractions when quantitating NADPH

oxidase activity. The need to add Mn^{+2} to demonstrate NADPH

oxidase activity was first questioned by Karnovsky (1973), and

more recent evidence shows that the Mn^{+2} requirement is not

physiological. For example, Curnutte, Karnovsky and Babior

(1976) have shown that virtually all of the characteristics of

the NADPH-dependent oxidase activity associated with the granule

fraction can be reproduced in a Mn^{+2} - requiring granulocyte-

free system employing xanthine oxidase (from milk) to generate

O_2^-. In these studies, they found that the pH optimum for xanthine oxidase was different from reported values, and that an unusual stoichiometry between the amount of NADPH oxidized and O_2^- produced existed - with the highest rates of O_2^- production very little NADPH was oxidized and vice versa. From this they concluded that the model xanthine oxidase system does not require enzyme interaction with Mn^{+2} or NADPH, and that a Mn^{+2} -catalyzed free radical chain reaction was responsible for the O_2^- production. Thus, Curnutte, Karnovsky and Babior (1976) have shown that a Mn^{+2} -dependent NADPH oxidation can occur in the absence of a granulocyte fraction. However, they state that as the non-enzymatic oxidation of NADPH in the presence of Mn^{+2} is initiated by O_2^-, then a true granule oxidase activity, which is NADPH-dependent, must provide the O_2^- required to initiate the reaction. It would appear that these authors cannot consider this as proof of the existence of a true O_2^- producing granule fraction NADPH oxidase, because Auclair et al (1976) have shown that O_2^- can be produced in a comparable system completely devoid of granulocytes.

Thus it must be concluded that any studies which were conducted on a granule fraction oxidase in the presence of Mn^{+2} are open to the criticism that an enzyme-catalyzed oxidation of NADPH may not have been measured at all.

I.3.6.2   The Mn^{+2}-catalyzed oxidation of NADPH by MPO

The problem with respect to the non-enzymatic oxidation of NADPH when Mn^{+2} is present has been revealed by Takanaka and O'Brien (1975). These workers have shown that granulocyte MPO (which would be present in most granule fractions from disrupted granulocytes) has an NAD(P)H oxidase

activity in addition to its classical peroxidase activity. This oxidase activity produces O_2^-, is enhanced greatly by Mn^{+2}, and is cyanide-insensitive although the classical peroxidase activity of MPO is not. As with the reaction described by Curnutte, Karnovsky and Babior (1976), the interaction between Mn^{+2} and MPO is mediated by O_2^-, is autocatalytic and involves a free radical chain reaction. From these studies it is clear that MPO may be the source of the NAD(P)H oxidase activity observed in granule fractions to which Mn^{+2} has been added.

As an argument against MPO as the source of the granule fraction NADPH oxidase activity, Patriarca *et al* (1976) and McPhail, DeChatelet and Shirley (1977a) compared the effects of various stimulators and inhibitors on the NADPH oxidase activity of several purified peroxidases (including MPO), and the granules from phagocytic cells. Although both groups found striking parallels between the effects of these agents on the two oxidase activities (and concede that there is strong evidence that the oxidase activity of the granule fractions may be due to MPO), they point out that there are several reasons for excluding MPO as being responsible:-

(i)   MPO-deficient patients have normal oxidase activity (reviewed by Patriarca *et al*, 1976; McPhail, DeChatelet and Shirley, 1977a)

(ii)   the critical oxidase is cyanide-insensitive while MPO is inhibited by cyanide (Hohn and Lehrer, 1975; Kakinuma and Chance, 1977)

(iii)   there is no difference in MPO activity between resting and phagocytosing granulocytes, whereas

phagocytosing granulocytes display an increase
in NADPH oxidase activity (McPhail, DeChatelet and
Shirley, 1977a).

Unfortunately these arguments against the granule
fraction oxidase activity being due to MPO are not conclusive,
because:-

(i)   it has not been definitively established that
       MPO-deficient granulocytes are completely lacking
       in MPO (see Patriarca et al, 1976)

(ii)   the NADPH oxidase activity of MPO has been shown
       to be cyanide-insensitive, while the peroxidase
       activity is not (Takanaka and O'Brien, 1975b)

(iii) Paul et al (1972) have found that MPO activity does
       increase with phagocytosis, just as NADPH oxidase
       activity does.

From the above discussion it can be seen that Mn^{+2}-
containing granule fractions catalyze an oxidation of NADPH which
must not be ascribed to a true NADPH oxidase. As outlined, the
oxidation in the presence of Mn^{+2} may be due to MPO (the studies
of Patriarca et al, 1976; McPhail, DeChatelet and Shirley, 1977a),
or it may be a completely non-enzymatic free radical chain
reaction (Curnutte, Karnovsky and Babior, 1976). Such studies
detailing a Mn^{+2}-stimulated oxidation of NADPH render many
earlier reports on CGD and normal granulocytes open to criticism,
for example, the studies of Rossi and Zatti, 1964; Patriarca
et al, 1971b; Hohn and Lehrer, 1975; DeChatelet et al, 1975;
Rossi, Romeo and Patriarca, 1972.

It is interesting to note that although research has been carried out in the absence of Mn^{+2} by several groups, the controversy over an NADH or NADPH oxidase still exists. With reference to these studies, some favour an NADH oxidase:-

> - Cagan and Karnovsky (1964)
>
> - Baehner and Karnovsky (1968)
>
> - Baehner and Nathan (1968)
>
> - Baehner, Gilman and Karnovsky (1970)
>
> - Mandell and Sullivan (1971)
>
> - Briggs *et al* (1975)
>
> - Segal and Peters (1976, 1977, 1978)

while some still favour an NADPH oxidase:-

> - Hohn and Lehrer (1975)
>
> - McPhail, DeChatelet and Shirley (1976)
>
> - McPhail *et al* (1977b)
>
> - Iverson *et al* (1977)
>
> - DeChatelet *et al* (1978)
>
> - Gabig, Kipnes and Babior (1978)
>
> - Auclair, Torres and Hakim (1978)

and some find evidence to support either NADH or NADPH:-

> - Hohn and Lehrer (1975)
>
> - Babior, Curnutte and McMurrich (1976)
>
> - Iverson *et al* (1977)

and again, some find neither oxidase is supported:-

> - Nathan, Baehner and Weaver (1969)
>
> - Babior, Curnutte and Kipnes (1975)

I.3.7   <u>The levels of the oxidase in granulocytes</u>

As an argument against the NADPH oxidase, Karnovsky

(1973) has pointed out that this enzyme activity does not exist

in granulocytes at the level claimed by Patriarca *et al* (1971a,b),

but only at one-tenth of that level, and therefore not enough

NADPH oxidase activity is available to explain the oxidative events

of phagocytosis. Patriarca and Rossi's group have agreed that

the level is too low, but they maintain that this is overcome by the

fact that they find the enzyme's K_m towards NADPH is lowered 10-

fold during phagocytosis (Patriarca *et al*, 1971b; Rossi, Romeo

and Patriarca, 1972). However, Hohn and Lehrer (1975), who have

conducted comparable experiments on the NADPH oxidase, found

that the K_m did not appreciably change with phagocytosis. The

experiments of Patriarca's group and those of Hohn and Lehrer

were conducted with Mn^{+2} present. In this regard, it has been

shown that a change in K_m with increasing O_2^- production (a

situation comparable to the observations of Patriarca's group)

can be duplicated by the Mn^{+2}-dependent, granulocyte-free system

studied by Curnutte, Karnovsky and Babior (1976). Therefore it

must be concluded that the change in K_m with phagocytosis may

have been artefactually produced by Mn^{+2} (see section I.3.6.1,

above).

I.3.8   <u>The rate of oxidase activity</u>

Although some groups of investigators report that

the oxidation of NADPH by resting or phagocytosing granulocytes

proceeded at a higher rate than the oxidation of NADH (Rossi,

Romeo and Patriarca, 1972; Hohn and Lehrer, 1975; Auclair,

Torres and Hakim, 1978), others have found just the opposite

(Evans and Karnovsky, 1961; Evans and Karnovsky, 1962). The study of Rossi, Romeo and Patriarca can be criticized because Mn^{+2} was present during measurements of NAD(P)H oxidase activity (section I.3.6.1). In addition, both studies finding a higher rate of NADPH oxidation can be questioned because the oxidase activity was quantitated in granule fractions only. It has recently become apparent that the plasma membrane contains a very significant part (if not all) of the NADH oxidase activity (see section I.4).

