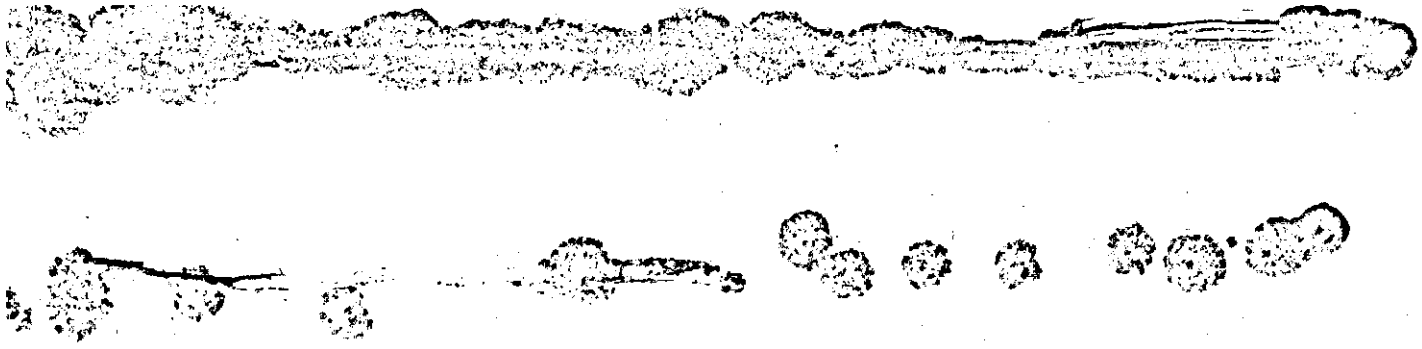
Studies of potentially inflammatory phospholipases A2 and the effects of chloroquine-like agents



STUDIES OF POTENTIALLY INFLAMMATORY PHOSPHOLIPASES A_2 AND THE EFFECTS OF CHLOROQUINE-LIKE AGENTS

by

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To my family

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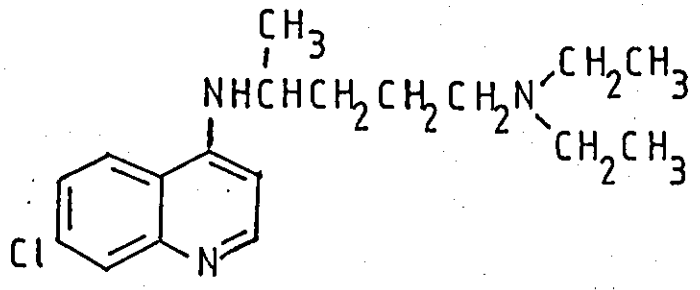

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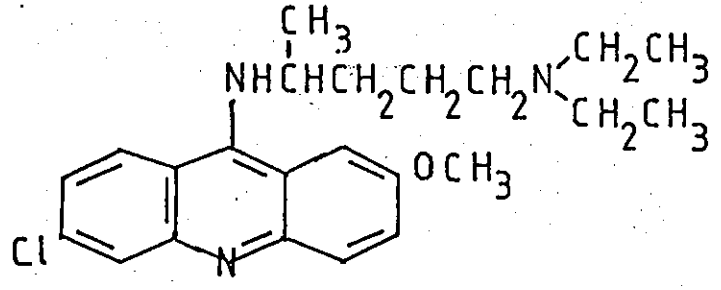

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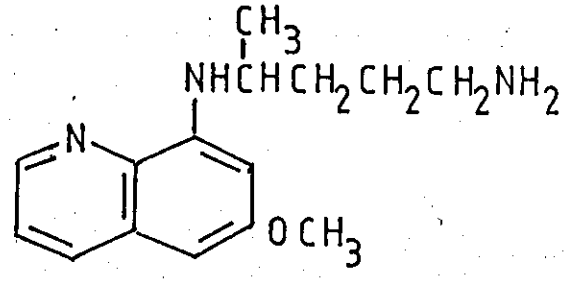

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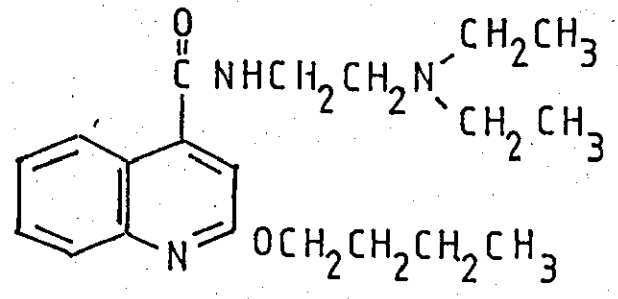

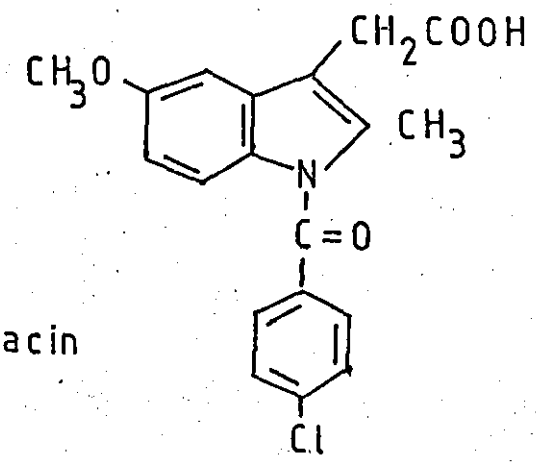

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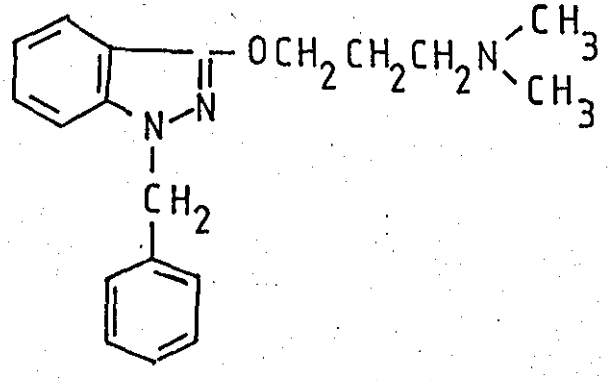
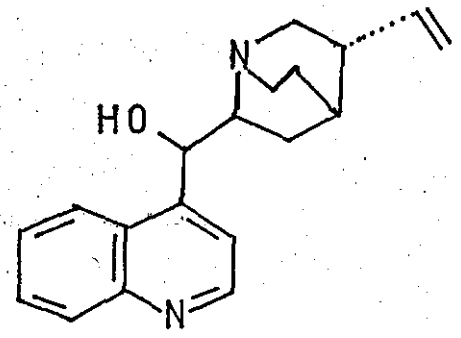


Benzydamine



Cinchonine

Chapter One

INTRODUCTION

PART A INFLAMMATION

1.1 Inflammation

Inflammation is an essentially normal and protective response to any noxious stimulus that may threaten the well-being of a host. The injurious stimulus may be a chemical agent (croton oil, carrageenan, glycogen), a physical agent (burns, ultra - violet light), or a biological agent (bacteria, fungi, viruses). In addition an endogenous factor may give rise to inflammation as is the case in autoimmune diseases.

Inflammation can vary from an acute, transient, and highly localised response such as a pin prick, to a complex sustained chronic response involving the whole organism as occurs with rheumatoid diseases.

When tissue injury is caused by a single event or a single exposure to a non - replicating agent, the inflammatory process progresses smoothly from injury to healing, and is termed acute inflamm - ation. If however, the injurious agent is self - replicating (parasites, bacteria and viruses), or attempts to remove the agent fail, then the inflammatory response becomes much more complex and will continue for as long as the agent itself persists. This gives rise to a chronic inflamm - atory process with much tissue damage due to the secretion of destructive lysosomal enzymes and may result in loss of function.

Inflammation is manifested by five cardinal signs of in - flammation. These are erythema, oedema, pain, heat and eventually loss of function. During an inflammatory response there is an initial brief cons - triction of the arterioles followed by prolonged dilation of the blood vessels. This produces an erythema which is then followed by leakage of

plasma protein from the blood causing an oedema. During the oedema phase the migration of leucocytes into the inflammed area is a characteristic feature of the inflammatory response. The liberation of mediators throughout the response leads to the production of pain and heat.

1.2 Mediators of Inflammation

The inflammatory process involves the liberation of many potent substances which can themselves duplicate the inflammatory response and lead to pain, heat and to the chronic condition, loss of function. Table 1.1 summarises the chemical nature and origin of these substances.

Each substance, as its involvement was proposed or demonstrated has been studied intensively and attempts have been made to link its actions to that of other mediators. Mediators generally have three properties : (1) they can induce some or all of the signs of inflammation, (2) they can be released during an inflammatory reaction, and (3) their release or action can be affected by anti - inflammatory drugs.

The importance of the mediators in the various types of inflammation depends upon the sensitivities of the tissues in which they are released and on the sequence of mediator release. For example, in anaphylactic shock there is an explosive and virtually simultaneous release of many mediators, for example histamine and SRS - A, whereas in the carrageenan - induced inflammatory response there is a sequential release of mediators.

1.3 Prostaglandins as mediators of inflammation

TABLE 1.1

MEDIATORS OF INFLAMMATION

AGENT	CHEMICAL NATURE	ORIGIN
1. HISTAMINE; SEROTONIN (5HT)	AMINE (STORED)	BASOPHIL MAST CELL; PLATELETS
2. SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS -A)	ACID LIPID	LEUKOCYTE
3. KININS	POLYPEPTIDES (SPLIT PRODUCTS)	PLASMA SUBSTRATE
4. PROSTAGLANDINS	ACID LIPIDS (NEWLY SYNTHESISED)	UBIQUITOUS INTRACELLULAR PRECURSORS
5. PLASMIN	PROTEASE (SPLIT PRODUCT)	PLASMA SUBSTRATE
6. HAGEMAN FACTOR (ACTIVATED)	PROTEASE	
7. COMPLEMENT	PLASMA PROTEINS AND SPLIT PRODUCTS	RETICULOENDOTHELIAL CELLS, LIVER
8. LYSOSOMAL ENZYMES	INTRACELLULAR PROTEINS (STORED)	PMN'S, MACROPHAGES MAST CELLS
9. LYMPHOKINES	INTRACELLULAR PROTEINS (NEWLY SYNTHESISED)	STIMULATED LYMPHOCYTES

(i) Discovery

In 1930 Kurzrock and Lieb observed that human myometrium tissue showed rhythmic contractions and relaxation when incubated with fresh human semen. This observation was confirmed by GOLDBLATT (1933) and Von Euler (1936) and the latter author identified the active component as an acidic lipid and named it "prostaglandin" thinking that it was produced in the prostate gland. In 1959, Eliasson showed that prostag - landins in human semen were derived from seminal vesicles and Bergstrom and Sjovall (1960) showed that the active substances were several closely related compounds. Prostaglandins are now known to be ubiquitous having been identified in almost every tissue.

Figure 1.1 shows the main prostaglandin related substa - nces and their biosynthesis from arachidonic acid. The subscript one (1) type products eg. PGE_1 and $PGF_{1\alpha}$ are biosynthesised from dihomo - γ - linolenic acid which contains 3 double bonds.

(ii) Release during inflammatory reactions

In several experimental models of inflammation prost - aglandin - like substances have been regarded as terminal mediators of the acute response, although one important exception is the anaphylactic response where discharge of mediators occurs simultaneously upon challenge with antigen.

(a) Carrageenan - induced inflammation of the rat paw

Carrageenan is a sulphated mucopolysaccharide derived from the Irish Sea Moss, Chondrus, and is able to induce an acute infl -

LEGEND TO FIGURE 1.1

Arachidonic acid can be oxygenated via 2 pathways:

1) CYCLOOXYGENASE Known to be present in all cell types except erythrocytes.

First PGG_2 is formed. This can be converted to PGH_2, which can break down enzymically or non-enzymically to a variety of other products (see figure). Inhibited by NSAIDS.

Enzymes PLA_2 = phospholipase A_2

TXS = thromboxane synthetase

PgS = prostaglandin synthetase

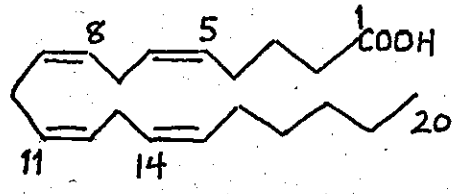
PcS = prostacyclin synthetase

3) LIPOXYGENASE Identified so far in lungs, platelets and leucocytes. An unstable hydroperoxide is formed (HPETE) which can then breakdown to the stable hydroxyacids or can be further tran - sformed to other products such as leukotrienes. Lipoxygenase is inhibited by BW 755C.

In lungs lipoxidase acting at C11 + C12

In platelets lipoxidase acting at C12

In leukocytes lipoxidase acting at C5.



ARACHIDONIC ACID

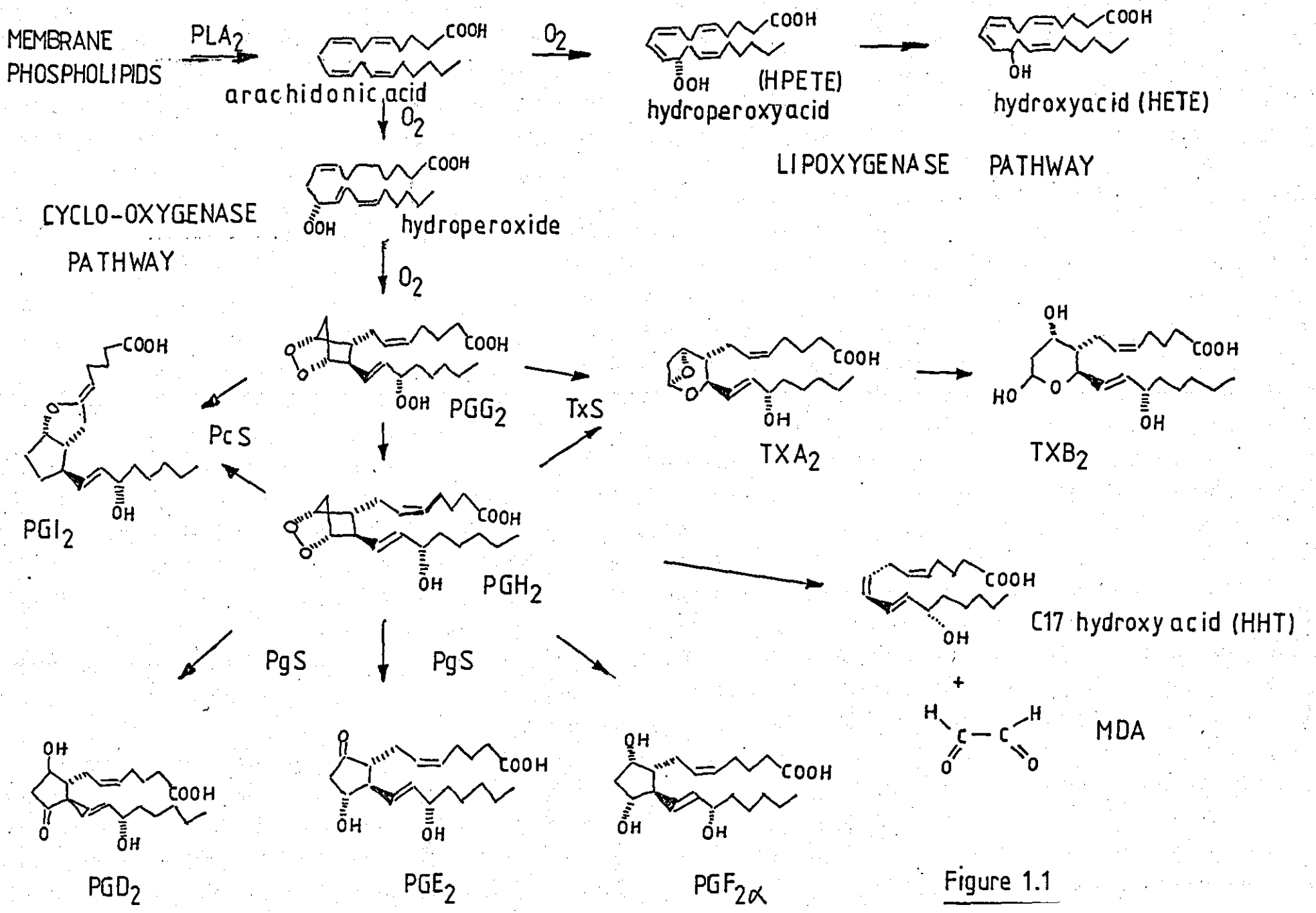

MEMBRANE PHOSPHOLIPIDS
PLA2
arachidonic acid
O2
COOH
OOH
(HPETE)
hydroperoxyacid
OH
hydroxyacid (HETE)
LIPOXYGENASE PATHWAY
CYCLO-OXYGENASE PATHWAY
hydroperoxide
PGG2
TxS
TXA2
TXB2
HO
PcS
PGI2
PGH2
C17 hydroxy acid (HHT)
+
MDA
PgS
PgS
PGD2
PGE2
PGF2α

Figure 1.1

ammatory response when injected into the paw of a rat. In this model which is used widely in the search for new anti - inflammatory agents, there appears to be distinct phases of mediator release (DiRosa et al, 1971a) (figure 1.2). It appears that during the first 1½hrs histamine and 5HT are the main mediators released. This was shown by the use of the anta - gonist compound 48 / 80 which depletes stores of histamine and 5HT. 48 / 80 Given over 4 days before carrageenan injection showed a marked reduction in paw oedema during the first 1½hrs after carrageenan injection. The second phase (1½ - 2½ hrs) of this reaction is mediated by Kinins. Simil - arly the use of cellulose sulphate, which lowers the plasma Kininogenen level by 50%, reduced the formation of the oedema during this phase. The use of 48 / 80 and cellulose sulphate together led to a supression of the oedema up to 2½hrs.