Interestingly, some investigators have recently stated that single oxidase which catalyzes both NADH and NADPH oxidations, but to different extents, may be involved. All of the following workers however, believe that the oxidase favours NADPH as the substrate:-

- Rossi, Romeo and Patriarca (1972)

- Allen *et al* (1974)

- Babior, Curnutte and Kipnes (1975)

- Johnston *et al* (1975)

- McPhail, DeChatelet and Shirley (1976)

- Iverson *et al* (1977)

In contrast, Takanaka and O'Brien (1975a) have examined a guinea pig oxidase which can utilize NADH or NADPH to virtually the same extent. They provide compelling evidence that the cyanide-insensitive oxidase activity is located in the plasma membrane of the granulocyte (see also section I.4).

I.4   <u>The intracellular location of the 0_2^- producing oxidase</u>

Since Iyer and Quastel's early investigations in 1963,

many other workers have found and studied the oxidase activity of

the granule fraction of disrupted cells. For example, Patriarca's

group found the oxidase activity of rabbit granulocytes was located

in a granule fraction which contained the azurophilic (myeloper-

oxidase-containing) granules (Patriarca et al, 1973). Other

groups have examined the oxidase activity of a comparable

granule fraction from fractionated cells (Hohn and Lehrer, 1975;

DeChatelet et al, 1975; McPhail et al, 1977b. However, not all

investigators agree on the exact location of this oxidase - one

group maintains that the oxidase is located in a granule fraction

which does not contain either the azurophilic or specific granules,

but contains instead large and dense granules (Iverson et al,

1977, 1978). Some of these investigators found no oxidase activity

in the supernatant (Rossi, Romeo and Patriarca, 1972; Hohn and

Lehrer, 1975), in agreement with the early studies of Karnovsky's

group (Evans and Karnovsky, 1961). However, a later paper by

Karnovsky's group explained that their laboratory had failed to

detect NADH oxidase activity in their supernatant preparations

because they had been too diluted by the procedure for cell

fractionation (Cagan and Karnovsky, 1964). They found that if

the granulocytes were homogenized in sucrose, then the oxidase

activity was equally divided between the granule and soluble

fractions of the cell, whereas in isotonic KCl all of the enzyme

was localized to the soluble fraction (Karnovsky, 1973). The

methodology used for cell fractionation is therefore quite crucial.

Workers who have found that the oxidase is largely in the

supernatant or soluble fraction include Cagan and Karnovsky,
1964; Baehner and Nathan, 1968; Baehner and Karnovsky, 1968;
Kakinuma and Chance, 1977.

It is interesting to note that recent work has
focused on a plasma membrane location for the oxidase. Early
workers suggested that the plasma membrane (Salin and McCord,
1974), or a site close to the cell surface (Root and Stossel,
1974) was involved because they detected large amounts of O_2^-
or H_2O_2 in the external medium surrounding the intact granulocyte.
As Salin and McCord emphasized, it is likely that the O_2^- comes
from the cell surface because the intracellular presence of super-
oxide dismutase would preclude the diffusion through the cell
of O_2^- produced intracellularly. Many investigators have now
detected and studied a plasma membrane oxidase activity. All
but Takanaka and O'Brien have examined the oxidase in human
granulocytes:-

- Takanaka and O'Brien (1975a, guinea pig granulocytes)

- Briggs *et al* (1975)

- Segal and Peters (1976, 1977, 1978)

- Cohen and Chovaniec (1977)

- Goldstein *et al,* (1977)

- Briggs, Karnovsky and Karnovsky (1977)

- Gabig, Kipnes and Babior (1978)

- Babior *et al* (1978)

- Tauber and Goetzl (1978)

- Dewald *et al* (1979)

In addition, some workers have found that their
investigations indirectly support a plasma membrane location for

the oxidase (Nakagawara *et al*, 1977 ; Root and Metcalf, 1977;

Jandl *et al*, 1978). It is relevant to note also that some

of the recent papers from investigators who had been studying a

granule-fraction oxidase state that their granule fractions were

not 'pure', but contained some plasma membrane contamination

(DeChatelet *et al*, 1975; Rossi, Romeo and Patriarca, 1976;

Iverson *et al*, 1978).

Some of the investigators have studied the nucleotide

specificity of the plasma membrane oxidase, finding it to be an

NADH oxidase (Briggs *et al*, 1975; Segal and Peters, 1976, 1977,

1978), or an oxidase with greater reactivity towards NADPH than

NADH (Tauber and Goetzl, 1978).

It seems certain that an oxidase is located in the

plasma membrane, but further study is required to determine the

characteristics of this enzyme. As mentioned in the Introduction

to this thesis, teleologically it would be sensible to have the

oxidase located in the plasma membrane because with phagocytosis

the enzyme would be internalized to become part of the phagocytic

vacuole membrane, thereby facilitating the delivery of O_2^- and

H_2O_2 to the contents of the phagosome.

I.5   Recent studies on the plasma membrane of CGD granulocytes

Studies of the granulocyte plasma membrane by

Segal and his associates have suggested that the human membrane

contains a novel cytochrome b which is also incorporated into

the phagosome membrane at phagocytosis (Segal *et al*, 1978;

Segal and Jones, 1978, 1979). These investigators believe that

this cytochrome is a component of the plasma membrane oxidase,

which they had previously shown to be located in the plasma

membrane (Segal and Peters, 1976, 1977, 1978). This plasma
membrane cytochrome, like the oxidase, was abnormal or undetectable
in CGD patients, two obligate heterozygotes (the mothers of two
affected sons), and both parents of a female CGD patient. Although
Segal *et al* have not proven that the abnormality in this cytochrome
is associated with the abnormal oxidative metabolism of CGD, their
suggestion that the abnormal cytochrome b may be the molecular
basis for the abnormal plasma membrane oxidase activities of their[3]
CGD patients seems reasonable. Only Segal's group and one other
have examined the granulocytes from CGD patients and subsequently
suggested that the actual defect in CGD is in the plasma membrane
oxidase (Segal and Peters, 1976, 1978; Gabig, Kipnes and Babior,
1978). The second group also provides some evidence that the
O_2^--producing oxidase requires flavin adenine dinucleotide (FAD) as
a co-factor for activity (Babior and Kipnes, 1977; Gabig, Kipnes
and Babior, 1978).

Segal and Peters (1978) point out that the plasma
membrane of CGD granulocytes is abnormal in other respects as
well - the Kell antigen system may be absent from these
granulocytes (Marsh *et al*, 1975), and fusion of the phagocytic
vacuole with the granules may be delayed (Gold *et al*, 1974). To
these may be added more recent reports indicating that the CGD
granulocyte plasma membrane is indeed abnormal:-

---

[3] It must be mentioned that Borregaard *et al* (1979) have not
found an absence or abnormality in this cytochrome b in the
granulocytes of three CGD patients.

(i)   CGD granulocyte cell surfaces are able to

        bind the normal amount of concanavalin A but then

        the granulocytes do not show the typical lectin-

        induced HMPS stimulation (Tsan *et al*, 1976 a,

        1977)

(ii)   CGD granulocytes show a very modest increase in

        ^{86}Rb efflux after phagocytosis whereas control

        granulocytes display a marked (6-fold) augmenta-

        tion of ^{86}Rb efflux from the cell surface

        (Lehrer, Olofsson and Ferrari, 1977).

(iii)   the studies of Nakagawara *et al* (1976 a, 1977)

        on CGD patients (described in section 4.4 of this

        thesis) indicate that the plasma membrane oxidase

        of CGD granulocytes is defective in function.