The appearance of prostaglandins in the inflammed paws was demonstrated at 2½ - 6hrs after carrageenan injection by Willis (1969), suggesting that prostaglandins may mediate this phase of the response. Compound 48 / 80 and cellulose sulphate had no effect on the oedema during this phase and neither did they affect associated migration of polymor - phonuclear (PMN) leucocytes into the area. However the depletion of com - plement by the use of antisera towards leucocytes or antigen - antibody complexes, reduced the oedema up to 6hrs. This demonstrated that comple - ment was required for the release of all the mediators and it was suggested that activation of complement, either by the irritant itself or fixation by altered tissue proteins was the event that led to the release of mediators and the progression of the inflammatory response.

(b) The carrageenan air - bleb

This is an experimental model in which an ordered release

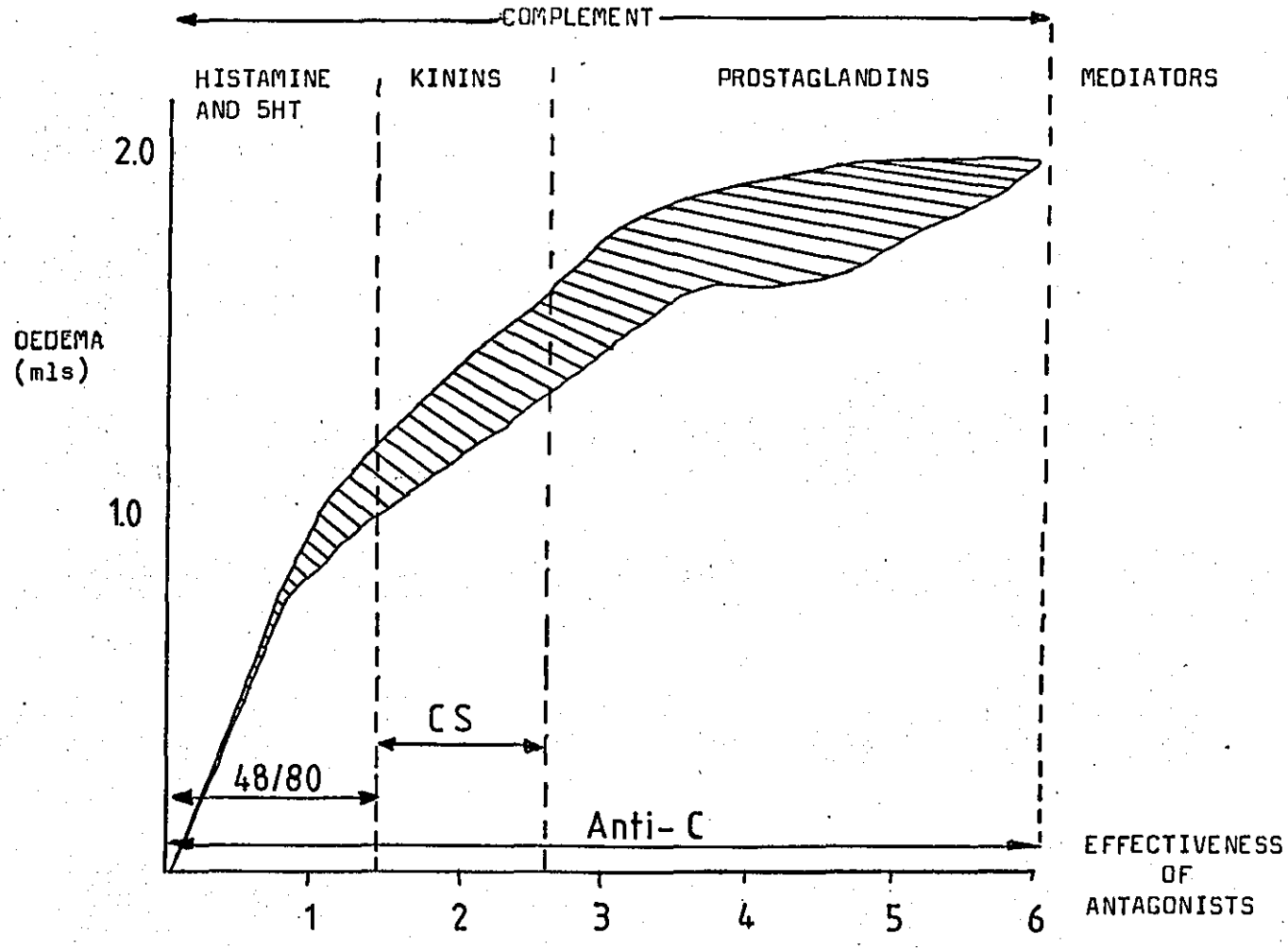


Figure 1.2 Time course of mediators in the carrageenan induced oedema of rat paw. The lined curve indicates intensity of vascular response as judged by accumulation of ^{131}I - albumin (from DiRosa et al, 1971 a).

of mediators occurs. A suspension of carrageenan is injected into a subcutaneous air - bleb formed on the back of a rat. An inflammatory reaction follows, and samples of the bleb - fluid can be withdrawn at certain time intervals and analysed. Again histamine and kinins are found shortly after carrageenan injection and prostaglandins, mainly PGE_2, appear after 3hrs and reach a maximum at 12-24hrs (Anderson et al, 1971). Carrageenan induces prostaglandin accumulation in a dose - dependant manner and the concentration of PGE_2 recovered at 24hrs (95ng/ml) was far in excess of that necessary to produce cutaneous inflammation. The lysosomal enzyme β- glucuronidase was found to increase in parallel with PGE_2, implying that appearance of prostaglandins was related to appearance of lysosomal enzymes.

(iii) Induction of inflammation

Prostaglandins are able to induce the signs of infla - mmation. These properties which will only be mentioned very briefly here have been reviewed in detail by Ferreira & Vane (1974) :-(i) Erythema can be produced by prostaglandins of the E series in low concentrations and the effects are long lasting. They can also counteract the vasoconstri - ction caused by agents such as noradrenaline and angiotensin. (ii) PGE, is the most powerful pyretic agent known and during fever the generation of a prostaglandin E - like substance in the central nervous system has been measured. (iii) Prostaglandins are not very good oedema producing agents on their own but sensitise blood vessels to the permeability effects of other mediators. (iv) The role of prostaglandins in pain pro - duction is to induce long lasting hyperalgesia ($\triangleq$ 2hrs), a state in which pain can be elicited by normally painless mechanical or chemical stimultation.

(iv) Effects of anti - inflammatory drugs

Inhibition of prostaglandin biosynthesis by indomethacin and aspirin was first shown by Vane and collegues (Vane, 1971; Smith and Willis, 1971; Ferreira et al, 1971). In the former report cell - free homogenates from guinea- pig lungs were prepared and the 900g supernatent used to synthesize prostaglandins from arachidonic acid. Indomethacin and aspirin both inhibited biosynthesis of PGE_2 and $PGF_{2\alpha}$ in a dose - dependent manner. Smith and Willis (1971) found that addition of the same drugs to washed human platelets which produce $PGF_{2\alpha}$ when incubated with thrombin, substantially reduced prostaglandin formation in a dose - related manner. Finally indomethacin and aspirin also abolished the release of prostaglandins observed when a perfused dog spleen is contracted by either catecholamines or nerve stimulation (Ferreia et al, 1971).

These observations were of importance since the effects found were at concentrations likely to be achieved in vivo and anti - inflammatory potency correlated well with the ability to inhibit prosta - glandin synthesis.

Since these findings inhibition of prostaglandin bio - synthesis by aspirin and related drugs has been shown in many other bio - logical systems (eg Tomlinson et al, 1972; Flower et al, 1972). However the steroidal anti - inflammatory drugs such as dexamethasone, although being more effective as anti - inflammatory agents than indomethacin, were shown not to directly inhibit prostaglandin synthetase, although they blocked release of prostaglandins in whole cell systems (Flower et al, 1972). These drugs are now known to be inhibitors of phospholipase activity and develop ments in their mode of action will be discussed later.

(v) Prostaglandins as modulators of inflammation

Prostaglandins of the E - type also have properties which are anti - inflammatory.

Lysosomal enzyme release by PMN leucocytes appears to be important in the pathogenesis of rheumatoid arthritis. PGE_1 and $PGE_{2\alpha}$, but not $PGF_{2\alpha}$, have been shown to reduce degrannulation and the release of lysosomal enzymes (Weissmann et al, 1972). Further, PGE_1 and $PGE_{2\alpha}$ have also been shown to prevent the release of histamine and SRS - A from basophils and lung fragments in vitro, when challenged with a presensitised antigen. These effects are thought to arise by the fact that PGE_1 and PGE_2, but not $PGF_{2\alpha}$, can increase intracellular cyclic adenosine 3',5'-monophosphate (cAMP), which when raised is known to inhibit lysosomal enzyme release.

In experiments using the carrageenan air - bleb technique, placement into the blebs of 200 μg of PGE_2 at the same time as carrageenan, reduced the rate at which leucocytes and lysosomal enzymes appeared at the site (Zurier et al, 1973). Also ultrastructural studies indicate that more lysosomes remain intact after carrageenan uptake in bleb leucocytes from PGE_1 treated animals than in leucocytes from control animals. These anti - inflammatory effects are observed at much higher concentrations of prostaglandins than those shown to potentiate the inflammatory response initiated by carrageenan (Ferreira and vane, 1974).

PGE_1 and PGE_2 have also been observed to suppress adjuvant induced arthritis and cartilage destruction in rats as measured by joint swelling (Zurier et al, 1973). The exact mechanisms involved in this action are not known although properties such as inhibition of

lysosomal enzyme secretion are thought to be important.

Prostaglandins therefore may have a complex role having both anti - and pro - inflammatory effects.

1.4. Involvement of Polymorphonuclear Leucocytes in Inflammation

(i) Function and morphology

Polymorphonuclear leucocytes, also referred to as neutrophils or granulocytes, are phagocytic cells primarily concerned with defence against foreign biological particles such as microorganisms. They originate from proliferating pools of precursor cells in the bone marrow where they differentiate and develop their characteristic granules. They make up the major portion of leucocytes in the blood (≙ 70%) and in response to chemotactic stimuli they can migrate into tissues and perform their function of phagocytosis and killing of biological material. PMN leucocytes live only for about one day in the circulation.

The mature circulating PMN leucocyte contains mainly two chemically distinct types of granules present in the cytoplasm called azurophil and specific granules. Azurophil granules are produced first during the promyelocyte stage of development, and arise from the inner, concave surface of the Golgi complex. These granules contain acid hydrolases (eg. β - glucuronidase), may be regarded as primary lysosomes found in other cells. Specific granules appear during the myelocyte stage of maturation and arise from the outer, convex phase of the Golgi complex. They are smaller in size than the azurophils and contain enzymes such as lysozyme. The protein composition of the membranes of these two granules have been found to be different and the azurophil granule also

contains a higher proportion of cholesterol in the membrane.

(ii) Release of lysosomal enzymes from PMN leucocytes during stimulation

During phagocytosis (particle ingestion or endocytosis) both types of granules discharge their contents, containing antibacterial agents and enzymes, into the phagocytic vacuole for the digestion of injested macromolecules. The specific granules preceed the azurophils in this process. Also during phagocytosis, probably because of incomplete closure of the phagocytic vacuole, a portion of the lysosomal constituents are released into the medium. *In vivo* the released hydrolytic enzymes would be free to act upon surrounding tissues and cause damage.

Mechanisms of the release process

Many *in vitro* studies have been carried out to examine the selective release of lysosomal enzymes from stimulated PMN leucocytes. A variety of stimuli have been used to elicite the release process. For example zymosan particles coated with complement or antibody, immune complexes, CSa, and N - formyl - L - methionyl - L - leucyl - L - phenylalamine.

An extensive study with the use of zymosan particles coated with complement (C3) or antibody (IgG) was carried out by Henson (1971) using peripheral rabbit leucocytes. In this case specific release of lysosomal enzymes occured in a time and concentration dependant manner. Maximal release of approximately 30% lysosomal enzymes occured with 5mg zymosan - complement particles. The release reaction was specific and mediated through receptors in the leucocyte

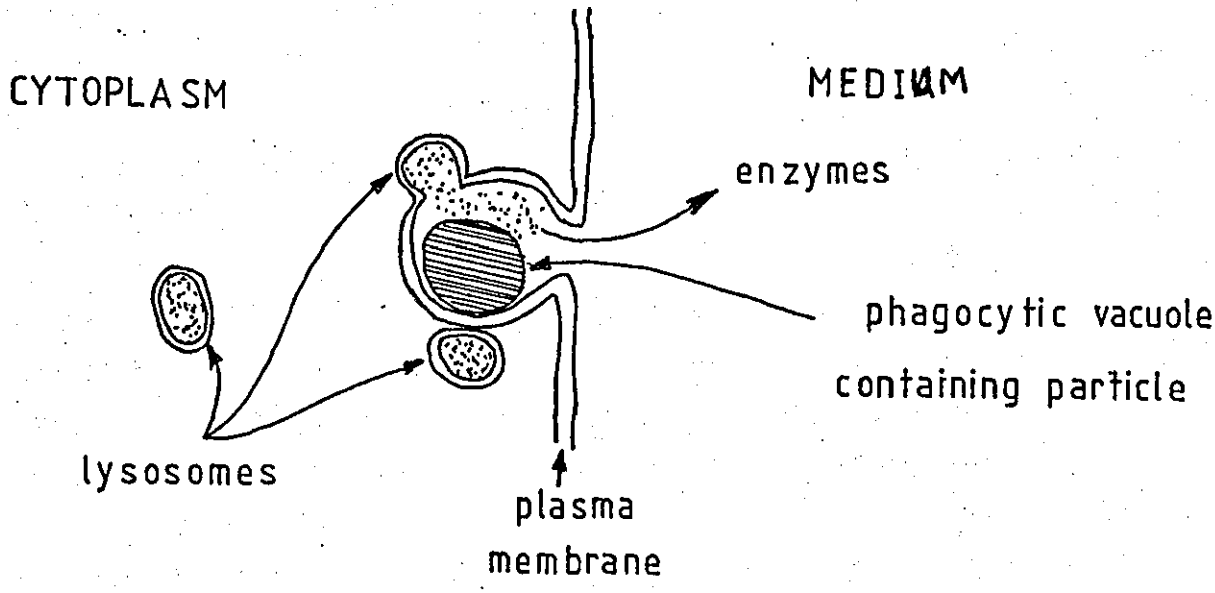
membranes which recognise complement (C3) and antibody (Fc portion of the IgG molecule), as no release was observed with zymosan particles alone. Release of lysosomal enzymes was also observed if antibody or complement or immune complexes were coated onto micropore filters which are too big to be ingested and hence provide a non - phagocytosable surface. Therefore as long as receptors on the leucocyte surface are stimulated, lysosomal enzyme release occurs whether or not phagocytosis occurs.

This finding is further supported by cytochalasin B treated human PMN leucocytes (Goldstein et al, 1975). This agent inhibits phagocytosis of particles by interference with microtubule function. When these leucocytes are exposed to either the complement component C5a, or immune complexes they act as secretory cells and release lysosomal enzymes without loss of cell integrity. The role of calcium ions has alone been investigated in this system. The complement component, C5a, in the absence of calcium ions is able to induce a significant release of lysozyme and β - glucuronidase. Addition of up to 1.5mM Ca^{2+} to the suspending media causes an increase in the release of both enzymes. However, further amounts of Ca^{2+} actually decreased β - glucuronidase release but had no effect on the release of lysozyme. Calcium itself only induced the release of lysozyme.

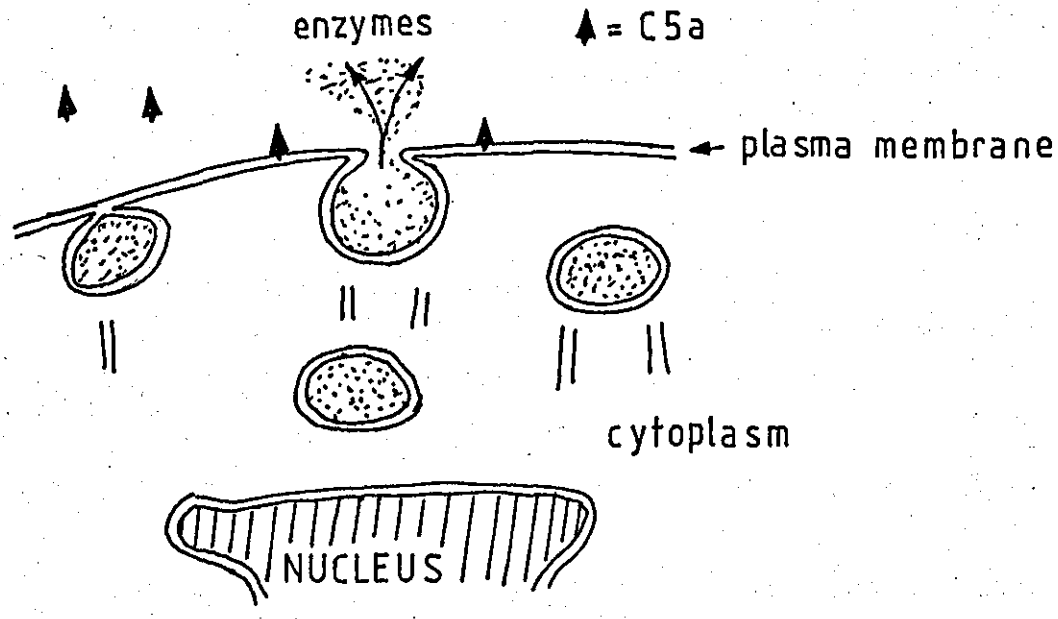
In another system, Northover (1977) using rabbit peritoneal neutrophils, demonstrated that calcium itself caused a time and concentration dependant release of both lysozyme and β - glucuronidase without release of the cytoplasmic marker enzyme lactate dehydrogenase. The non - steroidal anti - inflammatory drugs were found to affect the release of these two enzymes differently. The release of β - glucuronidase was inhibited by indomethacin, flufenamate and salicylate, and the

Figure 1.3. Mechanisms of lysosomal enzyme release from PMN leucocytes.