        In conclusion, while the evidence to support the

NADH oxidase has not been as strong as that to support the

NADPH oxidase, many of the arguments in favour of the NADPH

oxidase as the enzyme of the metabolic burst can be questioned

or refuted. Future studies of this enzyme will need to re-

examine the oxidase activity from pure granule fractions,

that is, granule fractions free of myeloperoxidase and plasma

membrane contamination. It is clear that further investigations

of the defective enzyme in CGD granulocytes are also required

before firm conclusions as to the nature of the oxidase of the

metabolic burst can be drawn.
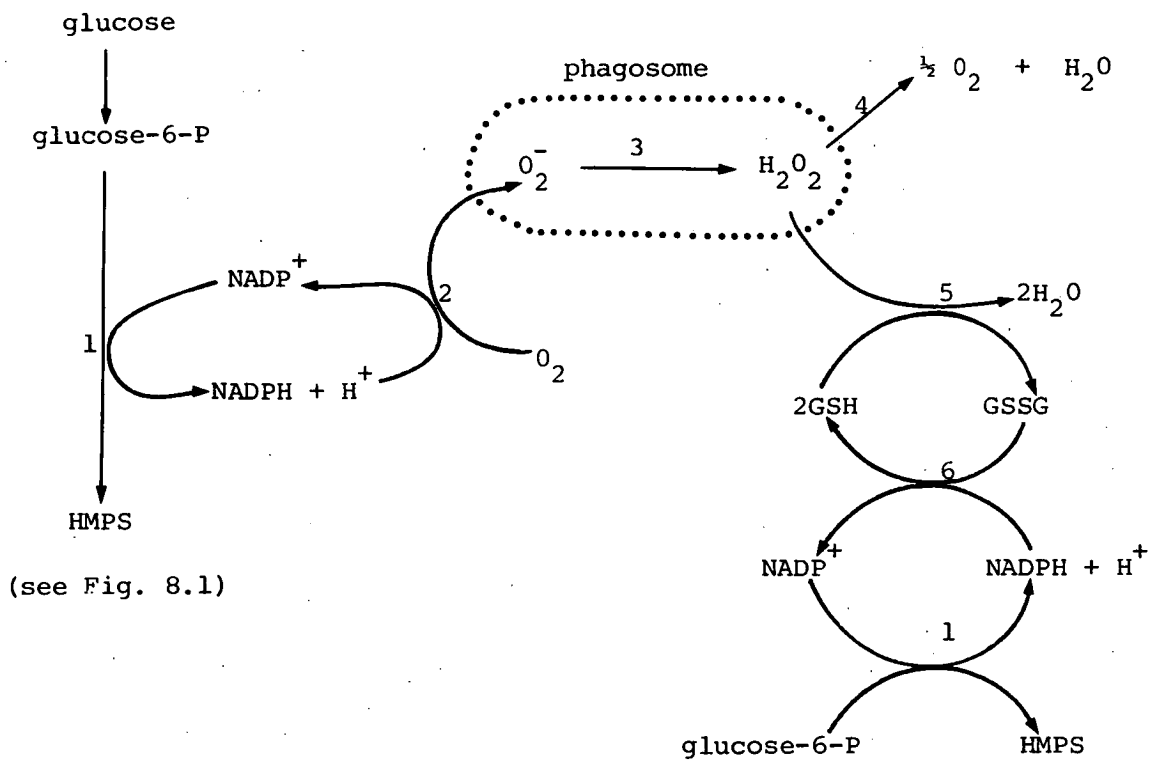
FIG. I.1   Activation of the HMPS by an NADPH-dependent
            oxidase. The enzymes involved are:-

1. glucose-6-phosphate dehydrogenase
2. NADPH oxidase
3. superoxide dismutase
4. catalase
5. glutathione peroxidase
6. glutathione reductase

and   HMPS = hexose monophosphate shunt
       GSH = reduced glutathione
      GSSG = oxidized glutathione

I.2   The Embden-Meyerhof pathway, as modified from Arese and Bosia
      (1973).   The enzymes involved are:-

   1. glucokinase (=hexokinase)

   2. phosphoglucose isomerase

   3. phosphofructokinase

   4. aldolase

   5. triosephosphate isomerase

   6. glyceraldehydephosphate dehydrogenase

   7. phosphoglycerate kinase

   8. phosphoglycerate mutase

   9. enolase

  10. pyruvate kinase

  11. lactate dehydrogenase

  12. glucose-6-phosphate dehydrogenase

Glucose

ATP ⟶ ⟍
ADP ⟵   1

Glucose-6-phosphate ⟶ 12 ⟶ HMPS (see Fig. 8.1)

↓ 2

Fructose-6-phosphate

ATP ⟶ ⟍
ADP ⟵   3

Fructose-1, 6-diphosphate

↓ 4

Glyceraldehyde-3-phosphate ⇌ 5 ⟶ Dihydroxyacetone-phosphate

NAD$^+$

Phosphate ⟶ ⟍ 6
⟶ NADH + H$^+$

1,3-Diphosphoglycerate

ADP ⟶ ⟍ 7
ATP ⟵

3-phosphoglycerate

↓ 8

2-phosphoglycerate

9 ⟶ H$_2$O

phosphoenolpyruvate

ADP ⟶ ⟍ 10
ATP ⟵

NADH + H$^+$ NAD$^+$

pyruvate ⟶ Lactate

11
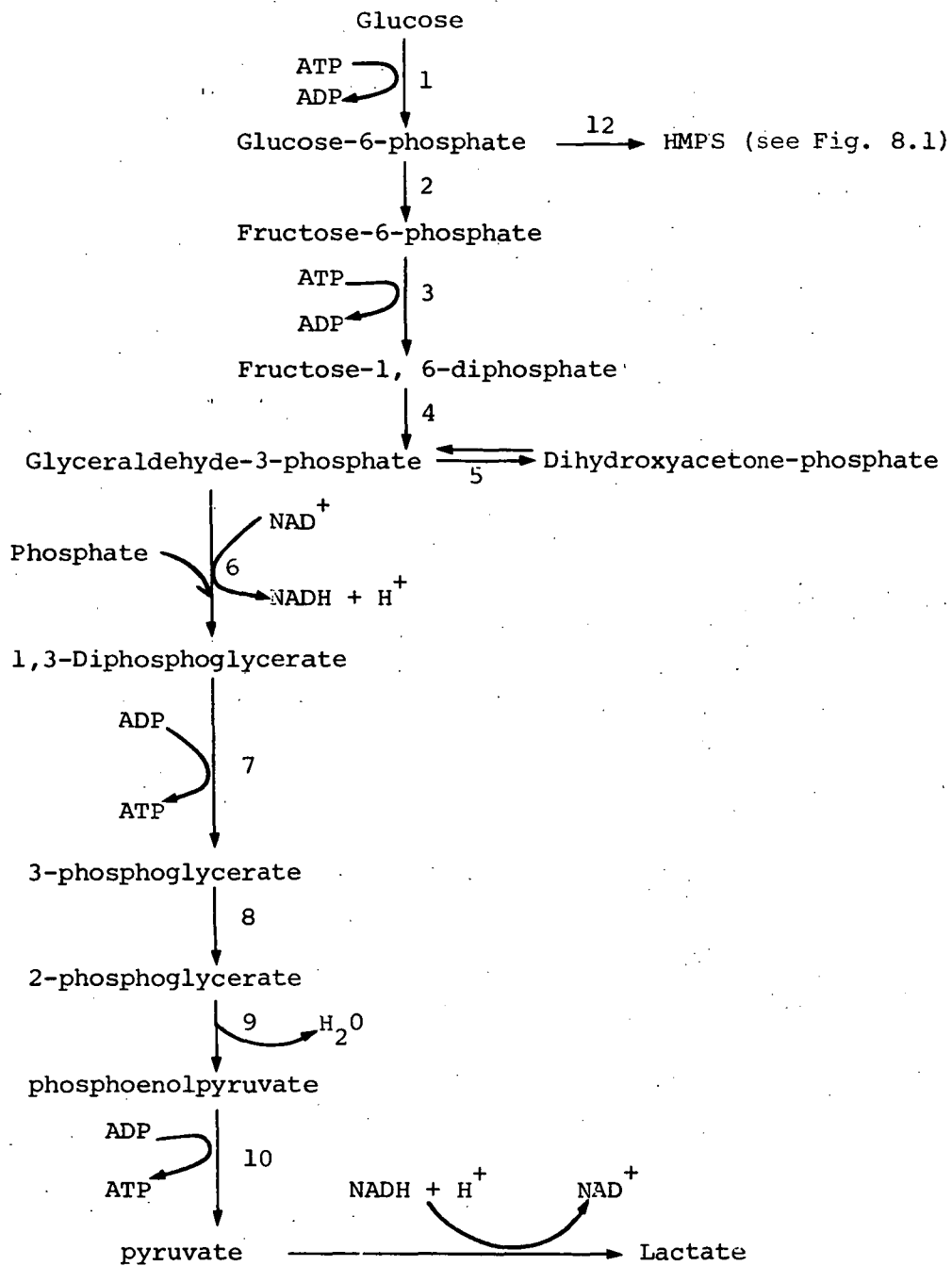
APPENDIX II

THE PREPARATION OF THE HEPES PHYSIOLOGICAL

SALINE SOLUTIONS

APPENDIX II   THE PREPARATION OF THE HEPES PHYSIOLOGICAL

SALINE SOLUTIONS

II.1   HEPES physiological saline solution (HSS)

HSS was prepared as described previously (Dewar *et al*,

1976). That is, 10 ml of a concentrated salt solution (II.4.1),

10 ml of glucose, 155 mM (II.5), 20 ml of HEPES buffer solution,

58.5 mM (II.6), and 90 ml of water were mixed daily for experiments.

The final composition of HSS was therefore 136.9 mM Na^+, 6.0 mM

K^+, 1.2 mM Mg^{+2}, 2.4 mM Ca^{+2}, 150.1 mM Cl^-, 11.9 mM glucose and

9.0 mM HEPES, with a pH of 7.4.

II.2 Ca^{+2} and Mg^{+2} free physiological saline solution (CMFH)

CMFH was also prepared as described previously (Dewar

et al, 1976). That is, 10 ml of a concentrated salt solution

minus Ca^{+2} and Mg^{+2} (II.4.2), 10 ml of glucose, 155 mM (II.5),

20 ml of HEPES buffer solution, 58.5 mM (II.6), and 90 ml of water

were mixed daily for experiments. The final composition of CMFH

was therefore 142.9 mM Na^+, 6.0 mM K^+, 148.9 mM Cl^-, 11.9 mM

glucose and 9.0 mM HEPES, with a final pH of 7.4.

II.3 Ca^{+2} free physiological saline solution (CFH)

CFH was prepared by mixing 10 ml of a Ca^{+2} free

concentrated salt solution (II.4.3), 10 ml of glucose, 155 mM

(II.5), 20 ml of HEPES buffer solution, 58.5 mM (II.6), and 90

ml of water daily for experiments. The final composition of

CFH was therefore 140.6 mM Na^+, 6.0 mM K^+, 1.2 mM Mg^{+2}, 149.1

mM Cl^-, 11.9 mM glucose, and 9.0 mM HEPES, with a pH of 7.4.

II.4   Concentrated salt solutions

        The following concentrated salt solutions were prepared every 2-3 weeks and stored at 4°C in Nalgene containers in between use.

II.4.1   Concentrated salt solution for HSS

NaCl	52.0 g
KCl	2.89 g
$MgCl_2 \times 6H_2O$	1.57 g
$CaCl_2 \times 2H_2O$	2.58 g

- made up to a volume of 500 ml with water.

II.4.2   Concentrated salt solution for CMFH

NaCl	54.3 g
KCl	2.89 g

- made up to a volume of 500 ml with water.

II.4.3   Concentrated salt solution for CFH

NaCl	53.4 g
KCl	2.89 g
$MgCl_2 \times 6H_2O$	1.57 g

- made up to a volume of 500 ml with water.

II.5   Glucose, 155 mM

        This solution was prepared fresh daily for experiments and contained 2.79 g of d-glucose (dextrose) per 100 ml of water.

II.6   HEPES buffer solution, 58.5 mM

        This solution was prepared by dissolving 13.94 g HEPES in approximately 700 ml water, adjusting the pH to 7.4 with 1 M-NaOH, and then adding sufficient water to make one litre. The buffer was prepared every 2-3 weeks and stored at 4°C in a Nalgene container in between use.

APPENDIX   III

ENZYME KINETIC ANALYSIS

APPENDIX III   ENZYME KINETIC ANALYSIS

The experiments on the variation in NBT reduction

rate with the concentration of NBT (section 3.2.4.3) were

analysed by using an enzyme kinetics approach. It was felt that

such an analysis of the data was necessary to determine if the

assay of the initial rate of NBT reduction was reproducible with

granulocytes isolated from different donors. For this analysis,

the principles of kinetic theory were reviewed by consulting

the detailed discussion of enzyme kinetics presented by Mahler

and Cordes (1966). These principles will be briefly outlined at

this point.

If the reduction of NBT to diformazan is thought of

as a simple chemical reaction, such as

$$O_2^- + O_2^- + NBT^{+2} \underset{k_{-1}}{\overset{k_1}{\longrightarrow}} NBT + 2O_2 \qquad (III.1)$$
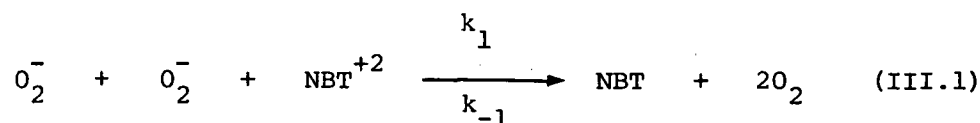
then the reaction can be characterised by describing how fast

NBT^{+2} is reduced to diformazan, that is, by the kinetics of the

reaction. The law of mass action specifies that the rate of a

reaction is proportional to the concentration of the participat-

ing reactants (or substrates), and thus

rate (or velocity)   = k_1 [reactants]

where k_1 is a proportionality constant. For the reaction

presented above, the rate of formation of diformazan from

$NBT^{+2} = k_1 \; [O_2^-]^2 \cdot [NBT^{+2}]$. The rate of this reaction is

dependent upon the concentration of three reactants (2 molecules

of O_2^- and one of NBT^{+2}), and therefore the reaction is third order.

However, reactions with 3 participants usually proceed by a series

of bimolecular and/or monomolecular steps (Mahler and Cordes,

1966). It seems reasonable to assume that the rate limiting,

or slowest step of the reaction would be the half reduction of

NBT^{+2} (by one molecule of O_2^-) to form the monoformazan (Eadie

et al, 1970). The reaction could be shown as

$$O_2^- \;+\; NBT^{+2} \quad \underset{k_{-1}}{\overset{k_1}{\xrightarrow{\hspace{2cm}}}} \quad NBT^{+1} \;+\; O_2 \qquad\qquad (III.2)$$
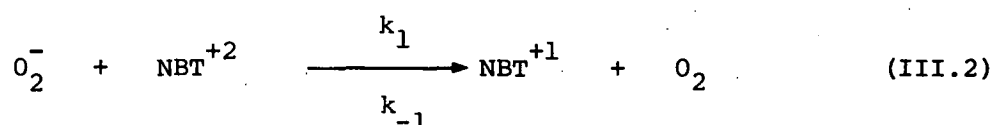
The rate of the overall reaction (III.1) would thus be second

order, with the rate (or velocity) = k_1 $[O_2^-]$ $[NBT^{+2}]$. If

however, we consider the reduction of NBT to formazan as an

enzyme catalyzed rather than a simple chemical reaction, we can

study the reaction by using an equation which was devised by

Michaelis and Menten (1913). They showed that for most enzyme

catalyzed reactions performed under carefully controlled

conditions, the variation of rate (velocity) with substrate

concentration can be described by the following equation:-

$$velocity \;=\; \frac{V_{max}\;[S]}{K_m \;+\; [S]} \qquad\qquad (III.3)$$

where V_{max} = the maximal velocity of the reaction

$[S]$ = the concentration of the substrate

(in this case, NBT^{+2})

and K_m = the concentration of substrate at which

the reaction proceeds half-maximally.

If the concentration of substrate is large with respect to the K_m then the equation can be rewritten in such a way as to reduce the kinetic order of the reaction (by one) to zero order. Thus,

$$\text{velocity} = \frac{V_{max} \, [S]}{K_m + [S]} \simeq \frac{V_{max} \, [S]}{[S]} \simeq V_{max}$$

That is, the velocity approaches V_{max} when [S] is very high, and therefore the velocity becomes independent of the substrate concentration. In the laboratory this is an important consideration because most studies are done on initial velocity (or initial rate), and in such studies the substrate concentration at any point during the reaction does essentially stay equal to the substrate concentration at zero time (i.e. these reactions are apparently zero order).

The shape of the plot of Michaelis-Menten data (variation of rate with substrate concentration) is a rectangular hyperbola (Fig. III.1). Such a graph is completely defined by the two kinetic parameters, V_{max} and K_m. These two parameters are both necessary and sufficient to define the kinetics of most reactions, provided the following criteria have been met:-

(i)   only a single substrate is involved (or if more than one is involved, the concentrations of all the other substrates are held constant).

(ii)   true initial rates (or initial velocities) of the reaction are measured by using different starting

concentrations of the substrate.

(iii) the concentration of the substrate at zero time is

much greater than the concentration of the enzyme

at zero time, and the same constant concentration

of the enzyme at zero time is used in all measure-

ments.

(iv) all other variables (temperature, buffer, etc.) are

held constant in all measurements.

Determining the K_m and V_{max} from the hyperbolic

curve is very difficult as it involves determination of an asymptote

as the substrate concentration approaches infinity. Therefore

the data from the Michaelis-Menten plot is usually replotted as

a straight line (Lineweaver and Burk, 1934), as described by the

following equation:-

$$\frac{1}{velocity} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (III.4)$$

The straight line from such plots (Fig. III.2) of

1/velocity versus 1/concentration can be used to determine the

K_m and V_{max} values.

With the foregoing as a guideline, the granulocytes

from 16 normal donors were used for 16 experiments on the

variation in NBT reduction with the concentration of NBT.

Five concentrations of NBT were tested in each experiment. The

data from these experiments gave the rectangular hyperbolae

described by the Michaelis-Menten equation. Two of the 16

plots are illustrated in Fig. III.3. These graphs were

transformed to straight lines (Lineweaver-Burk plots) by plotting

the data as 1/velocity versus 1/NBT concentration. The two

curvilinear plots of Fig. III.3 are shown as linear plots in

Fig. III.4.

The data was fitted to these straight lines by the

linear regression method using a Hewlett Packard calculator

(HP 27). From the calculator, the values for the y-intercept

(= $1/V_{max}$ value), slope (= K_m/V_{max} value) and 'r' (correlation

coefficient)[1] were obtained. By dividing the y-intercept

value by the slope, the x-intercept (= $1/K_m$ value) was obtained,

and the line could then be drawn through the two intercepts,

as shown in Fig. III.4. The 'apparent K_m',[2] V_{max}, and r

values for the plots from these 16 donors are given in Table

III.1. There was very good agreement in these values, suggesting

that the initial rate assay was reproducible with granulocytes

obtained from different normal donors.