(1) Release during phagocytosis, "regurgitation during feeding"



(2) Cytochalasin B treated leucocyte.
C5a contact with receptor leads to fusion of lysosomal granules with plasma membranes and release of enzymes.



Agents shown to inhibit lysosomal enzyme release include inhibitors of microtubule function, colchicine and vinblastine; The steroidal anti - inflammatory drugs and the non - steroidal anti - inflammatory drugs.

release of lysozyme was inhibited by salicylate, amidopyrine and oxyphen - butazone but not by indomethacin or flufenamate.

Mechanisms of the release process are also illustrated in figure 1.3.

It is apparent therefore that PMN leucocytes with their characteristic large number of granules have the necessary machinery for provoking all of the features of the acute inflammatory response. Indeed the appearance of large numbers of leucocytes (first PMN's and later monocytes) is a characteristic feature of inflammation. Increased activ - ities of lysosomal enzymes have been found in the rheumatoid synovium (Anderson, 1970) and have been shown to produce experimental tissue damage as when injected into the skin and joints of rabbits. (Weissmann et al, 1969).

(iii) The involvement of PMN leucocytes in the generation of prostaglandin - like materials

(a) Prostaglandins

The possibility that PMN neutrophils leucocytes may be important in generating prostaglandins that appear in the sites of acute inflammation was suggested by Anderson et al (1971). Using the carrageenan air bleb experimental model, these workers observed that the appearance of lysosomal enzymes β - glucuronidase and acid phosphatase paralleled the appearance of PGE_2 during 24hrs after carrageenan injection. It was suggested that during the phagocytosis of carrageenan by leucocytes, phospholipases are released and that these hydrolyse phospholipids of cell membranes to yield arachidonic acid. This fatty acid could then be

converted to PGE_2 by freely available tissue enzymes (prostaglandin synthetase).

Prostaglandins were shown to be released from rabbit peritoneal PMN leucocytes during phagocytosis of bacteria (Higgs and Youlten, 1972). The main prostaglandin released was PGE_2 (56%), and PGF_2 (28%), with some additional compounds that were unidentified (16%). Release of prostaglandins was greater in leucocytes undergoing phagocy - tosis than in resting controls.

In a later report (Higgs,McCall and Youlten, 1975) leucocytes undergoing phagocytosis were found to produce a chemotactic substance which was absent if indomethacin was included in the preparations. One of the prostaglandins produced was identified as PGE_1 by thin - layer chromatography and differential bioassay, and the chemotactic property of PGE_1 first shown by Kayley and Weiner (1971) was also con - firmed. Neither PGE_2 or $PGF_{2\alpha}$ were found to be chemotactic for PMN leucocytes. The production of PGE_1 by stimulated PMNs is of particular importance in inflammation as this would lead to further infiltration of leucocytes into the inflammatory site. The rate at which phagocytosing PMNs were found to produce prostaglandins could account for the levels observed in inflammatory exudates. It also appeared that homogenates from cells which had been pre - incubated with bacteria (ie phagocytosing leucocytes) showed twice as much prostaglandin synthetase activity as compared with homogenates from bacteria-free controls.

Similar release of prostaglandins has been demonstrated from human PMNs exposed to zymosan particles (Zurier and Sayadoff, 1975).

(b) Production of thromboxanes (TX) by PMN leucocytes

Thromboxane production was first shown in sensitised guinea - pig isolated perfused lungs when challenged with antigen (Piper and Vane, 1969). An additional activity which was different from histamine, SRS - A and prostaglandins was described in the perfusate. This caused the contraction of a rabbit aorta strip, and was termed rabbit aorta contracting substance (RCS). RCS was later identified to be a mixture mainly consisting of thromboxane A_2 (TXA_2) with smaller amounts of prostaglandin endoperoxides PGG_2 and PGH_2 (Hamberg, Swensson and Samuelsson, 1975).

TXA_2 - like activity is also produced by PMN leucocytes (Higgs et al, 1976). When prostaglandin endoperoxides PGG_2 and PGH_2 were incubated with homogenates of phagocytosing PMNs for as little as 2 minutes at 0°C, there was an increase in the rabbit aorta contracting activity and the coelic artery contracting activity. This increase of biological activity was attributed to the production of TXA_2 from evaluation of its biological half life ($T_{\frac{1}{2}}$ = 8 - 13 min at 0°C and less than 1 min at 37°C in aqueous solution), and in its greater potency in contracting the rabbit aorta.

An increase in RCS was only observed with homogenates of phagocytosing PMNs and not resting controls, and the conversion to TXA_2 was prevented by boiling the preparations and by the agent benzy - damine, an inhibitor of thromboxane synthetase. Approximately 25 - 30 % of the endoperoxides were estimated to be converted to TXA_2 - like activity, the remainder rearranging to prostaglandins which were measured by the contraction of the rat colon.

The generation of thromboxanes from PMNs has also been shown by Goldstein et al (1978) using cytochalasin B treated leucocytes exposed to opsonised zymosan particles. Thromboxane B_2 (TXB_2) which is the stable product of TXA_2, was released in a time and concentration dependant manner, with the earliest detection 1 - 2 mins after stimulation.

The generation of TXA_2 may be important. It is known to be a potent aggregator of platelets, and the aggregation of platelets leads to the release of more mediators into the system. It is now thought that intermediates of prostaglandin biosynthesis (in particular PGG_2) like TXA_2, are the more important mediators as these have more potent activity and shorter half lives than the more stable primary prostaglandins. Further support arises from the studies of the compound MK -447. This agent has anti - inflammatory properties like indomethacin but actually increases prostaglandin synthesis (Kuehl et al, 1977). In particular the peroxidase reaction is stimulated.

$$PGG_2 \longrightarrow PGH_2 + O^{\circ} \quad \text{(free radicals)}$$

$$PGH_2 \longrightarrow PGE_2 + PGF_{2\alpha}$$

The anti - inflammatory properties of MK - 447 are attributed to the removal of PGG_2 and its ability, to remove free radicals which are also toxic.

(c) Production of hydroxy acids by PMN leucocytes

In 1974 an alternate pathway of arachidonic acid oxygenation was described with the discovery of a platelet lipoxygenase. The product 12 - L - hydroxy - 5,8,10,14 - eicosa - tetraenoic acid

(HETE) is a potent chemotactic agent for PMN leucocytes and is therefore of importance in inflammation. Lipoxygenase activity has also been dete - cted in PMNs (Borgeat et al, 1976) with oxygenation occuring at C5 of arachidonic acid and C8 of dihomo - γ - linolenic acid. The indomethacin related drugs are not effective against lipoxygenase but BW 755C has been shown to inhibit both lipoxygenase and cyclooxygenase. This is probably why it has a significantly greater effect on leucocyte migration in vivo than indomethacin (Higgs et al, 1978).

The ability of PMN leucocytes to produce the various metabolites of arachidonic and dihomo - γ - linolenic acids suggests that they may be the source of prostaglandins found in some forms of inflammation, for example the carrageenan induced inflammatory response in the rat. However this may not be so in all cases. Glatt et al (1974) using urate crystals to induce inflammation of intertarsal joints of chickens, found that the appearance of prostaglandins (PGE_2 and $PGF_{1\alpha}$) peaked at 1 - 2hrs along with maximum production of oedema and maximum permeability of the vessels. However, PMNs were not found to arrive at the site in significant numbers until after 4hrs and reached a peak at 6hrs. The appearence of the lysosomal enzyme, lysozyme, exactly paralleled PMN infiltration. The prostaglandin content of the exudate actually decreased as numbers of PMNs were increasing. It was suggested in this case that other cells (eg. platelets) may be a more viable source of prostaglandins in this model.

1.5 Importance of Phospholipase A_2 in the Release of Prostaglandins.

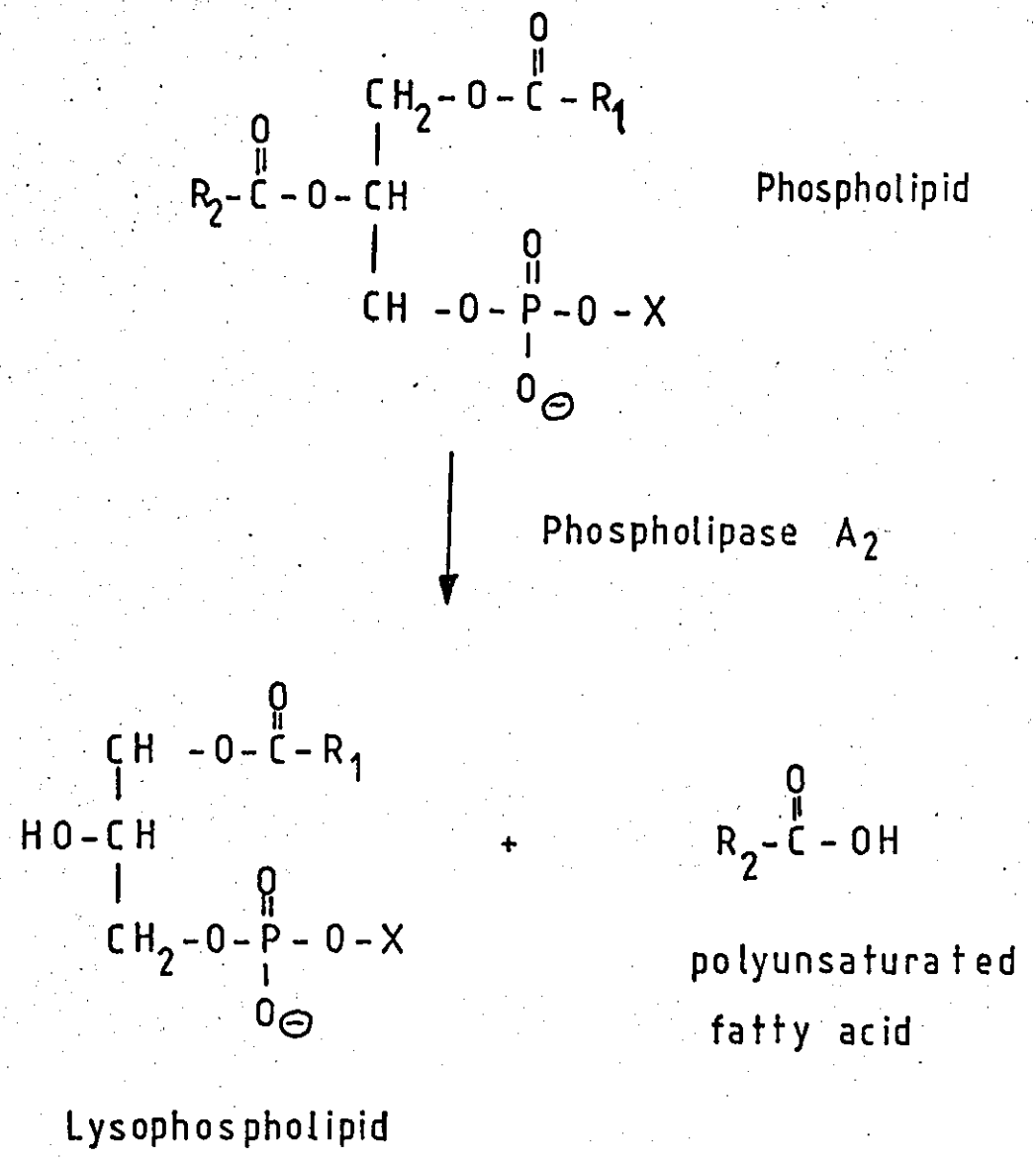
Prostaglandins are not stored to any considerable extent in mammalian tissues and it is believed that any increase in their levels

brought about by physiological stimulation is through rapid biosynthesis. The synthesis is therefore dependant upon the availability of precursor polyunsaturated fatty acids. The major portion of these fatty acids is present in the esterified form in tissue phospholipids which form a major constituent of membranes. Release of arachidonic acid therefore, occurs by the action of phospholipid splitting enzymes or phospholipases.

Phospholipases A, which are generally found in the lysosomes of most cells, are capable of bringing about the complete de - acylation of phospholipids. Arachidonic acid is a polyunsaturated fatty acid and generally occupies position 2 of phospholipids. Position 1 is normally esterified with a saturated fatty acid (figure 1.4). Therefore phospholipase A_2 (phosphatide 2 - acyl hydrolase E.C. 3.1.1.4.) is the important enzyme responsible for the release of arachidonic acid from phospholipids. The other product of phospholipase A_2 hydrolysis, lyso - phospholipid, is also important because this is a surface active agent and also cytotoxic if allowed to accumulate.

Experimental evidence suggesting the importance of phospholipase A_2 in prostaglandin biosynthesis has now been obtained in many tissues. A large proportion of the work was carried out on isolated guinea - pig perfused lungs although similar observations have been found in guinea - pig spleen (Flower and Blackwell, 1976), frog intestine (Bartels et al, 1970) and the thyroid gland (Haye et al, 1973).

The release of prostaglandin - like materials from sensitised, guinea - pig isolated perfused lungs when challenged with antigen has already been mentioned. This has also been shown by a variety of other stimuli using unsensitised lungs. The list includes bradykinin, mechanical trauma, rabbit aorta contracting substance -



X = base moiety (choline, ethanolamine, serine, inositol etc.)

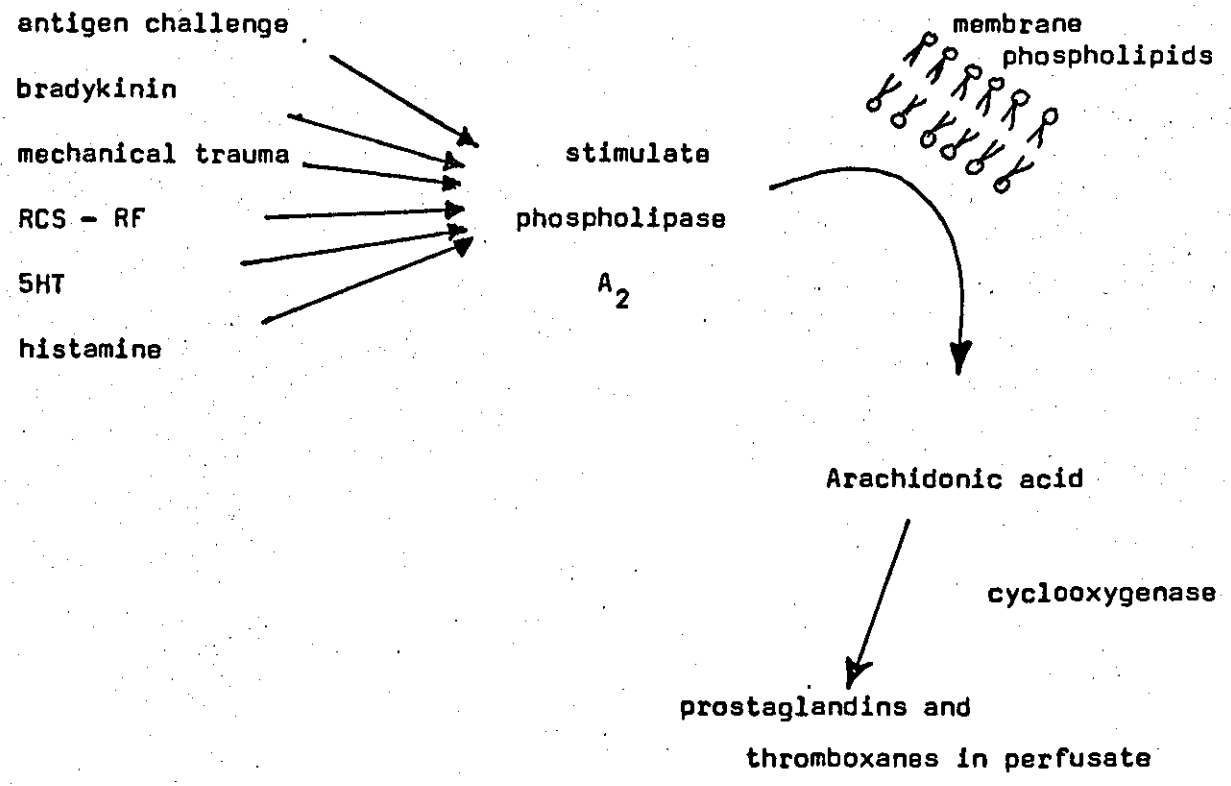
R_1 = saturated fatty acid

R_2 = unsaturated fatty acid.

Figure 1.4 Action of phospholipase A_2.

releasing factor (RCS - RF, thought to be a peptide) (Piper and Vane, 1969; Palmer et al, 1973), and 5HT and histamine (Alabaster and Bakhle, 1970). Further it has been shown that these stimuli cause the activation or increase the activity of a phospholipase A_2 which provides the fatty acid necessary for the production of RCS (Blackwell et al, 1978). Infusion of arachidonic acid, thereby by - passing the phospholipase step, also leads to the release of RCS (Vargaftig and Dao Hai, 1972). Indeed inhibitors of phospholipase activity such as anti - inflammatory steroids and mepacrine (see later) were shown to block the effects of these agents, again implying that they acted through effects on phos - pholipase activity.

In guinea - pig isolated perfused lung:-



1.6 Phospholipases of PMN leucocytes

Phospholipase activity of rabbit peritoneal PMN leucocytes was first described by Elsbach and Rizack (1963) who measured the release of fatty acids from dipalmitoyl phosphatidylcholine (PC) . The activity was optimal in the acid pH range and found mainly in the granule fraction. An acid active lipase activity was also detected. The granule free supernatant also contained an alkaline lysophospholipase activity.

In a later paper (Elsbach et al, 1965) use was made of substrates labelled with ^{32}P. The accumulation of monoacyl (^{32}P) phosphatidyl choline (lysophosphatidyl choline) from diacyl (^{32}P) phosphatidyl choline confirmed the presence of phospholipase A type activity. However the main product was glycerylphosphoryl choline indicating the presence of lysophospholipase activity in the homogenate. The phospholipase hydrolysed phosphatidyl ethanolamine (PE) to the same extent as PC and a direct transacylation activity, important for the synthesis of phospholipids, was also functional at high lysophospholipid concentration.

The difference in pH optima for the phospholipase A and lysophospholipase activities led to the suggestion that accumulation of lysophospholipids may be important during phagocytosis and digestion of bacteria by PMN leucocytes, as the pH of the phagosome is acid. lysophospholipids being lytic agents may help in the fusion of lysosomes with the phagocytic vacuole.

In 1972 phospholipid metabolism was examined with the use of leucocytes labelled during pre - incubation of the cells, for 1 hr at 37°C, with (^{32}P) lysophosphatidyl choline (Elsbach et al). The granulocytes lost less than 20% of incorporated radioactivity after 24hr.

at 37°C. This rate was not altered during phagocytosis, and the products were lysophosphatidyl choline, which was still associated with the cells, and glycerophosphoryl choline, which was released into the medium.

When incubated with live E.coli for 15 min in vitro, leucocytes were capable of killing 99% of (I - ^{14}C) palmitic acid labelled E.coli. However during this period only 30% of the phospholipids of E.coli were degraded, suggesting that killing of bacteria was associated with only a small degradation of phospholipids.

Certainly homogenates of leucocytes were found to readily degrade (^{32}P) PE, which is the major phospholipid of E.coli. The phosp - holipase A activity was maximal at pH 7.5, required Ca^{2+} and was almost all sedimentable at 8200g, indicating that it was present in the granules. Comparison of the leucocyte $\overset{PLA_1}{\lambda}$ with that from Crotalus Adamenteus venom demonstrated that the enzyme was of A_2 specificity. This enzyme was then shown to degrade all the phospholipids of E.coli equally well (Patriarca et al, 1972).

A more detailed examination of the phospholipase A and its cellular localisation in PMN leucocytes was presented by Fransen et al, 1974). Here extensive use of autoclaved E.coli which had been grown on (1 - ^{14}C) oleic acid was made as a substrate for phospholipase A. The (1 - ^{14}C) oleic acid was specifically incorporated in the 2 - position of E.coli phospholipids. Leucocyte homogenates and isolated phagosomes had a phospholipase A_2 activity which was optimum at both pH 5.5 and 7.5. The acid optima was inactive if liposomal suspensions of PE were used. Both activities required Ca^{2+}, a property which contrasts with lysosomal phospholipases of macrophages (Fransen et al, 1973). The phospholipase activities were associated with the azurophilic and

specific granules and were membrane bound. The activities were susceptible to product inhibition, and this inhibition was only partially reversed by the addition of albumin.

Further study of this phospholipase A_2 involved its purification (Weiss *et al*, 1975) and its comparison with a permeability increasing factor (PI) also located in the granules. PI is thought to cause the bactericidal effect of leucocytes on *E.coli*. The purification process which involved sulphuric acid extraction, dialysis and chromat - ography on CM - sephadex was unable to separate PI from phospholipase A_2. The preparation contained bactericidal activity but were clearly seper - ated from lysosomal enzymes such as lysozyme. Both phospholipase A_2 and PI are cationic and could possibly be a single protein. However PI act - ivity was inhibited by Mg^{2+} or Ca^{2+}, whereas phospholipase activity required Ca^{2+}.

PART B THE ANTI - RHEUMATIC EFFECTS OF CHLOROQUINE

1.7 INTRODUCTION

Chloroquine was developed principally as an antimalarial drug but has been used in the past 30 years in the treatment of Rheumatoid Arthritis and related diseases. It is a beneficial drug and actually slows down joint erosion in contrast to the milder non - steroidal anti - inflammatory drugs (NSAIDS eg aspirin and indomethacin) which although are very useful in relieving the symptoms, have no effect on the destructive nature of the disease. Unfortunately chloroquine also has many side effects, principal among which is retinopathy. This has limited its use in the United Kingdom, but it is still widely used in Sweden. Its anti - rheumatic effects are generally observed after 2 months treatment.

1.8 Tissue distribution and metabolism

A characteristic of chloroquine is its accumulation in tissues and very high concentrations have been found in the adrenal glands, liver, kidney, spleen, lungs and heart (Grundmann et al, 1972). Several reports have indicated that the drug persists in tissue long after discontinuation of therapy. Detectable amounts of chloroquine and metabolities are found in plasma and urine 3.5 - 5 years after the last administration (Rubin et al, 1963).

Upon administration chloroquine is completely absorbed in the gastro - intestinal tract and the drug is largely excreted unchanged (70%) in the urine.(Frisk - Holmberg et al, 1979).The half life measured from the plasma concentrations of a single oral dose

(0.31g chloroquine diphosphate) given to human patients was between 3 - 5 days. Various metabolites have been detected in the plasma and urine and it appears that metabolism involves the gradual degradation of the basic side chain of the chloroquine molecule so that eventually the 4 - amino - 7 chloroquinoline nucleus remains (Kuroda, 1962).

1.9 The anti - rheumatic mode of action of chloroquine

The mode of action of chloroquine is largely unknown but the drug also has many properties which do seem of relevence to its anti -rheumatic action. These include (a) effects on leucocyte migration, (b) effects on cartilage and collagen degradation and healing, and (c) lysosomotrophic activity and effects on lysosomal function. These have been adequately reviewed by Bresloff (1977) and only the actions of chloroquine important to the present work will be discussed.

1.10 Lysosomotrophic activity and effects on lysosome function

The relationship between chloroquine and the lysosomal system is central in explaining many of its actions. Lysosomal enzymes from phagocytic cells are important mediators of inflammation and are probably responsible for the connective tissue destruction in diseased joints. It is thought that chloroquine might exert its therapeutic effects through inhibition of lysosome function.

(i) Uptake into cells

Many cells exposed to chloroquine take it up avidly and achieve much higher intracellular concentrations (up to 400 times)

than in the surrounding medium. The selective uptake of a substance into the lysosomes of a cell from the surrounding medium is called lysosomotrophic activity (DeDuve et al, 1974). This rapid uptake is accompanied by a high degree of cytoplasmic vacuolation and evidence suggests that these are lysosomal. This has been shown in many cell types eg leucocytes of patients with sarcoidoses treated with chloro - quine (Fedorko, 1937), pancreatic cells of rats (Fedorko, 1968), cultured fibroblasts (Gaddioni et al, 1964), and mouse macrophages (Fedorko et al, 1968). Indeed lysosomotrophic activity is exhibited by most cationic amphiphilic drugs.

Initial uptake of chloroquine, which is also rapid, is known to be energy - independent and is followed by a slower energy dependent phase. The initial phase also raises the overall pH of the lysosomes (from pH 4.7 to 6.3 with 100 μM chloroquine, Ohkuma and Poole, 1978). The uptake process is also illustrated in figure 1.5.

Chloroquine has 2 ionisation constants (pKa_1 = 10.2, pKa_2 = 8.1) and at physiological pH, 18% of the drug is in the mono - protonated form which is soluble in lipid. This is able to pass through plasma membranes into a slightly more acid cytoplasm and to even more acid lysosomes, where the molecule becomes doubly protonated and incapable of passing back. The protonation of chloroquine in lysosomes or diges - tive vacuoles would deplete them of H^+ and result in a reduction of the acidity. Furth r uptake of chloroquine decreases unless more acid containing vacuoles are formed. The concentration gradient is further aided by the fact that chloroquine has been shown to bind to polar lipids (Seydel and Wassermann, 1976).

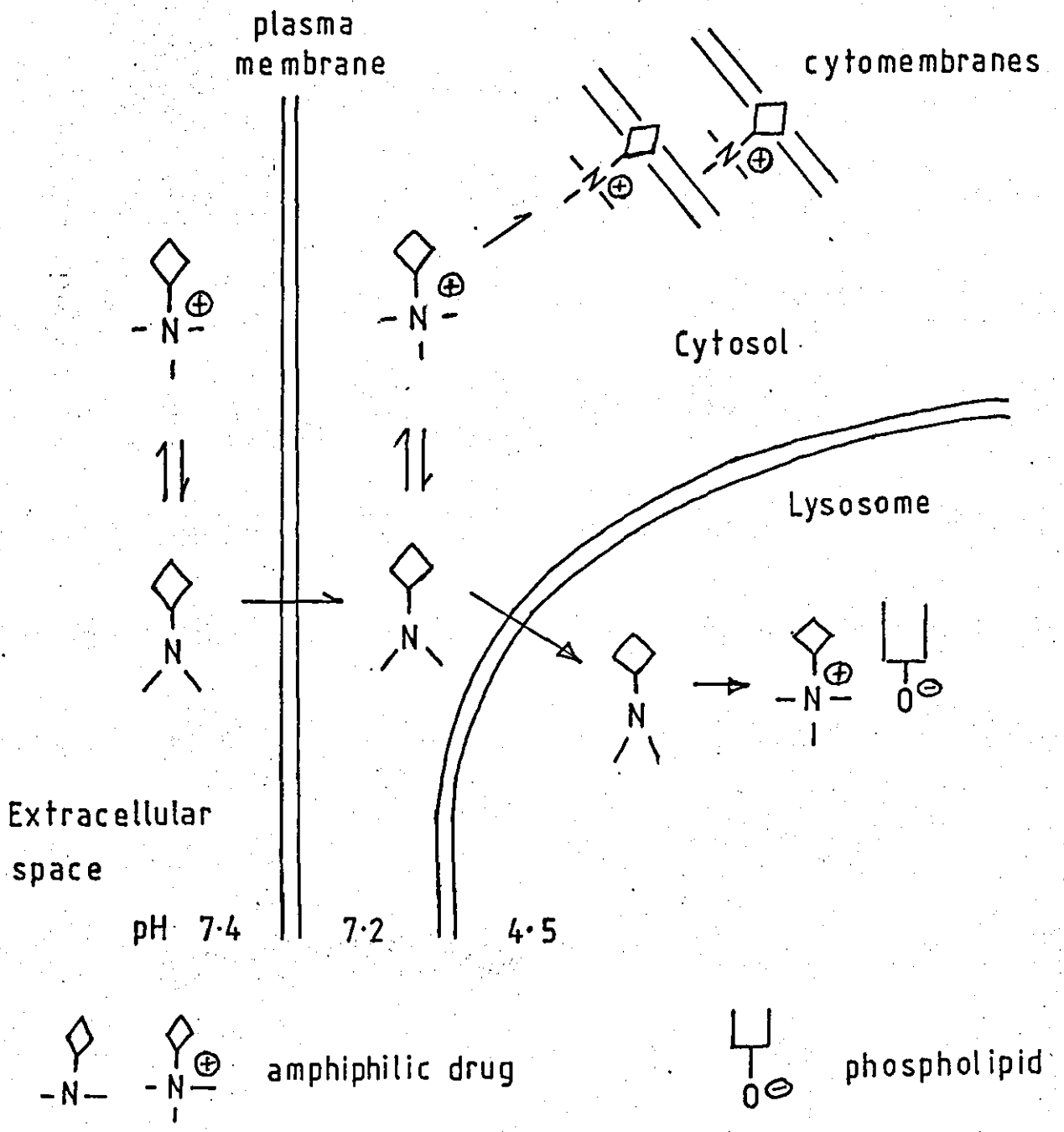


FIGURE 1.5.

Uptake of lysosomotrophic agents into cells.

(ii) Effects on lysosomal enzymes

Chloroquine in concentrations that could be achieved intracellularly ($\triangleq 10^{-4}$M) is able to inhibit a number of lysosomal enzymes important in causing damage to connective tissue. These include: chondromucoprotease of cartilage, cathepsin B, collagenases (although not lysosomal, therefore relevance not known) and neutral proteases (Cowey and Whitehouse, 1966; Ali et al, 1968; Mego and Chung, 1979). This has also been achieved in whole cell systems; human fibroblasts exposed to chloroquine ($1 - 2 \times 10^{-5}$M) in vitro are unable to digest proteins and mucopolysaccharides (Lie and Schofield, 1973), and macro - phages under the same conditions are also unable to degrade leucine radiolabelled bacteria. Raising the pH of the medium to pH 8.0 has been shown to have the same effect as chloroquine ie. inhibition of lysosomal enzyme activity and formation of vacuoles, on human fibro - blasts (Lie et al, 1972) and also on the malarial parasite, Plasmodium berghei, infected in erythrocytes (Homewood et al, 1972), indicating the importance of this property.

Inhibition or absence of lysosomal enzymes itself can be a sufficient cause for formation of autophagic vacuoles. Grossly enlarged lysosomes are observed with the addition of specific antibodies to lysosomal enzymes and also in several congenital storage diseases where a lysosomal enzyme is lacking (Hers and Van Hoof, 1969).

(iii) Actions on lysosomal membranes

Chloroquine being an amphiphilic molecule can be predicted to interfere with membrane properties as these are complex structures which have both lipophilic and hydrophilic constituents.

Many studies show stabilizing influences by chloroquine while others show contrasting effects.

In vitro studies on isolated lysosomes from liver and leucocytes (also peritoneal neutrophils) have indicated that chloroquine (10^{-5} - 10^{-3}M) and many other anti - inflammatory drugs inhibited the leakage of lysosomal enzymes, suggesting stabilization of the membranes (eg Weissmann, 1964; Ignarro, 1971).

Most anti - inflammatory drugs, whether steroidal or non - steroidal, also inhibit release of lysosomal enzymes from neutrophils and macrophages when exposed to various stimuli eg zymosan particles, immune complexes etc. However chloroquine does not possess this property and if anything it has been shown to increase the release of lysosomal enzymes at 10^{-4}M (Ringrose et al, 1975; Perper and Oronsky, 1974; Northover, 1977).

Chloroquine exposed to human fibroblasts not only causes vacuolation but also release of lysosomal enzymes in a dose - dependant manner (Weissmann et al, 1975). It also appears to inhibit the uptake of lysosomal enzymes into fibroblasts that are genetically deficient in these particular enzymes. This has been shown for aryl sulphatase (Weissmann et al, 1975) and ∝- L - iduronidase (Sando et al, 1979), and in both cases chloroquine interference with the specific binding of the lysosomal enzyme with the plasma membrane has been suggested.

(iv) Effects on lysosomal lipolytic processes

One of the unfortunate consequences of the lysosomo - trophic activity of chloroquine is lipidosis, and this is partly res - ponsible for the retinopathy seen in some patients on long term chloro - quine therapy. Lipidosis is a term used to describe a build up of polar lipids in lysosomes. Amphiphilic cationic drugs are particularly good lipidosis causing agents as they accumulate in lysosomes and bind to polar lipids. The drug polar lipid complexes are thought not to be susceptible to enzymic attack by phospholipases and hence a build up occurs. This phenomenon can have grave consequences in tissues, such as the retina, where a specially balanced lipid metabolism is essential for normal function. In experimental rats chloroquine has been shown to cause lipidosis in the neuroretinal cells of the retina (Drenckhahn and Lullmann - Rauch, 1978), in the cornea and in the lens which can then develope a cataract (Drenckhahn and Lullmann - Rauch, 1977).

Another important property of chloroquine which contributes to retinopathy is its ability to bind strongly to the pigment melanin. This binding can again lead to increased melanin con - centrations in the retina which can have a toxic effect on the optic nerve.

1.11 Effects of chloroquine and related drugs on lipolytic enzymes

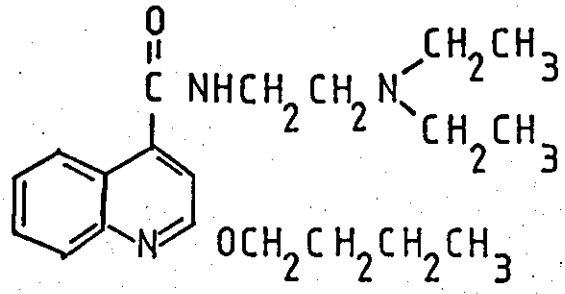
Chloroquine (up to 6×10^{-3}M) along with quinine, quinacrine, hydroxychloroquine and primaquine were shown to inhibit lipolysis in rat epididymal fat pads by Markus and Ball (1969). The drugs inhibited the enzyme lipase whether it was activated by adrenaline, released by heparin or just present in homogenates. Chloroquine has

also been shown to inhibit phospholipase A_2 of Plasmodium bergei, a fact important as this enzyme provides fatty acids essential for the malarial parasite to survive (Canedella et al, 1969).