---

[1] Correlation coefficients are used to guage the 'goodness
of fit' of the data to a straight line, perfect fit
being a correlation coefficient of +1.00 for these lines.

[2] The term 'apparent K_m' rather than K_m is preferable when
the experiments have been conducted with an impure enzyme,
for example, with intact granulocytes.

velocity

V_{max}

$\dfrac{V_{max}}{2}$

K_m $3/K_m$ $5/K_m$ $7/K_m$ $9/K_m$ [S]

$1/velocity$

$2/V_{max}$ .... SLOPE = K_m/V_{max}

$1/V_{max}$

$-1/K_m$ $1/K_m$ $2/K_m$ $1/[S]$
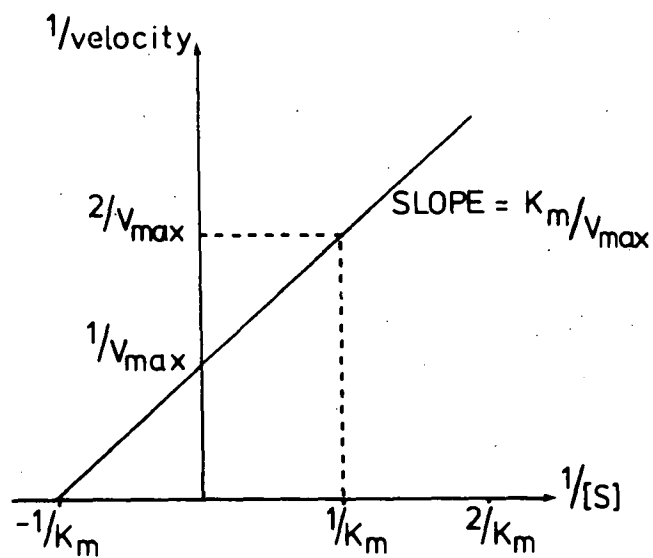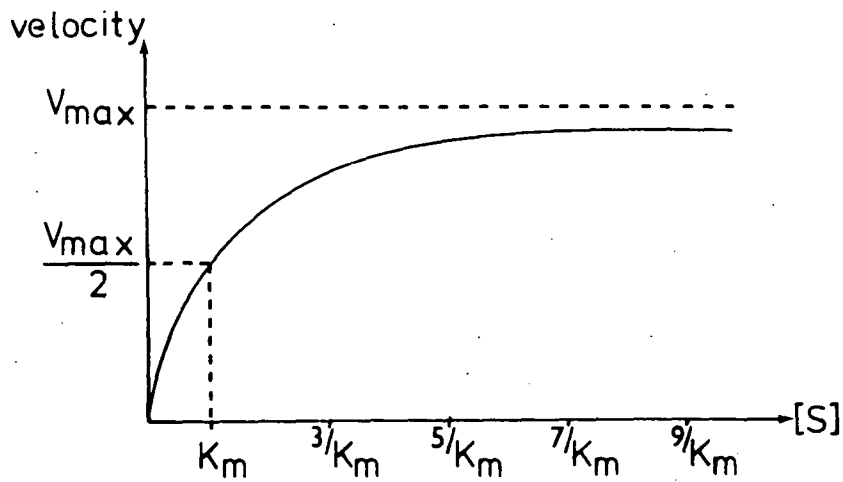
FIG. III.1. The plot of velocity with respect to substrate concentration, according to the Michaelis-Menten equation (top). The graph is completely defined by the two kinetic parameters, K_m and V_{max}.

FIG. III.2. Lineweaver-Burk plot of $1/velocity$ versus $1/substrate$ concentration (bottom). As shown, the y-intercept = $1/V_{max}$, the x-intercept = $-1/K_m$ and the slope is K_m/V_{max}.
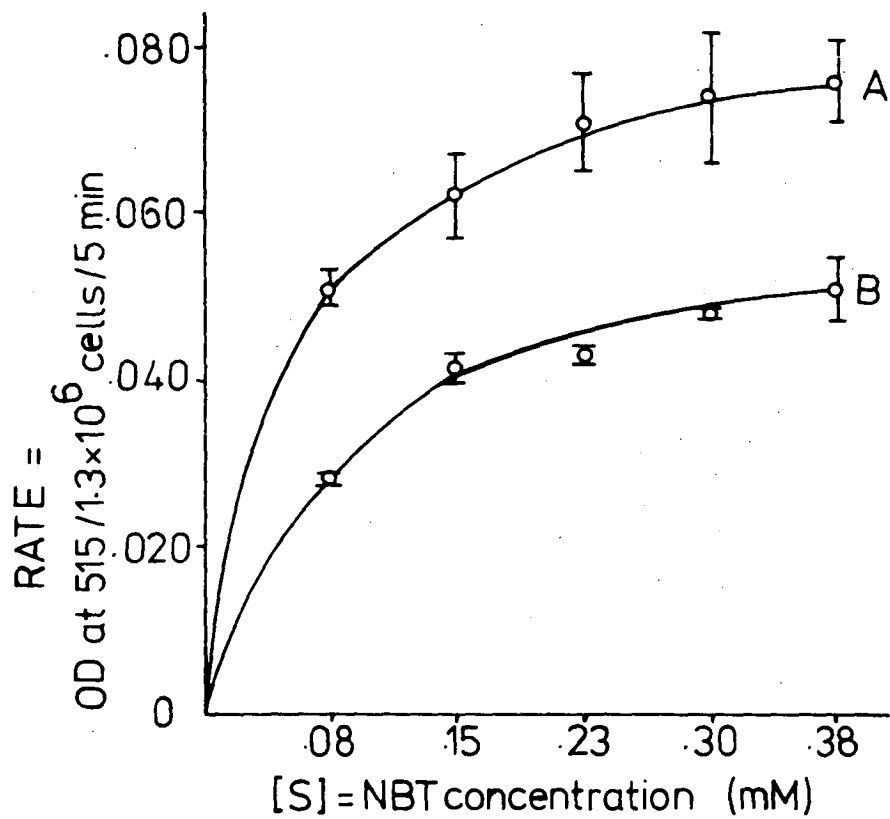
FIG. III.3. The data from 2 experiments (2 of 16 donors) on

the rate of NBT reduction as a function of the

concentration of NBT dye. Each point is the

$\bar{X} \pm$ SEM of 3 determinations.
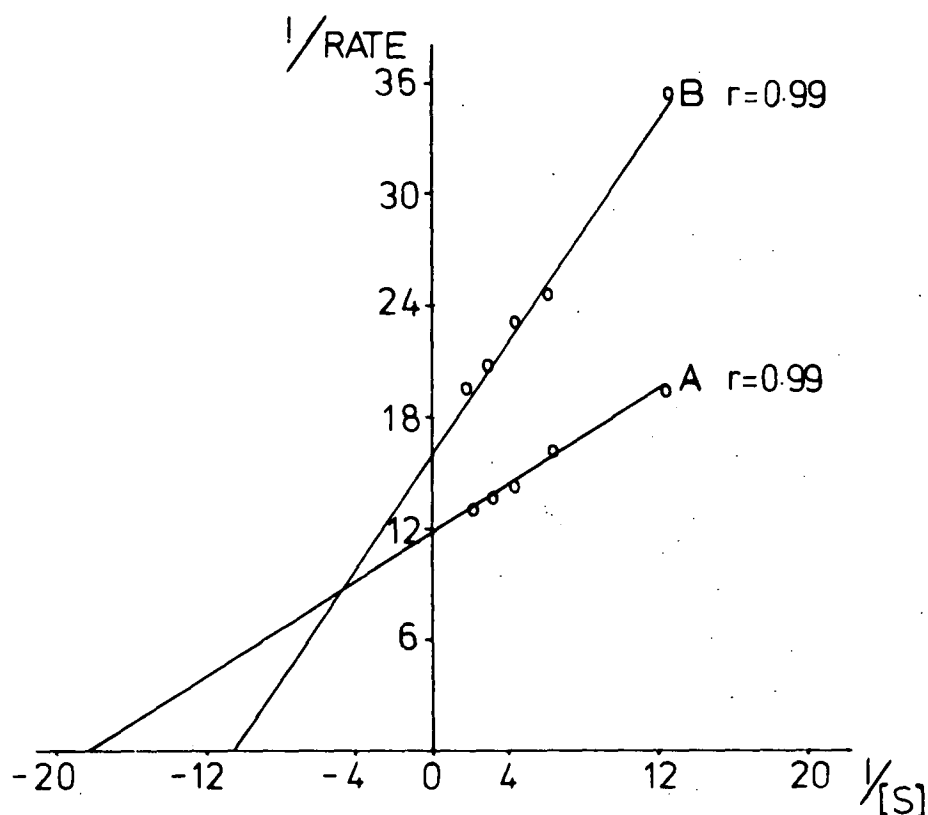
FIG. III.4. The data from FIG. III.3 replotted as a Lineweaver-Burk plot. For line 'A' the x and y-intercepts were 18.68 and 11.58, giving an apparent K_m and V_{max} of 0.054 mM and 0.086 OD_{515} units/1.3 x 10^6 granulocytes/5 min, respectively. For line 'B' the x and y-intercepts were 10.61 and 15.81, giving an apparent K_m and V_{max} of 0.094 and 0.063 OD_{515} units/1.3 x 10^6 cells/5 min, respectively (see also the data collected in Table III.1).