Particularly important in inflammation is the study of Vargaftig and Dao Hai (1972), who showed that mepacrine (quinacrine) blocked the release of RCS from isolated guinea - pig perfused lungs when injected with bradykinin but not when arachidonic acid was injected. This indicated that the release of fatty acids from phospholipids was the site of action of mepacrine. Indeed bradykinin was later shown to cause an increase in phospholipase A_2 activity in this system (Blackwell et al, 1978).

Chloroquine and related drugs also resemble the local anaesthetics, eg dibucaine, in structure and indeed possess local anaesthetic activity themselves (Jindal et al, 1960), (see also page xii).

Dibucaine



This is of relevance as local anaesthetics have been shown to inhibit the activity of phospholipases (of pancreatic and seminal plasmal origin), triglyceride lipase (from Rhizopus arrhizus), lysophospholipases and cholesterol esterases (Kunze et al, 1976; Traynor and Kunze, 1976; Scherphof and Westenberg, 1975). All of these enzymes are not only involved in lipid metabolism, but can provide polyenoic fatty acids for prostaglandin biosynthesis.

1.12 Aims of the project

In the light of the above introduction PMN leucocytes play an extremely important role, not only as phagocytic cells but capable of providing prostaglandin related substances at sites of infla - mmation. Phospholipases are therefore particularly important as they can provide necessary precursors used to synthesiseprostaglandin related substances.

The work intends to investigate the ability of peritoneal neutrophil leucocytes, when stimulated, to contribute to any phospholipase A activities present in cell - free inflammatory exudates.

The biochemical properties and possible control mechanisms of any phospholipase A released during stimulation will be examined using bacterial membranes labelled with (1 - ^{14}C) oleic acid, as substrate, and compared with those of any phospholipase A present in the cell - free inflammatory exudate.

The interactions of chloroquine - like agents with these enzymes and other phospholipase A will be examined in an attempt to gain more imformation on the mode of anti - rheumatic action of these agents.

Chapter Two

MATERIALS AND METHODS

SECTION I MATERIALS

1.1 Chemicals and Reagents.

All laboratory reagents and solvents used were of analytical grade and used without further purification.

Bovine serum albumin (BSA), zymosan A from S.cerevisiae (yeast), N - 2 - hydroxyethyl piperazine - N'- 2 - ethanesulphonic acid (HEPES), phenolphthalein mono -β- glucuronic acid sodium salt, 2 - amino - 2 - methyl - 1 - propanol, Micrococcus lysodeikticus (dried cells), β - nicotinamide adenine dinucleotide reduced form and heparin (170 units per milligram) were obtained from Sigma Chemical Company Limited (Poole, U.K.).

Enzymes

Phospholipase A_2 from the snake venoms Crotalas adamenteus (Eastern Diamondback Rattlesnake) and Naja naja sputatrix (Malay Cobra) were also from Sigma Chemical Company, Poole. A partially purified phospholipase A_2 enzyme from pig pancreas was obtained from Bohringher (Mannheim, GFR.).

Phospholipase A_2 substrate

Escherichia coli (E. coli) strain W 8666. This was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

E. coli phospholipids were labelled using (1 - ^{14}C) fatty acids.

(1 -^{14}C) oleic acid (specific activity 57 mCi.mmol.$^{-1}$), (1 -^{14}C) linoleic acid (specific activity 61 mCi.mmol.$^{-1}$), and (1 -^{14}C) palmitic acid (specific activity 57 mCi.mmol.$^{-1}$) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

Lipid standards.

Oleic acid, L -α- phosphatidyl ethanolamine dipalmitoyl, L - α- phosphatidyl glycerol dipalmitoyl and L - α- lysophosphatidyl choline were obtained from Sigma Chemical Company.

Drug substances

Chloroquine sulphate, mepacrine hydrochloride and sodium aurothiomalate were kind gifts from May and Baker Ltd. Chloroquine diphosphate, D - penicillamine (free base) and primaquine diphosphate were obtained from Sigma Chemical Company.

Liquid scintillation fluids.

Unisolve 1 and KL 372 (for radioactive counting of aqueous samples) were purchased from Koch - Light Laboratories Ltd. Liquid scintillation counting was carried out on a LKB 1215 Rackbeta Liquid Scintillation Counter.

Thioglycollate medium

Thioglycollate medium (United States Pharmacopeeia, 18th revision 1970) was supplied by Oxoid Limited.

1.1 Media and Buffers.

(i) Triethanolamine medium.

E. coli for labelling were grown in a minimal media buffered with triethanolamine. The composition of this medium was as follows.

	$g.l.^{-1}$
$(NH_4)_2\ SO_4$	2.0
$FeSO_4.7H_2O$	0.0005
KCl.	0.075
Triethanolamine	7.5
$NaH_2PO_4.H_2O$	0.138
$MgSO_4.7H_2O$	0.2
Sodium succinate	5.0

The pH was adjusted to 7.9 - 8.0 with dilute hydrochloric acid.

Sterilization of the media (less $FeSO_4.\ 7H_2O$) was carried out by autoclaving at 120°C and 2.7 kg/cm^2 for 15 minutes. $FeSO_4.7H_2O$ was sterilised by membrane filtration and added to the media prior to use.

(ii) Thioglycollate medium (U.S.P. 18th revision, 1970)

This was used to elicit an inflammatory response in the peritoneal cavity of rabbits. The composition of this mediumis as shown overleaf.

	$g.\ l^{-1}$.
Yeast extract	5.0
Tryptone	15.0
Dextrose	5.5
Sodium thiglycollate	0.5
Sodium chloride	2.5
L - Lystine	0.5
Resazurin	0.001
Agar no. 1	0.5

pH approximately 7.1

29.5g of the medium was dissolved in 1 litre distilled water and sterilized by autoclaving at 120°C and 2.7 Kg/cm^2 for 15 minutes.

(iii) HEPES buffer (Ca^{2+} free suspending medium.)

For studies of the release of lysosomal contents from leucocytes. The composition was as follows:

	mM	$g.l^{-1}$.
HEPES (N - 2 - hydroxyethyl - piperazine - N^1 - 2 - ethane - sulphonic acid)	5	1.192
NaCl	150	8.766
Glucose	10	1.802
KCl	3	0.224

and adjusted to pH 7.4 with NaOH.

SECTION II GENERAL PROCEDURES

PART A ASSAY METHODS

(i) Protein Estimation

Protein estimation was carried out using the method of Lowry et al (1951) using 0 - 200 µg bovine serum albumin as standard. Figure 2.1 shows the standard curve obtained.

(ii) Lipid Extractions

All extractions of lipids of radiolabelled E.coli were carried out using a modification of the method of Bligh and Dyer (1959).

E.coli (representing 5 - 10 x 10^8 cells) were suspended in 0.5ml water. 3ml Chloroform : methanol (1:2) was added and the mixture was vigorously shaken. 50µl 0.5M HCl and 1ml chloroform was further added and the mixing repeated. Then 1ml water was added and after more vigorous shaking the organic and aqueous layers were separated by cent - rifugation at 600g for 5 minutes. The organic layer was then removed and the aqueous layer further washed with 1ml chloroform. The organic extracts containing bacterial lipids were then combined, evaporated to dryness under nitrogen and redissolved in a small volume of chloroform - methanol (2:1). The extracted lipids were then subjected to thin - layer chroma - tography (TLC) on Keiselgel G plates for separation of individual lipid species. Two types of solvent systems were used.

(I) chloroform / methanol / glacial acetic acid

65 : 25 : 6 v/v

(II) petroleum ether (60 - 80) / diethyl ether / formic acid

60 : 40 : 1 v/v

System I was used for separation of phospholipid species and system II for separation of neutral lipid species.

When radioactive samples were to be determined neutral and phospholipid standards were also applied on the same plates as markers as the amounts of lipids present in 10^9 cells of E.coli was not detectable. The lipid species were visualised by exposure of the plates to iodine vapour and after evaporation of the iodine the fractions were scraped directly into counting vials for determination of radioactivity by liquid scintillation counting. This was achieved in a medium of Unisolve 1 (7mls) plus water (3mls) which when mixed together formed a stable gel in which the radioactive Keiselgel was counted at an efficiency of 83%.

(iii) Phospholipid determination

This was performed using the method of Raheja et al (1973), which is a colourimetric method for the quantitative estimation of phospholipids without acid digestion. A calibration curve (figure 2.2) was constructed using L - α - phosphatidyl choline dipalmitoyl (0-800 μg phospholipid representing 0 - 33 μg P or 0 - 1089 nmoles P).

Phospholipid determination of radiolabelled E.coli was carried out on 5 - 10 x 10^9 cells after extraction of lipids by the method of Bligh and Dyer (1959) as previously described. Estimation of bacterial numbers was determined using Brown's opacity tubes

FIGURE 2.1.

Calibration curve for protein estimation.

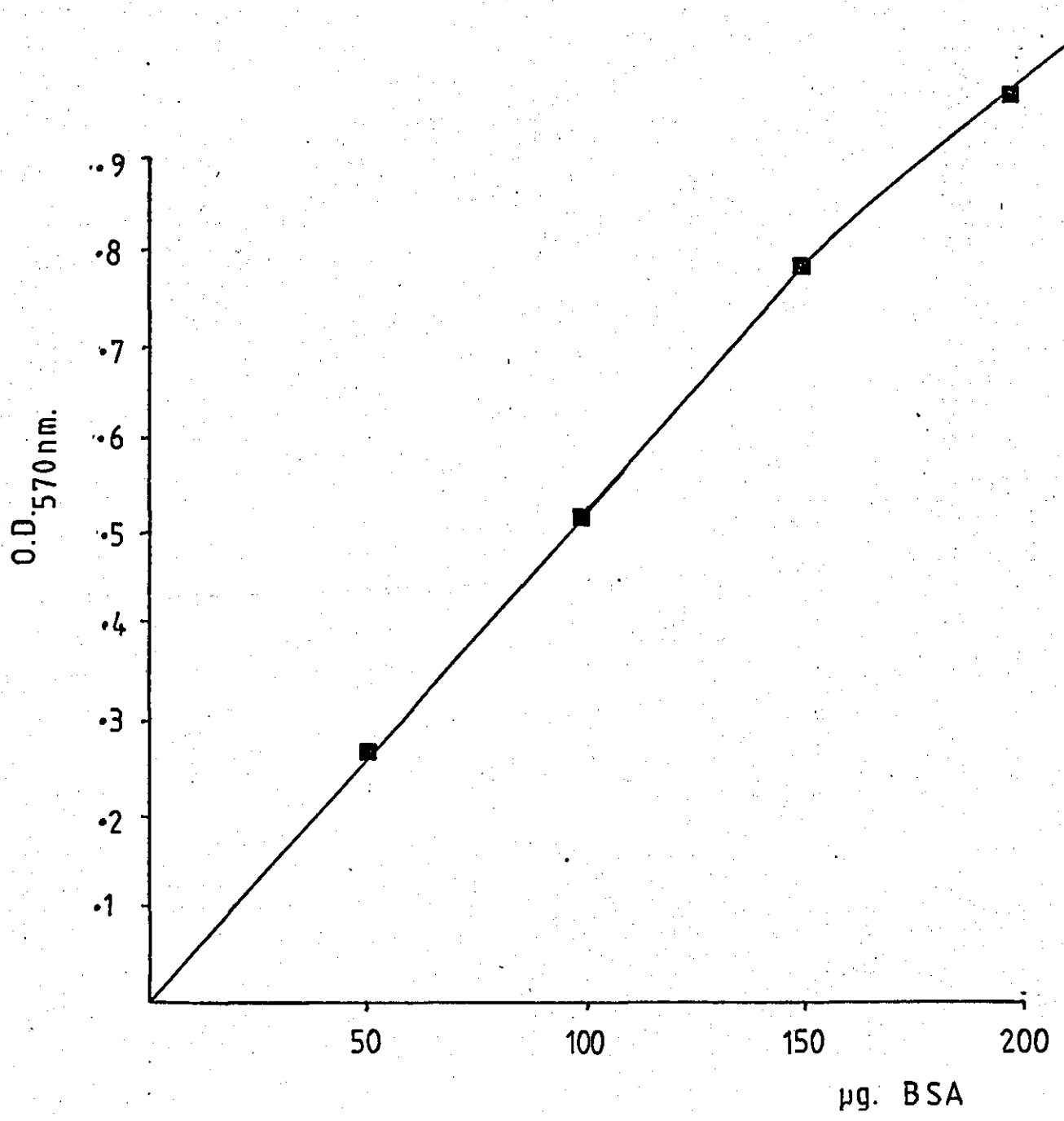

O.D.570nm.
·9
·8
·7
·6
·5
·4
·3
·2
·1
50
100
150
200
µg. BSA

FIGURE 2.2.

Phosphorus determination by the method of Raheja et al., 1973.

The method involves heating isolated phospholipids with a chromogenic solution and reading the absorbance of the resulting coloured complex at 710 nm.

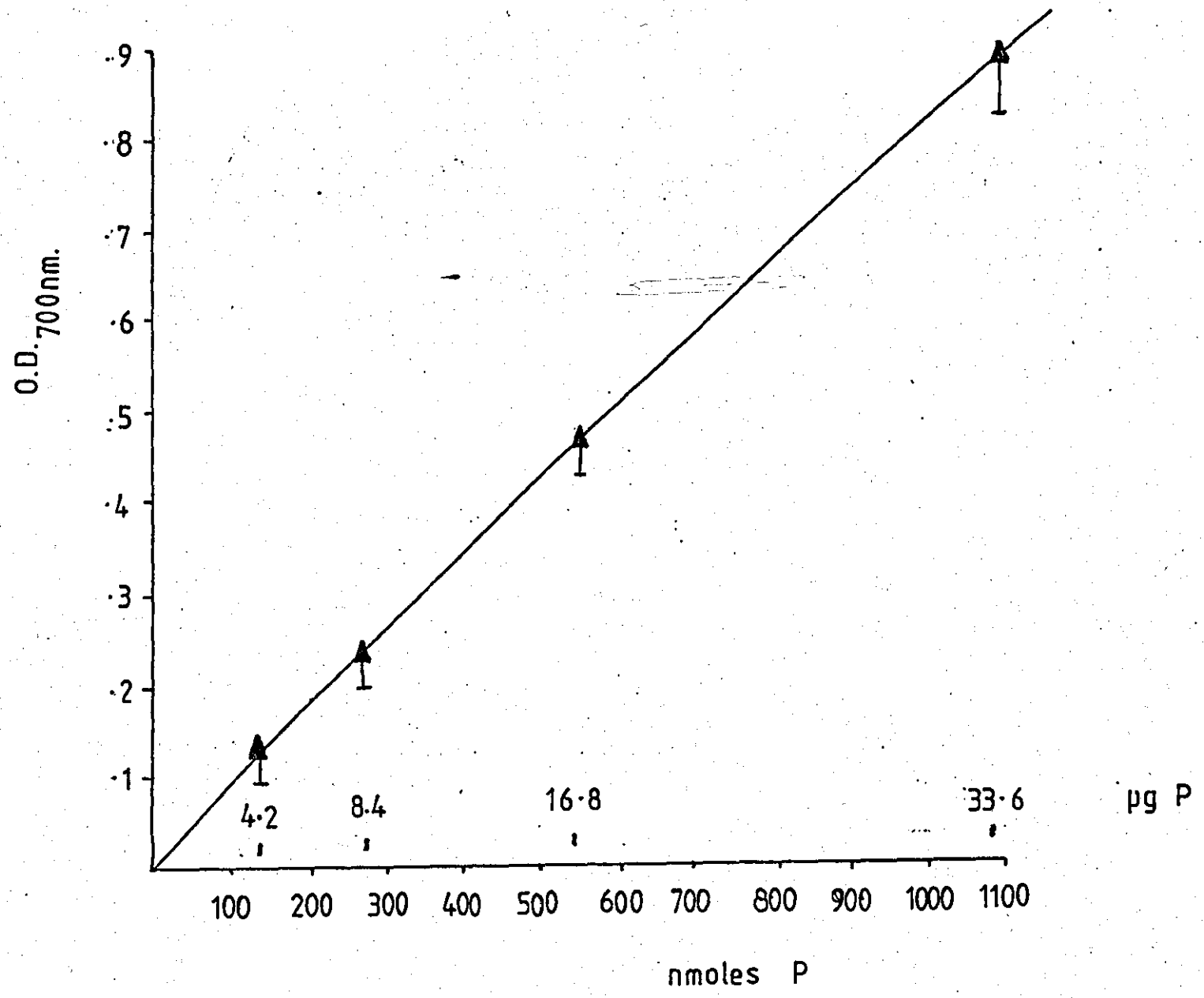
Chromogenic solution

16 g of ammonium molybdate is dissolved in 120 ml of water to give solution I. 40 ml of concentrated HCl and 10 ml of mercury are shaken with 80 ml of solution I for 30 min to give, after filtration, solution II. 200 ml of concentrated H_2SO_4 is added carefully to the remainder of solution I. To the resultant solution is added solution II to give solution III. 45 ml of methanol, 5 ml of chloroform, and 20 ml of water are added to 25 ml of solution III to give the chromogenic solution.

Method

0.1 ml of the chromogenic solution is added to 0.4 ml of chloroform containing 1-10 µg P, in a glass tube. The tube is placed in a boiling water bath for 1-1.5 min. After cooling to room temperature 5 ml of chloroform are added and shaken gently. The absorbance at 710 nm is read after 30 mins.

Points represent average ± S.D. of 4 determinations.


O.D.700nm.
·9
·8
·7
·6
·5
·4
·3
·2
·1
4·2
8.4
16·8
33·6
µg P
100
200
300
400
500
600
700
800
900
1000
1100
nmoles P

A relationship between cell numbers and phospholipid content was evident.

Bacterial cell number	PHOSPHOLIPID DETERMINATION	
	Absorbance 710nm	Nanomoles Phospholipid
1×10^{10}	0.192 ± 0.01 (3)	203
5×10^{9}	0.1 ± 0.02 (3)	100

The number of determinations are shown in the parenthesis.

(iv) Estimation of endogenous calcium in rabbit peritoneal fluid.

This was carried out using an EEL Flame Photometer fitted with a calcium filter. The instrument was calibrated using 0 - 75 parts per million calcium nitrate dissolved in triple distilled water (5.8920 g/l Ca $(NO_3)_2$ 4 H_2O is equivalent to 1000ppm).

(v) Partition coefficients (Log P values) of anti - malarial drugs.

Partition coefficients of chloroquine, mepacrine and primaquine were determined between chloroform and 40mM tris - malic acid buffer pH 6.0 as the aqueous system. Tris - malic acid buffer was used as this was the medium used in phospholipase enzyme assays. 3ml of a 0.01mM solution of antimalarial drug was whirlimixed for 5 minutes with 3ml chloroform. The aqueous phase was seperated from the organic phase by mild centrifugation and the optical density was measured at the appropriate λ max (chloroquine, 343nm : primaquine, 260nm : mepacrine, 281).

Log P values were calculated from the concentration of drugs partitioned between the organic and aqueous phases. Preliminary experiments showed that at the concentrations of antimalarial drugs

used there was a linear relationship between concentration of drug and optical density at the appropriate λmax.

$$\text{Log } P = \log \frac{\text{O.D. chloroform}}{\text{O.D. aqueous.}}$$

PART B COLLECTION OF BIOLOGICAL SAMPLES

(i) Collection of polymorphonuclear leucocytes (neutrophils) from rabbits

Female rabbits of the New Zealand white strain weighing from 2 to 3kg were used. 100ml sterile thioglycollate medium (USP) was injected intraperitoneally through a 0.8mm external diameter stainless steel needle while the rabbit was under light anaesthesia with ether. After 17 to 22hr the rabbit was anaesthetised with 80mg/kg sodium pent - obarbitone intraven ously and 200ml of sterile 0.15M sodium chloride solution containing heparin (4 in. /ml) was injected intraperitoneally. After lightly massaging the abdomen the fluid was allowed to drain th - rough a multi - perforated 2.5 inch 16 gauge plastic catheter.

The cells in the peritoneal fluid (10^8 - 10^9) were sedimented by centrifugation in plastic tubes at 200g for 6 min at 22°C. The sedimented cells were washed twice by resuspension and recentrifu - gation at 200g for 6 min at 4°C in HEPES buffered medium. Any contamina - ting erythrocytes which were occasionally present in the peritoneal fluid were lysed by resuspending the cells in 0.9% ammonium chloride pH 7.2 for 5 minutes at 22°C (Henson, 1971) and then washing the remaining neu - trophils in HEPES buffered medium. The resulting cells were 95% neutrophils as determined by differential cell counts. The cell concentration was adjusted to 1×10^7 cells ml^{-1}.

(ii) Collection of rabbit cell - free inflammatory peritoneal exudate

The inflammatory peritoneal fluid was collected, after induction of a sterile peritonitis in female New Zealand white rabbits, exactly as described above. The cells in the fluid (95% neutrophils) were sedimented by centrifugation at 3000g for 6 minutes and the supernatant was used as a source of phospholipase A activity without further purification.

SECTION III EXPERIMENTAL METHODS

PART A PHOSPHOLIPASE SUBSTRATE

1.0 Preparation of labelled substrate for determination of phospholipase A_2 activity

Phospholipids of E.coli were labelled with (1 - ^{14}C) oleic acid using a modification of the method described by Patriaca, Beckerdite and Elsbach, (1972). E.coli was grown in triethanolamine medium overnight at 37°C. Aliquots of the overnight culture were diluted ten - fold with fresh medium and further incubated with shaking for 2.5 hrs at 37°C. The bacteria in 30mls of culture (approximately 2 - 3 x 10^{10} cells) were sedimented by centrifugation at 3000g and resuspended in 10ml of fresh triethanolamine medium. To this was added 0.4ml of a 10% BSA solution in triethanolamine to which had been complexed 2.5 μCi of (1 - ^{14}C) oleic acid (0.2 μmoles) by incubation with shaking at 37°C for 30 minutes. This mixture was then incubated for 3hrs at 37°C with shaking to allow the label to be taken up by the bacteria.

Following this labelling period the cells were sedimented by centrifugation at 3000g for 15 minutes, washed in fresh media, recentrifuged and again resuspended in 10.4 ml triethanolamine media containing 0.4ml 10% BSA, without any complexed fatty acid. This was further incubated for 1.75 to 2hrs to allow the label to become fully incorporated into cellular phospholipids. After this period the cells were again collected, washed once with saline and then resuspended in 5ml saline.

The radiolabelled E.coli were autoclaved for 15 minutes at 120°C and 2.7 Kg/cm^2 pressure. This procedure destroys bacterial pho - spholipases but does not destroy the membrane phospholipids, and these were then used as the substrate for phospholipase A_2 assays.

Procedures using (1-^{14}C) linoleic and (1-^{14}C) palmitic acids were also performed.

1.1 Determination of the position of the (1 - ^{14}C) oleic acid label in E.coli phospholipids

This was carried out by the complete hydrolysis of E.coli phospholipids using a specific phospholipase A_2 present in the snake venom Crotalus adamenteus (van Deenan and De Haas, 1966). The assay mixture contained 5 x 10^8 autoclaved radiolabelled E.coli (representing 10 nanomoles of phospholipid), 5mM Ca^{2+} 40mM tris - HCl buffer, pH 8.0 and 0.5mg Crotalus adamenteus venom in a total volume of 0.5ml. The mixture was incubated at 25°C with shaking for 2hrs. The reaction was stopped by the addition of 3ml chloroform / methanol (1:2) and the lipids were extracted by the method of Bligh and Dyer (1959) and quantified as previously described.

PART B STUDY OF INFLAMMATORY PHOSPHOLIPASES

2.1 Release and properties of a phospholipase A_2 from rabbit polymorphonuclear leucocytes (neutrophils)

The release of phospholipase from neutrophils was exa - mined using two types of stimuli ; a phagocytosable particle zymosan

with bound complement (zymosan - complement) (Henson, 1971), and the soluble stimulus calcium ions (Northover, 1977).

Neutrophils were collected from rabbits as described earlier (page 44).

Zymosan - complement (ZC) was prepared by incubating zymosan previously washed with HEPES buffer, with rabbit serum (10mg/ml) for 30 mins at 37°C in order to allow complement, in particular C3 to fix (Henson, 1971). The particles were then washed extensively with HEPES buffered medium at room tempreture.

Neutrophils (1×10^7 cells) in 1ml HEPES buffered medium were incubated with shaking at 37°C and either varying amounts of ZC (0 - 5mg) or Ca^{2+} (0 - 5mM final concentration) were added in a volume of 0.2ml. In control experiments 0.2ml HEPES buffered medium alone was added to the neutrophils. Incubations were carried out for 60 mins except when incubation time itself was varied, after which the tubes were cooled in melting ice. The cells were sedimented and the supernatants removed for assays of released contituents. The enzymes assayed were phospholipase A, the lysosomal enzymes β - glucuronidase and lysozyme, and the cyto - plasmic enzyme marker lactate dehydrogenase. Total release of enzymes were obtained by treatment of the cells with 0.2% Triton x -100 or by sonication (6μm for 3mins). Control experiments showed both treatments to be equivalent. Both treatments were necessary as Triton x - 100 is an inhibitor of phospholipase activity (Franson et al, 1974).

	Activity of sonicated preparation compared with 0.2% Triton prep. Triton preparation = 100%.
β - glucuronidase	98.2% ± 4.0 (6)
lysozyme	95.5% ± 5.0 (6)

Brackets indicate number of determinations.

In experiments using zymosan - complement the uptake of zymosan particles into the neutrophils was also determined using phase - contrast microscopy. The percentage cells containing particles and the number of particles per phagocytosis - positive cells, were dete - rmined by observing 100 cells.

Figure 2.3 summarises the procedure used.

Determination of enzyme activities

Phospholipase A activity in the supernatants was assayed using the E.coli labelled with (1 - ^{14}C) oleic acid. The reaction mixture contained 7.5×10^8 radiolabelled E.coli (representing approx - imately 15 nmoles of phospholipid), 40 mM tris - malic acid buffer pH 6.0 5×10^{-3}M Ca^{2+} and an appropriate aliquot of the supernatant. Total volume was 0.5ml and incubations were carried out for 60 mins at 37°C. Reactions were stopped by the addition of 3ml of chloroform / methanol (1:2) and the lipids analysed as described earlier.

This enzyme was also characterised with respect to pH

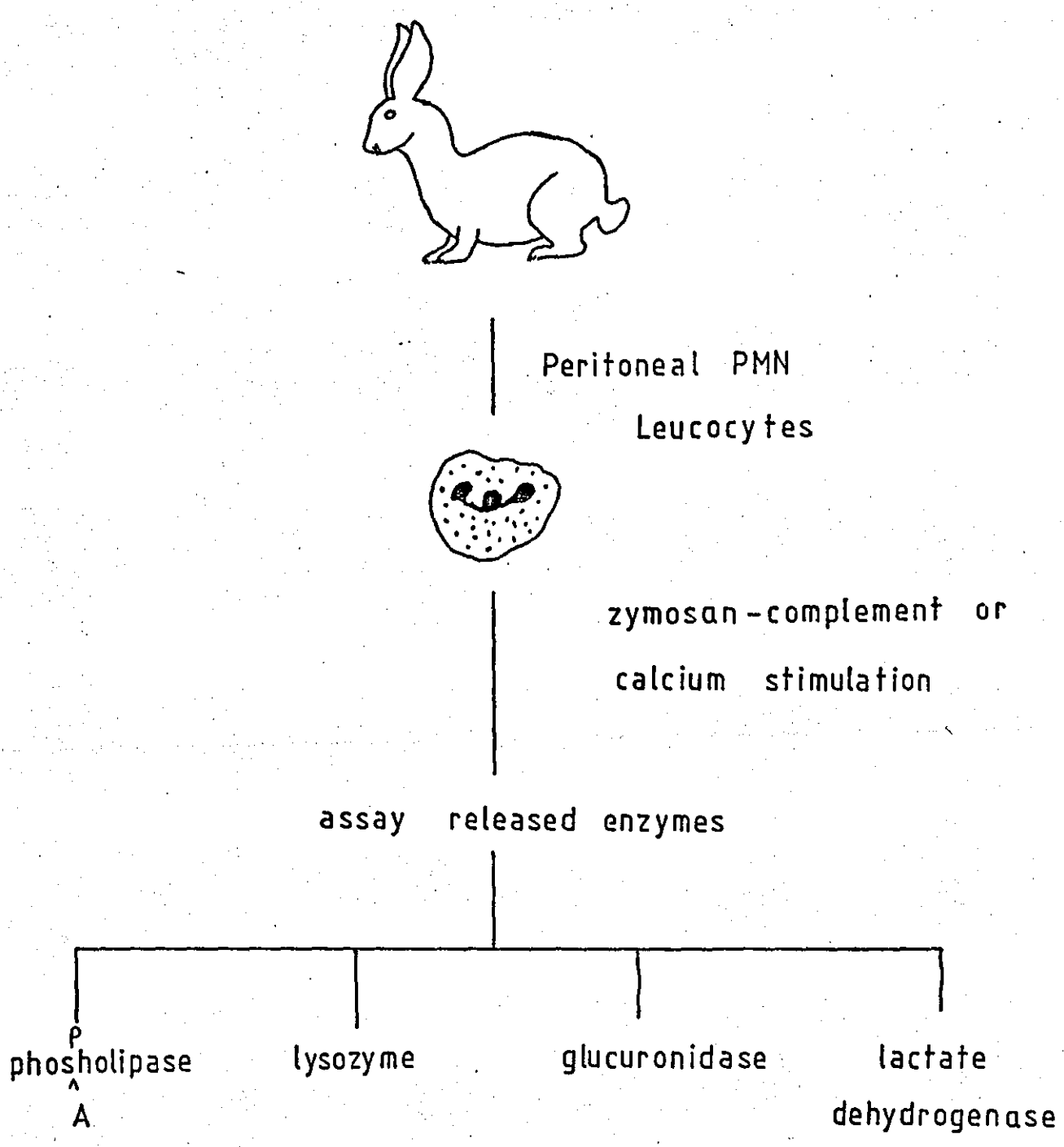


FIGURE 2.3

Outline of procedure for measurement of release of neutrophil constituents during stimulation.

profile and Ca^{2+} requirement, such parameters were varied as indicated.

β- Glucuronidase activity of the supernatants was carried out using the method of Fishman (1955). Briefly this involved measure - ment of the release of phenolphthalein from phenolphthalein glucuronide after 4hrs incubation at 56°C.

Lysozyme was determined by the rate of lysis of Micrococcus lysodeiktius measured by the decrease in absorbance at 510nm as carried out by Smolelis and Hartsell (1949). Incubations were carried out for 20 mins at 25°C.

The cytoplasmic enzyme marker lactate dehydrogenase was measured by the method of Wroblewski and La Due (1955). Pyruvate was converted to lactate in the presence of the reduced form of β - nicotin - aminde adenine dinucleotide (β- NADH) and the enzyme assay was monitored by the oxidation of NADH to NAD^+ at 366nm.

All enzyme assays were normally carried out in triplicate or at least in duplicate, and reactions were linear for the conditions employed. No enzyme blanks were always included and appropriate corrections made.

2.2 Properties of a phospholipase A present in the inflammed peritoneal exudate of the rabbit

Phospholipase A activity of the rabbit inflammatory peritoneal fluid (after removal of the cells by centrifugation, page 45)

was also characterised using radiolabelled E.coli. The incubation medium contained, unless stated otherwise, 40mM tris - buffer, 5mM Ca^{2+}, 0 - 1mg enzyme protein and 5×10^8 autoclaved radiolabelled E.coli, in a total volume of 0.5ml. Incubations were normally carried out for 5 mins at 37°C. Reactions were stopped and hydrolytic activity was determined as before (page 49). Various parameters were varied in order to characterise the enzyme activity.

3.0 PART C EFFECTS OF CHLOROQUINE - LIKE AGENTS ON INFLAMMATORY AND OTHER PHOSPHOLIPASES.

The effects of chloroquine - like agents were examined on phospholipases obtained from different sources. Preliminary work was carried out with a crude phospholipase A_2 enzyme from Crotolas adamenteus venom and a purified enzyme from pig pancreas, against egg - yolk phos - pholipids. Inflammatory phospholipases from rabbit peritoneal fluid and from peritoneal neutrophils were tested using the (1 - ^{14}C) oleic acid labelled E.coli as substrate.

3.1 Effects of chloroquine - like agents on phospholipase A_2 from Crotolas adamenteus venom and pig pancreas

Enzyme activity in these highly active sources was assessed by direct titration of the fatty acids released from egg - yolk phospholipids with time. The amount of alkali needed to keep the pH constant at 8.0 in the presence of phospholipase A_2, was recorded automatically using a Radiometer TTT - 1c Titrator equipped with a SBU - 1a syringe and a combined glass - calomel electrode. Rate of

hydrolysis was linear with time for at least 3 minutes after injection of enzyme.

Procedure

One egg - yolk was stirred to homogeneity in 100ml of distilled water for 10 minutes, and 20ml of a 160mM solution of calcium chloride added. The emulsion was then freed from the yolk bags by filtration through cheesecloth. This preparation was stable for up to 3 days.

In a reaction vessel maintained at 25°C, were added 6ml of egg - yolk emulsion containing Ca^{2+}, 1ml 20mM sodium deoxycholate and the volume was made up to 20ml with distilled water. The pH was brought up to 8.0 and the blank consumption of 10mM NaOH needed to maintain this pH was recorded. Then 10µl of a 1mg/ml solution of Crotalas adamenteus venom in water was added and the alkali consumption was again recorded. In this system all the fatty acids released were titrated with 100% efficiency (Nieuwenhuizen, Kunze and De Haas, 1974), so the number of micromoles of alkali used was equivalent to the number of micromoles of fatty acids released. The experiments were repeated in the presence of varying concentrations of chloroquine diphosphate.

Determinations using the enzyme from pig pancreas were carried out using an identical procedure but at 37°C. Drugs screened against this enzyme were chloroquine sulphate, dibucaine hydrochloride, cinchonine sulphate, salicylic acid and indomethacin.

Dose - response curves and ED_{50} values were determined using least squares analysis of data.

3.2 Effects of chloroquine - like agents on the activity of peritoneal fluid phospholipase A

Studies of the effects of the antimalarial drugs chloroquine, mepacrine and primaquine formed the major part of this work. The drugs were first screened on the peritoneal fluid phospholipase activity towards E.coli phospholipids since this was a more stable and a more active source of enzyme activity than the neutrophil source. Other agents tested were D - penicillamine, indomethacin, aspirin, salicylic acid and benzydamine.