TABLE III.1   Kinetic parameters for the reduction of
NBT by intact granulocytes

Sample	K_m [a]	V_{max} [b]	r
1	.083	.078	.99
2	.054	.075	.99
3 [c]	.094	.063	.99
4	.068	.052	.93
5	.071	.075	.91
6	.050	.085	.90
7	.118	.093	.94
8 [d]	.054	.086	.99
9	.103	.083	.94
10	.185	.102	.99
11	.105	.087	.92
12	.192	.069	.91
13	.102	.081	.95
14	.146	.093	.93
15	.127	.089	.92
16	.087	.090	.81
$\bar{X}$ ± SEM	.102±.011	.081±.003	.94±.01

a)   apparent K_m for NBT, in mM concentration units

b) V_{max} in units of $OD_{515}/1.3 \times 10^6$ granulocytes/5 min

c)   corresponds to line B in Fig. III.4

d)   corresponds to line A in Fig. III.4

r = correlation coefficient

APPENDIX   IV

MICROTUBULES AND MICROFILAMENTS

APPENDIX IV   MICROTUBULES AND MICROFILAMENTS

IV.1   MICROTUBULES

Cytoplasmic microtubules[1] are straight hollow cylinders with an external diameter of 25 nm, a wall thickness of 5 nm and a variable length (Ledbetter and Porter, 1963; Slautterback, 1963). These structures were first seen in cilia by Fawcett and Porter (1954) but they had been postulated to exist by many scientists in the late 1800's, who believed that 'fibrils' would explain the elastic, tensile and architectural properties of cytoplasm (reviewed by Burnside, 1975). By advocating the use of glutaraldehyde as a fixative for electron microscopy, Sabatini et al (1963) greatly assisted the study of microtubules. With this fixative microtubules have been found in every eukaryotic cell which has been examined closely by electron microscopy (Burnside, 1975; Snyder and McIntosh, 1976).

Microtubules are probably most familiar as the mitotic apparatus of dividing cells and the fibrils of cilia, flagella, centrioles and basal bodies. They have been classed as *stable* (the microtubules in cilia and flagella) or *labile* (those in the cytoplasm of plant and animal cells and comprising the mitotic apparatus) by Wilson (1974). Stable microtubules can be isolated as intact organelles and are not dissociated by chemical agents which bind to and destroy labile microtubules (discussed in section IV.1.1). In addition, stable microtubules do not appear to be in equilibrium with a subunit pool. In

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[1] The name 'microtubule' was first used in 1963 by Slautterback.

contrast, labile microtubules are considered to be in a state

of 'dynamic equilibrium' with subunits (Inoué and Sato, 1967),

are easily disrupted by 'spindle poisons' (discussed in section

IV.1.1), and cannot be easily isolated intact from cells because

of their lability (Wilson, 1974).

In cross section the wall of the microtubule is

almost invariably composed of 13 subunits (Snyder and McIntosh,

1976), as illustrated in Fig. IV.1. When viewed longitudinally,

13 helically-wound protofilaments can be seen running parallel

to the long axis of the microtubule (Fig. IV.1). Ledbetter and

Porter (1964) were the first to demonstrate the existence of the

13 subunits. The unique class of proteins making up microtubules

were called tubulins by Mohri (1968). They are dimeric molecules

with molecular weights of 115,000 daltons, known as 6S tubulin.

With protein denaturation, two similar subunits called α and β

tubulin (3 - 4S) are formed. The amino acid composition of these

subunits is remarkably similar (Snyder and McIntosh, 1976).

In vivo the labile or cytoplasmic microtubules appear

to exist in a state of dynamic equilibrium between the polymerized

form (the microtubule) and a soluble subunit pool (Inoué, 1964;

Inoué and Sato, 1967; Stephens, 1973; Borisy *et al*, 1974). The

mechanism whereby the cell is able to assemble and disassemble the

microtubules is not completely understood, but nucleotides, calcium,

magnesium, microtubule-associated proteins, and microtubule-

organizing centres appear to play a role in the regulation of the

process (reviewed by Snyder and McIntosh, 1976; Stephens and Edds,

1976). The redox state of the cell and in particular the

sulfhydryl groups may be involved as well, because microtubule

function can be impaired by oxidized glutathione (Kuriyama and Sakai, 1974).

IV.1.1   Microtubule disrupting agents

Microtubule-disrupting agents, or microtubule inhibitors, are used as pharmacological tools in studying microtubule-mediated cellular phenomena. These agents have long been called 'spindle poisons' (Wilson et al, 1974) because they inhibit mitosis by impairing microtubule (spindle) function. Colchicine, vinblastine and vincristine are the most widely used microtubule-disrupting agents. Their chemical structures are shown in Figs. IV.2, IV.3 and IV.4, respectively. These drugs bind specifically to tubulin[2] and thereby shift the dynamic equilibrium towards depolymerization of the microtubules (Taylor, 1965; Borisy and Taylor, 1967; Deysson, 1968; Dustin, 1972; Margulis, 1973; Wilson et al, 1974).

Colchicine binds to the tubulin which is in soluble pools (Wilson and Meza, 1973; Wilson and Bryan, 1974) and thus blocks microtubule polyermization. The colchicine binding sites are masked on assembled (polymerized) microtubules. One mole of colchicine binds tightly and very specifically to one mole of tubulin (Wilson and Bryan, 1974) in a non-covalent, slow and irreversible fashion (Wilson and Meza, 1973; Wilson and Bryan, 1974; Wilson et al, 1974). Colchicine binding is strongly temperature-dependent - at $0^{o}C$ the binding is extremely slow while at $37^{o}C$ it is optimal (Wilson et al, 1974).

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[2] Most studies have used isolated brain tubulin (Snyder and McIntosh, 1976).

Irradiation of colchicine with ultraviolet light
converts colchicine (by first-order kinetics) into a mixture of
β and γ lumicolchicines[3] (Wilson et al, 1974), as illustrated
in Fig. IV.2. The change in colchicine is at the seven-membered
C ring, which is altered to form two smaller rings. As the C
ring is crucial for colchicine's binding to tubulin, lumicolchicine
is inactive as an anti-mitotic agent (Wilson and Bryan, 1974).
Lumicolchicine does not prevent colchicine's binding to tubulin
however (Wilson and Friedkin, 1967). Lumicolchicine is extremely
useful as a control for studies of microtubule-mediated cellular
functions. It can be assumed that a response is not dependent
upon microtubule integrity if both colchicine and lumicolchicine
treatments produce the same result.

The vinca alkaloids (vinblastine, vincristine and
desacetylvinblastine) also disrupt microtubules by binding to
tubulin. Of these, the most studied agent is vinblastine.
Bensch and Malawista (1969) were the first to observe that the
interaction of vinblastine with tubulin results in the formation
of highly regular crystals. These crystals contain one mole of
bound vinblastine per mole of tubulin (Bryan, 1972). The binding
of vinblastine to tubulin is strong and rapid, with two binding
sites per tubulin dimer (Wilson, 1975; Wilson, Creswell and Chin,
1975). However, the vinblastine-tubulin complex is not very
stable (Wilson, Creswell and Chin, 1975). The binding sites for

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[3] In the literature the photoproduct is usually referred to
as lumicolchicine, rather than β and γ lumicolchicines.

the vinca alkaloids, like colchicine's binding sites, are masked

on polymerized tubulin, and like colchicine, the binding of the

vinca alkaloids to soluble tubulin will block microtubule poly-

merization (Wilson, Creswell and Chin, 1975). Unlike colchicine

binding, however, vinblastine binding *in vitro* is relatively

insensitive to temperatures between 0 and 37°C. Vincristine and

desacetyl-vinblastine completely inhibit the binding of vinblastine

to tubulin because the vinca alkaloids all share the same binding

site (Wilson, Creswell and Chin, 1975). This site is distinct

from the binding site for colchicine, and in fact the binding

of vinblastine can stabilize the more labile colchicine-binding

site on tubulin (Wilson, 1970).

Microtubules also have two guanine nucleotide binding

sites per dimer (which are separate from the vinca alkaloid-binding

site, according to Wilson, Creswell and Chin, 1975), and the vinca

alkaloids can competitively inhibit GTP from binding (Snyder and

McIntosh, 1976). Studies on hog brain tubulin have shown that

GTP and magnesium in equimolar amounts are required for maximal

assembly of microtubules *in vitro* (Olmsted and Borisy, 1975).

Vinblastine in high concentrations will precipitate

tubulin *in vitro*, but this is probably a non-specific effect,

as vinblastine can also precipitate a large number of other

acidic proteins, as well as double-stranded DNA (Wilson *et al*,

1970). For this reason (discussed by Wilson *et al*, 1974;

Wilson, Creswell and Chin, 1975) it is very likely that there are

two classes of binding sites for vinblastine on tubulin: a high

affinity, biologically important class and a low affinity,

biologically unimportant class. The doses of the vinca

alkaloids which result in microtubule disruption (that is, the doses which are biologically important) in mammalian cells are ~ 7 - 8 x 10^{-8} M (Wilson, Creswell and Chin, 1975). As one would expect, plasma concentrations of this magnitude are achieved *in vivo* during cancer chemotherapy with the vinca alkaloids (Owellen *et al*, 1976).

Podophyllotoxin and griseofulvin also disrupt microtubules by inhibiting microtubule polymerization (Wilson *et al*, 1974; Snyder and McIntosh, 1976), but these drugs are not routinely used as experimental tools. Their chemical structures are shown in Figs. IV.5 and IV.6, respectively. Podophyllotoxin binds to tubulin, competing with colchicine for the colchicine-binding sites. The binding is reversible, rapid, and not temperature-dependent. Griseofulvin, on the other hand, is thought to inhibit microtubule assembly by binding to proteins associated with microtubules, and not to the tubulin dimer itself (Roobol *et al*, 1977).

IV.1.2   Microtubule-mediated cellular functions

By using the anti-mitotic agents (discussed above) as experimental tools, investigators have shown that microtubules play a role in many cellular functions. Among the cellular phenomena which have been shown to depend upon microtubules are:-

(i)   the function of the mitotic apparatus or spindle

during cell division (Rebhun and Sander, 1967;

Sato, 1969).

(ii) the maintenance of cell shape (reviewed by Porter, 1966).

(iii) cell movement (Olmsted and Borisy, 1973).

(iv) feeding in ciliates (Tucker, 1972) and ingestion in phagocytes (reviewed by Stossel, 1975).

(v) humoral secretion, for example the release of insulin from pancreatic B-cells (Malaisse et al, 1975), and lysosomal enzyme release in granulocytes (Malawista, 1975; Weissmann et al, 1975a,b).

(vi) the translocation of materials in nerve fibres by a process known as axonal transport (Ochs, 1974).

(vii) intracellular movement of organelles such as chromosomes, lysosomes, phagosomes and pigment granules (reviewed by Burnside, 1975), and finally

(viii) the control of cell surface topography (reviewed by Berlin et al, 1975). The role of microtubules in the control of cell membrane organization has been discussed in the introduction to this thesis, and in section 6.4.

IV.1.3 Models depicting a contractile activity of microtubules

Many of the above-mentioned roles imply motion or movement, and as microtubules themselves are not contractile, models have been proposed to allow for such a contractile activity. The models have usually attempted to depict a contractile-like capability of microtubules during mitosis, and include:-

(i)   the 'dynamic equilibrium' model of Inoué and Sato (1967),

       where an increase or decrease in length (through polymeri-

       zation or depolymerization) can provide the necessary force

       for a contractile event, for example, to move the

       chromosomes poleward during mitosis.

(ii)   a sliding filament theory, where adjacent microtubules

       slide against each other in a fashion akin to the sliding

       of myosin upon actin in striated muscle (McIntosh *et al*,

       1969; Warren, 1974; Murphy and Tilney, 1974; Murphy,

       1975). In this model a dynamic equilibrium between sol-

       uble subunits and polymerized microtubules [as proposed

       in model (i) above] is thought to be involved initially

       in establishing and then later disassembling microtubules.

       In contrast to model (i) however, cross-bridges between

       adjacent microtubules rather than changes in microtubule

       length are thought to facilitate the motion.

(iii) A zipper hypothesis, where chromosomes are moved by a

       cyclical lateral interaction between parallel spindle

       fibres and non-parallel sets of microtubules (Bajer,

       1973), and

(iv)   a model where microtubules act as guiding tracks for

       the transport of materials or organelles along their

       length, and where the actual propelling along of these

       materials or organelles is carried out by other linear

       elements, such as microfilaments (Ochs, 1972; Rebhun,

       1972; Forer, 1974; Burnside, 1975). Since this

       particular model was proposed, many investigators have

       shown that microfilaments are indeed often found closely

associated with microtubules in the cell cytoplasm (reviewed by Nicolson, 1976).

IV.2   MICROFILAMENTS

When viewed by electron microscopy microfilaments appear as a randomly oriented meshwork of ~ 5 - 8 nm diameter filaments with a variable length up to several μm (Pollard and Weihing, 1974; Nicolson, 1976). These filaments are composed of two helically wound polymers of actin (reviewed by Pollard and Weihing, 1974; Schreiner and Unanue, 1976; Nicolson, 1976). The monomers which make up cytoplasmic or non-muscle actin are single polypeptide proteins of ~ 43,000 molecular weight which contain calcium or magnesium and ATP (reviewed by Nicolson, 1976).

Microfilaments are often seen in the cortical cytoplasm, closely apposed to the plasma membranes of a variety of cell types (reviewed by Nicolson, 1976). Microfilaments can also be found in the cytoplasm in regions of cellular extension, such as pseudopodia (Wessells et al, 1971). Like microtubules, the microfilaments are apparently labile - able to assemble into helical microfilaments (F-actin) and disassemble into soluble globular subunits (G-actin) without requiring protein synthesis (Godman et al, 1975).

Applying the classical technique of heavy meromyosin binding to chick embryo cells, Ishikawa et al (1969) were the first to demonstrate the existence of actin in non-muscle cells. Since their discovery, actin has been found in a variety of cell types (reviewed by Pollard and Weihing, 1974), including phagocytes (Allison et al, 1971; Senda et al, (1975). It was noted by early investigators that this actin had the same cellular

distribution as the cytoplasmic microfilaments (reviewed by

Pollard and Weihing, 1974). Using a fluorescent antibody

technique, Lazarides and Weber (1974) and Goldman *et al* (1975)

were able to demonstrate conclusively that the non-muscle actin

was indeed located in the microfilamentous regions. Further

characterization has shown that the cytoplasmic actins purified

from several types of phagocytic cells show strikingly homologous

structural and functional properties with respect to each other and

to striated muscle actin (reviewed by Pollard and Weihing, 1974).

In addition to actin, another protein which bears a remarkable

similarity to striated muscle myosin has been found in a variety

of non-muscle cell types, including brain cells, fibroblasts,

platelets and granulocytes (Pollard and Weihing, 1974). Like

muscle myosin this protein possesses ATPase activity and is able

to bind actin.

The myosin in these various cell types differs from

the actin however, in that it appears to be heterogeneous

(Miranda *et al*, 1974a,b; Pollard and Weihing, 1974). Myosin has

been found in association with microfilaments (Pollard and

Weihing, 1974) but is also localized elsewhere in the cytoplasm

and on the cell surface (reviewed by Nicolson, 1976). The myosin

is composed of heavy (~ 200,000 molecular weight) protein chains

and two classes of light chains (~ 16,000 and 19,000 M.Wt.)

according to Adelstein, Conti and Anderson (1973).

IV.2.1   <u>Microfilaments as contractile structures</u>

The discovery of intracellular actin and myosin similar
in structure and function to muscle actin and myosin led some
workers to propose a role for these contractile proteins in cellu-
lar phenomena of a contractile nature (Allison, 1973; Reaven and
Axline, 1973; Pollard and Weihing, 1974). These workers also
suggested that the contractile events (such as phagocytosis,
movement, and adherence of cells to substrate) might be controlled
in a manner analogous to the regulation of muscle contraction. To
briefly review, striated muscle contraction is regulated by cyto-
plasmic calcium concentrations and the proteins troponin and
tropomyosin. These proteins bind to muscle actin, and dissociate
in the presence of calcium. This process allows actin (in the
presence of magnesium) to facilitate the hydrolysis of ATP by
myosin. ATP then provides the chemical energy required for myosin
filaments to slide upon actin to produce contractile force (Huxley,
1969). Unfortunately a role for calcium in activating actin-
myosin interaction in non-muscle cells has not been clearly
established, although several observations indicate that there are
similarities in non-muscle and muscle contraction. As in muscle,
cytoplasmic myosin can hydrolyze ATP, and filaments of actin-myosin
are able to contract in the presence of ATP and magnesium (Pollard
and Weihing, 1974). Furthermore, one of the regulatory proteins
(tropomyosin) has been found in platelets (Cohen and Cohen, 1972).