Determinations of phospholipase activity in the presence and absence of drugs were carried out as those used to determine the properties of the peritoneal fluid enzyme (page 51).

3.3 Effects of chloroquine - like agents on the phospholipase activity of PMN leucocytes.

Studies of the effects of the antimalarial drugs chloroquine, mepacrine and primaquine on the phospholipase activities of PMN neutrophils was tested on cell suspensions that had been sonicated to release all of the cellular enzymes.

Leucocytes suspended in HEPES buffered medium at 4°C at a concentration of 7×10^6 cells ml^{-1} were sonicated at 6μ for 2 x 2 mins with 2 minutes separating each sonification period. The suspension was then centrifuged at 3000g for 10 mins at 4°C and the supernatant was used as a crude source of phospholipase activity. Incubations were

carried out using 10^9 (1 - ^{14}C) oleate labelled E.coli, 5mM Ca^{2+}, 40mM tris - malic acid buffer pH 6.5 and 0.1mg 3000g neutrophil supernatent for 30 mins at 37°C. Termination of assays and analysis of lipids was carried out as before.

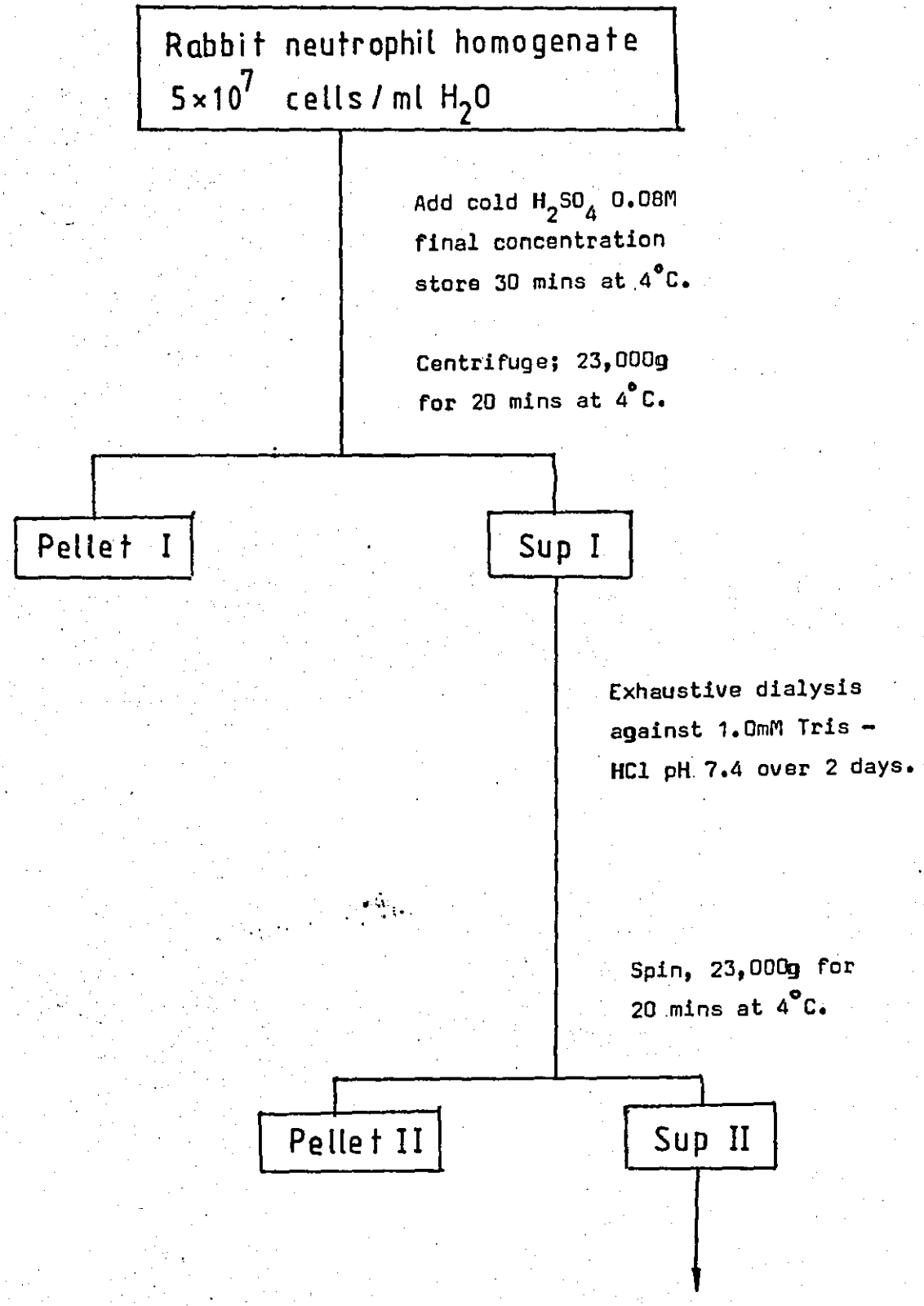
The effects of the antimalarial drugs were also tested after partial purification of the neutrophil phospholipase. The purifi - cation was carried out by the method of Weiss et al (1975) using sulph - uric acid to extract the enzyme as outlined in fig 2.4. Washed neutrophils were resuspended in distilled water (4°C) at a concentration of 5×10^7 cells/ml. Then ice - cold sulphuric acid was added to a final concentr - ation of 0.08M and the suspension was kept at 4°C for 30 mins. After this time a white precipitate was apparent and this was centrifuged at 23,000g for 20 mins. The supernatant (designated Sup I) was then exhaustively dialysed against 1mM tris - HCl buffer pH 7.4. The pellet (pellet I) was resuspended in an equivalent volume of distilled water (equal to the supernatant removed). After dialysis of Sup I a further precipitate formed and was again separated by centrifugation at 23,000g for 20 mins. The resulting supernatant (Sup II) was used as the partially purified preparation of phospholipase.

3.4 Interactions of heparin with antimalarial drugs

Interactions of heparin with the antimalarial drugs chloroquine, mepacrine and primaquine were examined by measuring optical densities at 700nm after mixing solutions of heparin (50 units/ml final concentration) with varying amounts of antimalarial drugs (0 - 5mM), in a total volume of 10ml. All three drugs showed very little absorbance alone at 700nm (less than 0.02 O.D. units) in the concentration ranges used.

FIGURE 2.4.

Partial purification of rabbit neutrophil phospholipase A.



3.5 Membrane actions of antimalarial drugs on guinea-pig red blood cells

The effects of antimalarial drugs on the haemolysis of guinea - pig red blood cells were examined. Such compounds are amphiphilic molecules and thus may exert their effects on enzymes such as phospholip - ases by membrane stabilizing or labelizing action.

Fresh heparinised whole guinea - pig blood was used. Firstly a 50% lysis figure was obtained using 0.05ml blood and 5ml of varying concentrations of sodium chloride. After exactly 30 mins incubation at room temperature the mixtures were centrifuged at 1000g for 5 mins. The haemoglobin content of the supernatant was measured at 543nm on a Unicam S.P. 500 spectrophotometer, and was used as a measure of cell lysis. Complete cell disruption was caused by incubation with distilled water.

50% Lysis was found at 0.425% NaCl. The experiments were then repeated in the presence of varying concentrations of anti - malarial drugs dissolved in 0.425% NaCl. Mepacrine and primaquine both showed absorbances at 543nm, and so optical densities of the haemoglobin content in the presence of these drugs were corrected by the use of appropriate blanks.

4.0 PART D STUDIES OF AN ENDOGENOUS INHIBITOR OF PHOSPHOLIPASE A_2 IN THE 8200g SUPERNATANT OF NEUTROPHIL LEUCOCYTES

4.1 Preparation and determination of inhibitory activity in the 8200g supernatant of neutrophil leucocytes

Neutrophil leucocytes were collected from New Zealand White rabbits as described earlier (page 44). The washed leucocytes were suspended in ice - cold 0.34M sucrose at a concentration of 1×10^8 cells/ml and stored at 4°C for 30mins. The cell suspension was then homogenised with a Potter - Elvejham type homogeniser until cell disruption was complete as monitored by phasecontrast microscopy. The homogenate was then subjected to centrifugation at 8,200g for 20 mins to separate the nuclei, cellular membranes and lysosomal granules from the cytosol. The 8,200g pellet was used as the granule phospholipase preparation and the 8,200g supernatant was used without further purification as the source of an endogenous inhibitor of phospholipase activity.

In this work the inhibitory activity towards the granule phospholipase at acid pH was examined and the 8,200g supernatant inhibitor was screened against the phospholipase A present in the rabbit peritoneal fluid exudate (PE) after the neutrophils had been sedimented out. Assays were again carried out using (1 - ^{14}C) oleate labelled E.coli as substrate (1.5×10^9 cells containing approximately 30nm of phospholipid), 5mM Ca^{2+}, 40mM tris buffer, 0.35mg peritoneal fluid or 0.5mg granule protein as the enzyme source, and varying amounts of 8,200g supernatant as the inhibitor. (0 - 400 µg protein) in a total volume of 0.5ml. Incubations were normally carried out for either 5 or 10 mins depending upon the amounts of enzyme

protein and inhibitor used.

A pH profile of the phospholipase inhibitory capacity of the 8,200g supernatant on the peritoneal fluid enzyme was also deter - mined. Tris - malic acid buffer was used for pHs between 5.5 and 7.5 and tris - HCl was used for pHs between 7.5 and 9.0.

4.2 Effects of the PMN 8,200g supernatant on the phospholipases from pig pancreas, Crotalus adamenteus venom and Naja naja venom

Inhibitory effects of the 8,200g supernatant fractions were also tested against commercially available phospholipases from different sources. These sources were pig pancreas and the snake venom s Crotalus adamenteus and Naja naja.

Incubations were carried out as before for 10 mins and at pH 7.5. Sufficient enzyme proteins were used to give approximately 20% hydrolysis of E.coli phopholipids as control values. Actual amounts of enzyme used to achieve this was 0.05ng protein for the pig pancreas enzyme, 1 μg protein for the venom Crotalus adamenteus and 0.25 μg protein for the venom Naja naja.

The possibility that inhibition of phospholipase A_2 activity by the 8,200g supernatant could be due to a non - specific protein effect was also examined as phenomena of this type had already been shown in the case of phospholipase of the liver (Weglicki et al, 1974). To examine this each of the 4 phospholipases examined were also assayed in the presence of an equivalent concentration of BSA.

Chapter Three

RESULTS

PART A PHOSPHOLIPASE A_2 SUBSTRATE

1.0 Radiolabelling of E.coli with (1 - ^{14}C) oleic acid

For adequate uptake of (1 - ^{14}C) oleic acid by E.coli very specific conditions were found to be necessary. These were:-

(i) The (1 - ^{14}C) fatty acid must be complexed with albumin prior to contact with the E.coli otherwise the radiolabel is not adequately taken up, as shown by the values obtained below:-

(a) Without prior complexing ie (1 - ^{14}C) oleic acid added together with BSA and E.coli and shaken vigorously.
Radioactivity (cpm) obtained per 5×10^8 cells = 1441 ± 207 (5 determinations)

(b) With prior complexing ie BSA + (1 - ^{14}C) oleic acid incubated at 37°C for 30 mins before adding E.coli.
Radioactivity (cpm) per 5×10^8 cells = 16882 ± 4634 (7 determinations)

(ii) The initial number of E.coli exposed to the radiolabel had an effect on the usefulness of the preparation as a substrate for phospholipase A_2 assays (see table below). An initial count of $2 - 3 \times 10^{10}$ organisms in log phase of growth per 10.4ml incubation mixture was found to be suitable as this provided approximately 5×10^{10} cells (after losses during washings in the procedure), with an adequate amount of radioactivity and sufficient for approximately 50 enzyme assays.

Initial bacterial count	Bacteria recovered at end of procedure	Radioactivity (cpm) per 5×10^8 cells
2.3×10^{10}	5×10^{10}	16882
5×10^9	1×10^{10}	14565
5×10^{10}	NT	7000

The use of smaller initial viable E.coli numbers (ie 5×10^9 cells) led to a smaller recovery of cells at the end of the procedure and therefore useful for a smaller number of PLA_2 assays. The uptake of radioactivity per 5×10^8 cells was adequate.

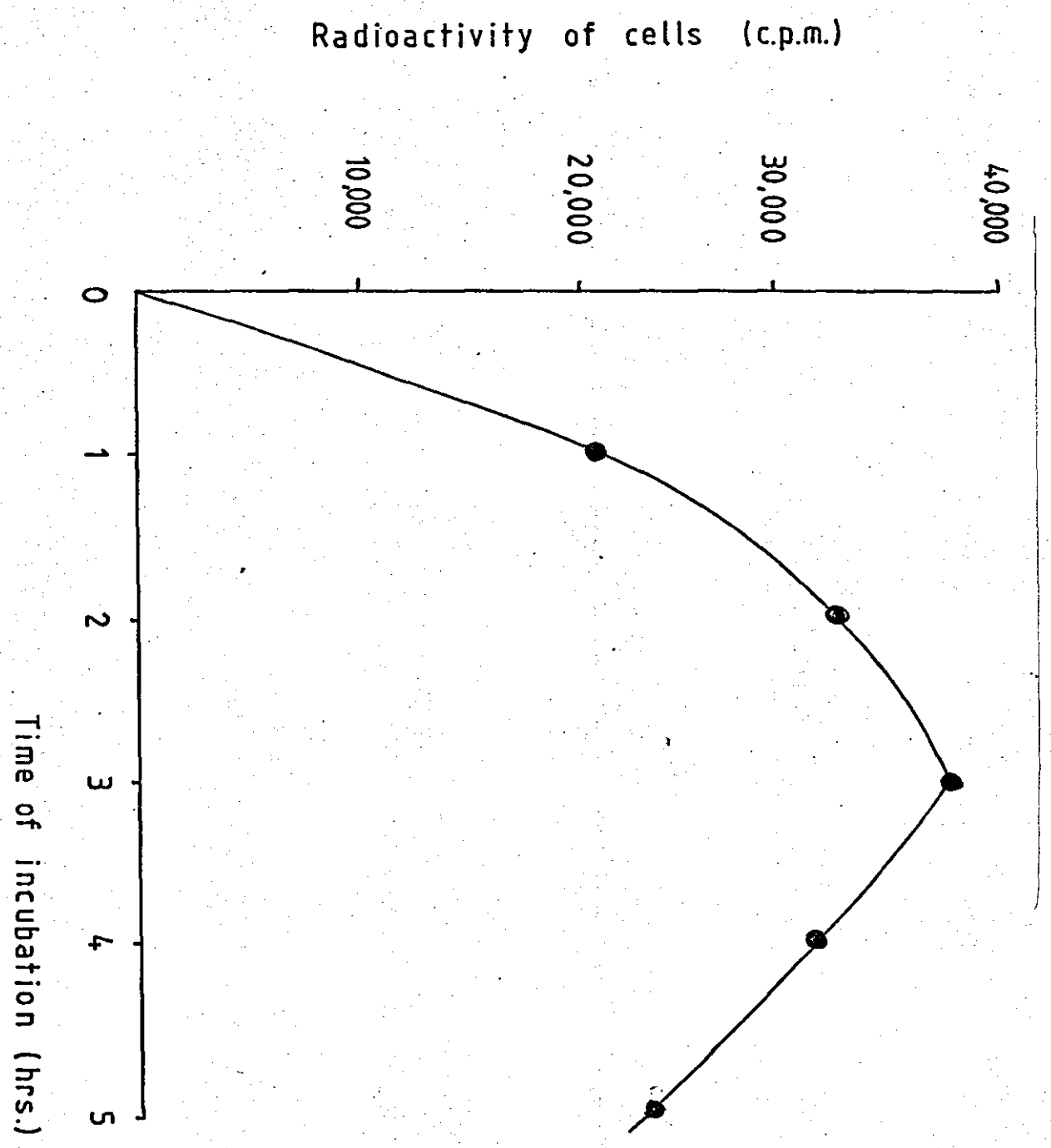
Conversely if the initial count was larger than 3×10^{10} organisms, ie 5×10^{10}, then although sufficient cell numbers were recovered at the end of the procedure, the uptake of radioactivity per cell decreased.

(iii) The time of the labelling period was also important. Figure 3.1 shows the uptake of the label into the cells during the labelling period. Uptake occurs very rapidly during the first hour of incubation and reaches a maximum after 3hrs. Thereafter the amount of radioactivity in the cells actually decreases.

(iv) A further 1.75 - 2hr incubation after the labelling period in the absence of any further (1 - ^{14}C) oleic acid was necessary to acheive maximum incorporation of the fatty acid into cellular phospholipids. Examination of the distribution of radioactivity after the labelling period revealed 53% $\pm$ 7.5 (4 determinations) to be within E.coli phospholipids (phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin) and 47% $\pm$ 7.5 (4 determinations) still withinfatty acid.

FIGURE 3.1.

Uptake of radioactivity, (1 - ^{14}C) oleic acid, into E.coli W during the labelling period. 0.2 ml of culture was taken and the cells were seperated by cent - rifugation and then counted by liquid scintillation counting.
Points represent mean of duplicates.


Radioactivity of cells (c.p.m.)
10,000
20,000
30,000
40,000
0
1
2
3
4
5
Time of incubation (hrs.)

The further incubation period improved the distribution of radioactivity to approximately 94% in phospholipids (see below).