More recent investigations have shown that cytoplasmic
actin binds to a protein called α-actinin (Hartwig and Stossel, 1975;
also   Boxer *et al*, 1976), which may control the formation and

binding of microfilaments to each other (Mooseker and Tilney, 1975).

IV.2.2   Microfilament-disrupting agents

Microfilament organization can be disrupted by a related group of fungal metabolites, whose structure is shown in Fig. IV.7 (reviewed by Nicolson, 1976). Therefore these agents, known as cytochalasins[4], have been used in micromolar doses as pharmacological tools to study the role of microfilaments in cellular phenomena. Cytochalasins A and B have been isolated from *Helminthosporium dematioideum,* and C and D from *Metarrhizium anisopliae* (Aldridge *et al*, 1967). Of the four cytochalasins only B and D have received widespread use in the laboratory. Cytochalasin B is the more useful of the two experimentally because of its reversibility of action and low cellular toxicity (Carter, 1967; Wessells *et al*, 1971).

It is thought that the cytochalasins disrupt the architecture of microfilaments by inducing the clumping together of dysfunctional actin (Wessells *et al*, 1971). These drugs apparently have no effect on cytoplasmic microtubules (Schroeder, 1970; Wessells *et al*, 1971; Bhisey and Freed, 1975; Weissmann *et al*, 1975a,b). Miranda *et al* (1974a, b) have put forth an alternative mode of action of the cytochalasins. From experiments with cytochalasin D (which they found to be ~ 10 times as potent as cytochalasin B) these authors suggest that the cytochalasins

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[4] The name 'cytochalasins' comes from cytos (meaning cell), and chalasis (meaning relaxation) and was first used by Aldridge *et al* (1967).

cause an initial hyper-contraction of the filaments followed by

clumping and dysfunction as a result of the unrestricted contrac-

tion. The further suggest that with low doses of the cytochalasins

disassembly of the filaments does not occur, but there is enough

contraction to produce a response. Studies by others have shown

that the cytochalasins do not appear to act on isolated F-actin

(assembled actin) *in vitro* (Forer *et al*, 1971) or interfere with

the binding of myosin to actin *in situ* (Schroeder, 1973).

IV.2.3   Microfilament-mediated cellular functions

By virtue of their sensitivity to the cytochalasins,

microfilaments have been implicated in a variety of cellular

functions. As suspected by the early workers, the cellular

functions involved are primarily those where the generation of

contractile force appears to be required, namely:-

(i)   cytoplasmic streaming, membrane ruffling and membrane

asymmetry, and cell movement (reviewed by Wessells

et al, 1971; Allison, 1973; Pollard and Weihing,

1974; Miranda *et al*, 1974 a,b; and Nicolson, 1976).

(ii)   secretion of lysosomal enzymes from granulocytes

(Boxer *et al*, 1974; Sajnani *et al*, 1976; Becker,

1976), and insulin release from pancreatic B-cells

(Orci *et al*, 1972; Malaisse *et al*, 1975). It is

interesting to note that these secretory events

are stimulated when microfilaments are disrupted by

the cytochalasins, whereas other types of secretion

are inhibited under the same circumstances. For

example, thyroid secretion (Williams and Wolff,

1971), growth hormone secretion (Schofield, 1971),

and histamine release from mast cells (Orr *et al*, 1972) are inhibited by the cytochalasins.

(iii)   plasma membrane receptor redistribution (reviewed by Nicolson, 1976; Schreiner and Unanue, 1976).

(iv)   cell-cell interactions, for example, the binding of erythrocytes to human T cells (Cohnen, Fischer and Brittinger, 1975).

An undesirable side-effect of the cytochalasins is their inhibition of the transport of glucose, deoxyglucose, glucosamines, and nucleosides, which is probably not mediated by microfilament disruption (reviewed by Allison, 1973; Pollard and Weihing, 1974; and Nicolson, 1976).

It is not unreasonable to envisage that micro-filaments would require points of anchorage in order to generate the contractile force, as Stossel (1975) and Wilkinson (1976) have appreciated. A model wherein microtubules provide such an anchoring system for the microfilaments in their con-tractile role in the movement of plasma membrane proteins has been proposed and is widely accepted (Poste, Papahadjopoulos and Nicolson, 1975; Nicolson and Poste, 1976; Nicolson, 1977). This model for the role of cytoplasmic microtubules and micro-filaments in the control of plasma membrane phenomena has been presented in the introduction to this thesis (section 1.7).
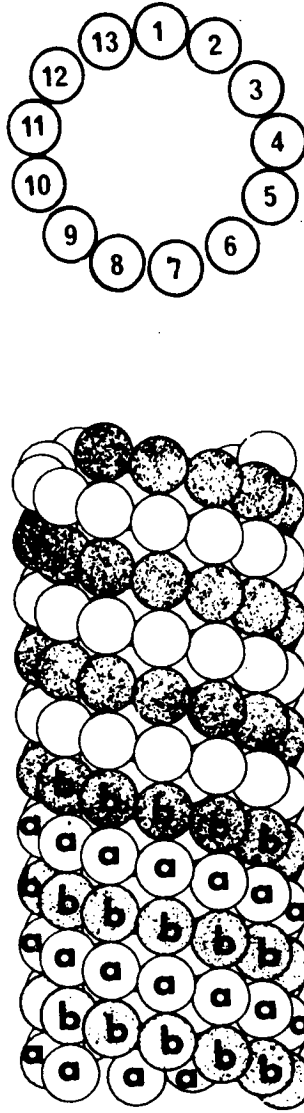
FIG. IV.1.   Cross-sectional diagram of a microtubule having 13

subunits (top).   A longitudinal view (bottom) depicts

the 13 helically-wound protofilaments of α-tubulin

(a) and β-tubulin (b), running parallel to the long

axis of the microtubule [from Fujiwara K and Tilney

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FIG. IV.2. The structures of colchicine and lumicolchicine, taken from Paulson JC and McClure WO (1975). *Ann. NY. Acad. Sci.* 253: 517.

FIGS. IV.3 and IV.4. The structures of vinblastine and vincristine, differing only in the group attached to nitrogen (arrows). From Wilson (1975).

FIGS. IV.5 and IV.6. The structures of podophyllotoxin and griseofulvin, taken from Paulson and McClure (as above).

FIG. IV.7. The structure of the cytochalasins, as shown by Aldridge *et al*, 1967:

	R^1	R^2
Cytochalasin A	O	H,OH
" B	H,OH	H,OH
" C	O	H,OAc
" D	H,OAc	H,OAc
" E	O	O
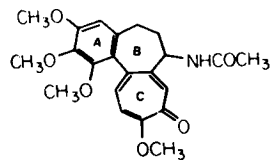
Colchicine
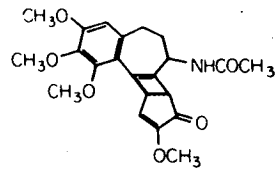
Lumicolchicine

Vinblastine

Vincristine

Podophyllotoxin

Griseofulvin

Cytochalasins

REFERENCES

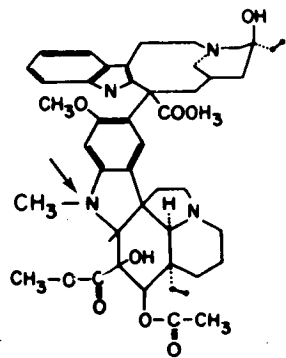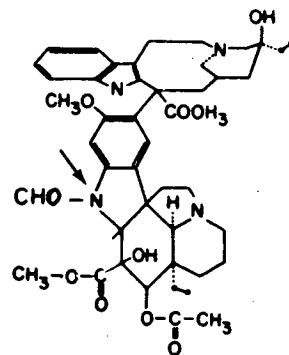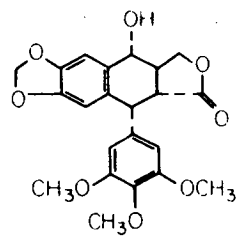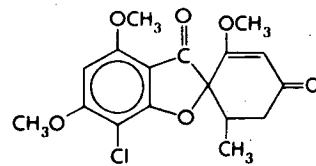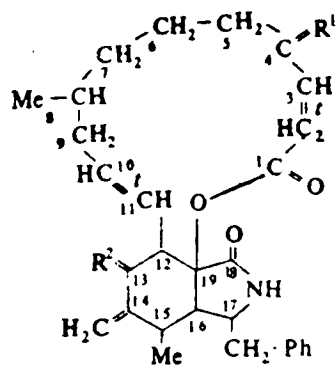Adelstein RS, Conti MA and Anderson W (1973). Proc. Natl. Acad. Sci. (USA) 70: 3115.

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