The (1 -^{14}C) oleate labelled E.coli was autoclaved so that bact - erial phospholipases were destroyed and the procedure also rendered the membrane phospholipids more accessible to attack by phospholipases. (Fransen et al, 1973).

1.1 Distribution of label among lipids of E.coli

Reproducibly 70% of the total radioactivity present in E.coli was extracted by the method of Bligh and Dyer (1959) (table 3.1). Further lipid extractions did not improve this figure suggesting that approximately 30% of the total radioactivity present in E.coli was contained in non - extractable lipid or non - lipid molecules.

Separation of the extracted lipids by TLC revealed the distribution of label shown in table 3.1. Greater than 94% of the total recoverable lipid radioactivity was associated with phospholipids. The most heavily labelled components were phophatidyl ethanolamine ≥ phosphatidyl glycerol ≥ cardiolipin, these are also the major phosp - holipids of E.coli (Ames, 1968, Wurster et al, 1971). The autoclaving procedure did not appear to destroy the phospholipids since the distri - bution of radioactivity closely resembled the composition of lipids of E.coli.

1.2 Position of (1 -^{14}C) oleic acid in E.coli phospholipids

Hydrolysis with the specific phospholipase A_2 of Crotalas adamenteus venom resulted in a large increase of radioactivity

TABLE 3.1

DISTRIBUTION OF (1-^{14}C) OLEATE AMONG LIPIDS OF E.COLI

LIPID	PERCENTAGE COMPOSITION OF E.COLI PHOSPHOLIPIDS*	% RADIOACTIVITY	
PHOSPHOLIPIDS			
PHOSPHATIDYL ETHANOLAMINE	69	59 ± 4 (4)	94%
PHOSPHATIDYL GLYCEROL	19	19.5 ± 1.5 (4)	
CARDIOLIPIN	6.5	10 ± 1.0 (4)	
PHOSPHATIDIC ACID		3.5 ± 0.3 (4)	
LYSOPHOSPHOLIPIDS		2.0 ± 0.14 (4)	
NEUTRAL LIPIDS			
FATTY ACIDS		3.5 ± 0.3 (4)	6%
TRIGLYCERIDES		1.0 ± 0.1 (4)	
DIGLYCERIDES		1.5 ± 0.11 (4)	

The values shown are obtained after extraction of 10^9 cells Total extracted count = 15456 ± 479.9 (11)dpm Original E.coli radioactivity = 22089 ± 558(5) dpm. Therefore 69.9% ± 4 of the total radioactivity is extracted by the method of Bligh and Dyer (1959). Figures in brackets indicate the number of determinations. *Ames (1968).

RF values of lipids (solvent system chloroform/methanol/HAc). Fatty acid 1.0, Cardiolipin 0.8 - 0.85., PG 0.66., PE 0.4 - 0.45., Lyso PE 0.1 - 0.15. Phosphatidic acid 0.9. Also P.T.O.

Rf values in solvent system pet. ether / diethyl ether / formic acid.

Triglycerides 0.7., Fatty acids 0.5., Diglycerides 0.35.,

Phospholipids 0.0.

TABLE 3.2

HYDROLYSIS OF OLEATE LABELLED PHOSPHOLIPIDS OF E.COLI BY A SPECIFIC PHOSPHOLIPASE A_2 FROM THE SNAKE VENOM OF CROTALUS ADAMENTEUS

FRACTION	% RADIOACTIVITY		
	E.COLI ALONE	+ SNAKE VENOM	DIFFERENCE
PHOSPHATIDYLETHANOLAMINE, PHOPHATIDYLGLYCEROL AND CARDIOLIPIN	90.3% ± 1.6 (3)	8.9% ± 0.9 (3)	-81.4%
FATTY ACID	6.2 ± 0.45 (3)	85.3% ± 1.8 (3)	+79.1%
LYSOPHOSPHATIDYL - ETHANOLAMINE	3.5 ± 2.05 (3)	5.8% ± 2.8 (3)	+2.3%

97% of the enzymically hydrolysed radioactivity appeared in the fatty acid fraction. The number of determinations are given in brackets.

Reaction mixtures contained 5×10^8 autoclaved labelled E.coli, 5mM Ca^{2+}, 40mM tris - HCl buffer pH 8.0 and with or without 0.5mg Crotalus adamenteus venom. Incubations were carried out for 2hrs at 25°C.

TABLE 3.3

INCORPORATION OF RADIOLABELLED FATTY ACIDS IN E.COLI PHOSPHOLIPIDS

FATTY ACID	NATURE OF FATTY ACID	% INCORPORATION INTO POSITION :- 1	2
PALMITIC ACID	C_{16} SATURATED	77	23
OLEIC ACID	C_{18} 1 DOUBLE BOND	3	97
LINOLEIC	C_{18} 2 DOUBLE BONDS	13	87

Percentage incorporation was determined using a specific phospholipase A_2 of Crotalus adamenteus venom.

in the fatty acid fraction with only a small increase in the lysophos - pholipid fraction (table 3.2). The results demonstrate that 97% of the (1 - ^{14}C) oleic acid was in position 2 of the phospholipids.

Procedures using (1 - ^{14}C) linoleic acid resulted in less labelling of the 2 position (87% in pos^{n} 2 of phospholipids).

Use of (1 - ^{14}C) palmitic acid resulted in 77% of the fatty acid incorporated into position 1 and 23% in position 2 of the bacterial phospholipids. Table 3.3 shows a comparison of these values.

Routinely (1 - ^{14}C) oleate labelled E.coli were used.

PART B STUDY OF INFLAMMATORY PHOSPHOLIPASES

2.0 Release and properties of a phospholipase A_2 from rabbit polymorphonuclear leucocytes (neutrophils)

2.1 Release of phospholipase A_2 during phagocytosis of zymosan - complement particles

Neutrophil leucocytes incubated with zymosan - complement (ZC) particles, at 37°C over 60 mins, showed uptake of the particles and release of granule enzymes known to be markers for lysosomes.

Phospholipase A_2 release (fig. 3.2) was shown to be :- (i) concentration dependant, (ii) linear up to 0.5mg ZC, (iii) reach a maximum of approximately 30% at 5mgZC and (iv) follow the release of the lysosomal marker enzymes β - glucuronidase and lysozyme. The cytoplasmic enzyme lactic dehydrogenase was released only to a minor extent during the reaction. This demonstrated that release of phospholipase along with β - glucuronidase and lysozyme was specific and not due to cell break - down as this would also release the enzyme lactate dehydrogenase to a comparable extent.

The release of phospholipase A_2 activity was paralleled by the uptake of zymosan - complement particles as shown in table 3.4. As phospholipase activity was released, the percentage of cells containing zymosan particles increased and also the number of particles ingested by each cell increased.

FIGURE 3.2.

Release of enzyme activities from neutrophils by zymosan - complement particles.
Neutrophils were incubated in HEPES buffered medium in the presence of varying amounts of ZC. After 60 minutes at 37°C aliquots of the suspending medium were taken for enzyme assays as described in Materials and methods.

circles = β - glucuronidase
triangle = phospholipase A
crosses = lactate dehydrogenase.

Vertical bars represent 1 S.E.

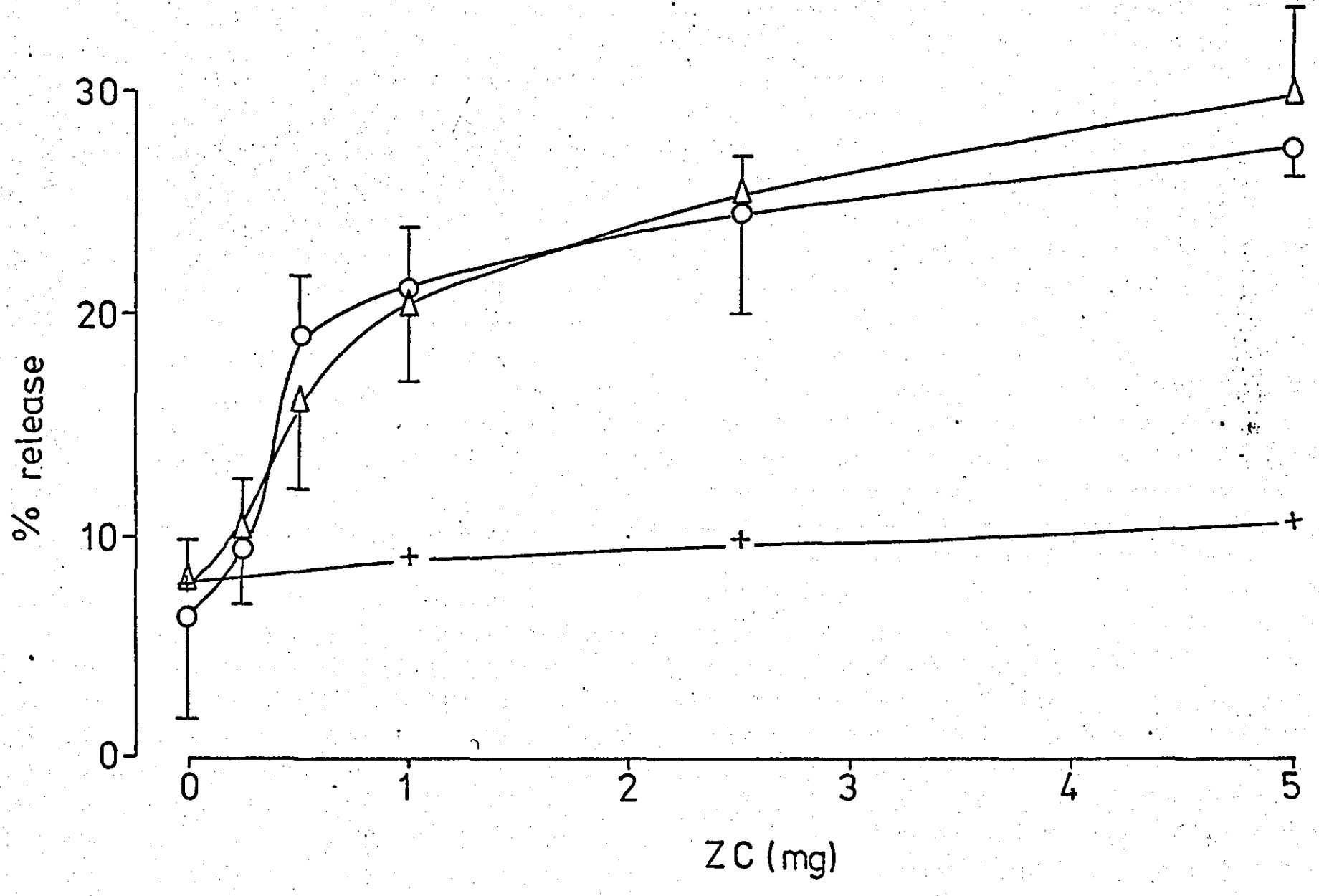

30
20
10
0
% release
0
1
2
3
4
5
ZC (mg)

TABLE 3.4

RELEASE OF PHOSPHOLIPASE A_2 AND PHAGOCYTOSIS OF ZYMOSAM - COMPLEMENT PARTICLES (ZC)

ZC (mg)	PLA_2 RELEASE (%)	PHAGOCYTOSIS - POSITIVE CELLS (%)	No. OF PARTICLES PER POSITIVE CELL (%)
0	8.3 ± 2.0 (3)	0	0
0.25	10.1 ± 2.8 (3)	53	1.4 ± 0.5
0.5	16.0 ± 4.0 (3)	62	2.0 ± 1.2
1.0	20.3 ± 4.8 (3)	83	2.4 ± 1.7
2.5	25.0 ± 2.0 (3)	85	2.8 ± 1.2
5.0	30.0 ± 4.0 (3)	96	3.2 ± 1.5

Brackets indicate the number of determinations.

Phagocytosis - positive cells and no. of particles per positive cell were determined by counting 100 cells.

Neutrophil leucocytes (1×10^7) were incubated in the presence of varying amounts of ZC for 1hr at 37°C. Phospholipase A activity at pH 6.0 was assayed as described in Materials and Methods.

FIGURE 3.3.

Time dependancy of neutrophil enzyme release with zymosan - complement.

Neutrophils were incubated in HEPES buffered medium containing 2.5mg ZC at 37°C. Aliquots of the suspending medium were taken for enzyme assays (see text) at times shown.

circles = β - glucuronidase

triangles = phospholipase A

squares = lysozyme.

Vertical bars represent 1 S.E.

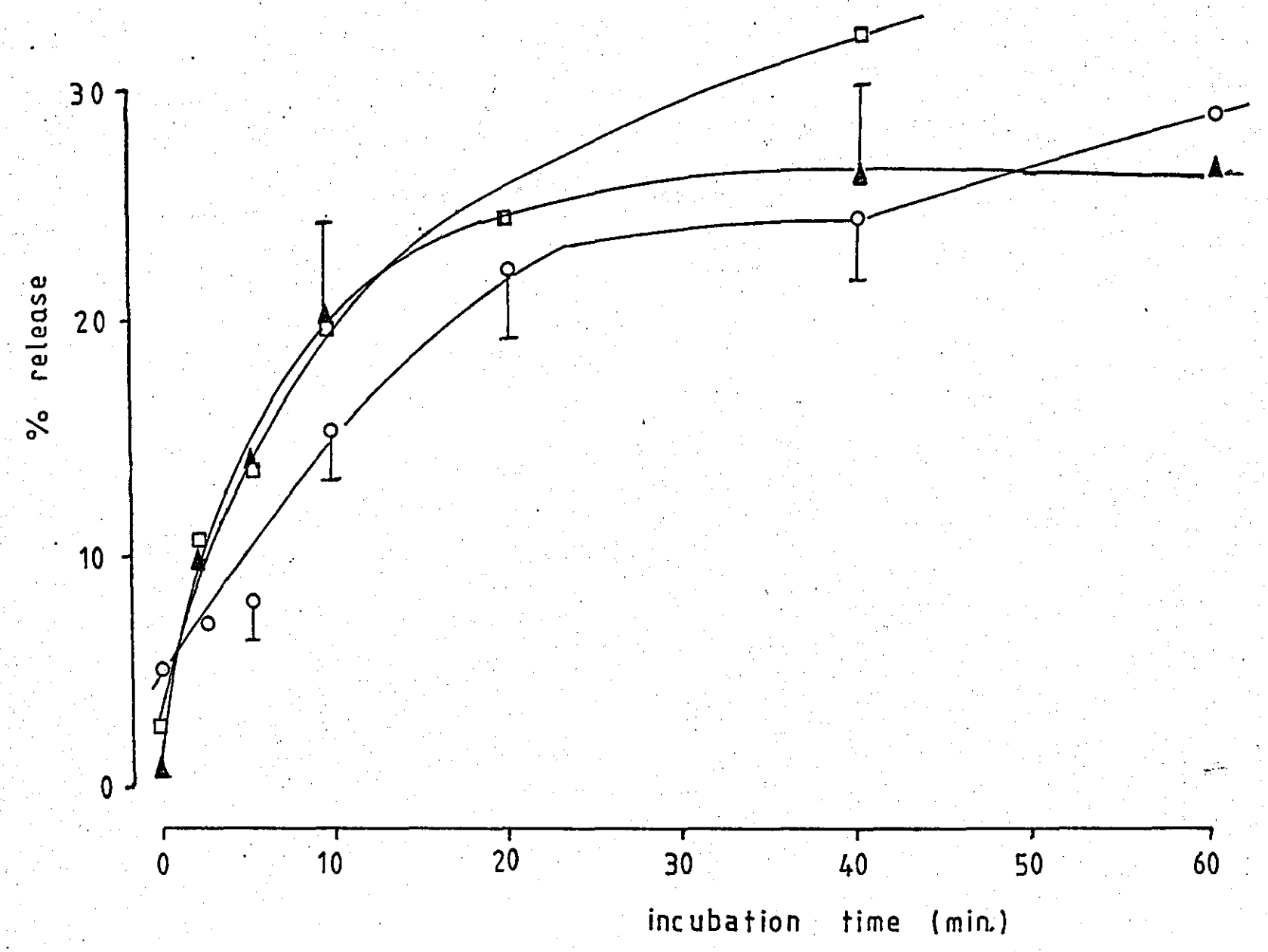

30
20
10
0
% release
0
10
20
30
40
50
60
incubation time (min.)

TABLE 3.5

TIME DEPENDENT RELEASE OF PHOSPHOLIPASE A AND PHAGOCYTOSIS OF ZYMOSAN – COMPLEMENT PARTICLES (ZC)

TIME	PHOSPHOLIPASE A RELEASE (%)	PHAGOCYTOSIS – POSITIVE CELLS (%)
0	1.0 (0, 2.0)	0
3	10.3 ± 3.0 (3)	33
5	14.8 (12.6,17.0)	39
10	20.7 ± 4.7 (3)	58
40	26.0 ± 4.0 (3)	84

Single figures are means of duplicate determinations (given in brackets). Figures including standard deviations state number of determinations in brackets. 100 cells were counted to determine phagocytosis – positive cells. Neutrophil leucocytes (1×10^7) were incubated with zymosan – complement (2.5mg) for varying times at 37°C. Phospholipase A activity was assayed as described in Materials and Methods.

FIGURE 3.4.

Release of neutrophil enzymes in the presence of Ca^{2+}.

Neutrophils were incubated in HEPES buffered medium in the presence of varying amounts of Ca^{2+}. After 60 mins. at 37°C aliquots of the supernatant were examined for enzyme release as described in text.

circles = β - glucuronidase
triangles = phospholipase A
squares = lysozyme
crosses = lactate dehydrogenase.

Points represent means of duplicate determinations.

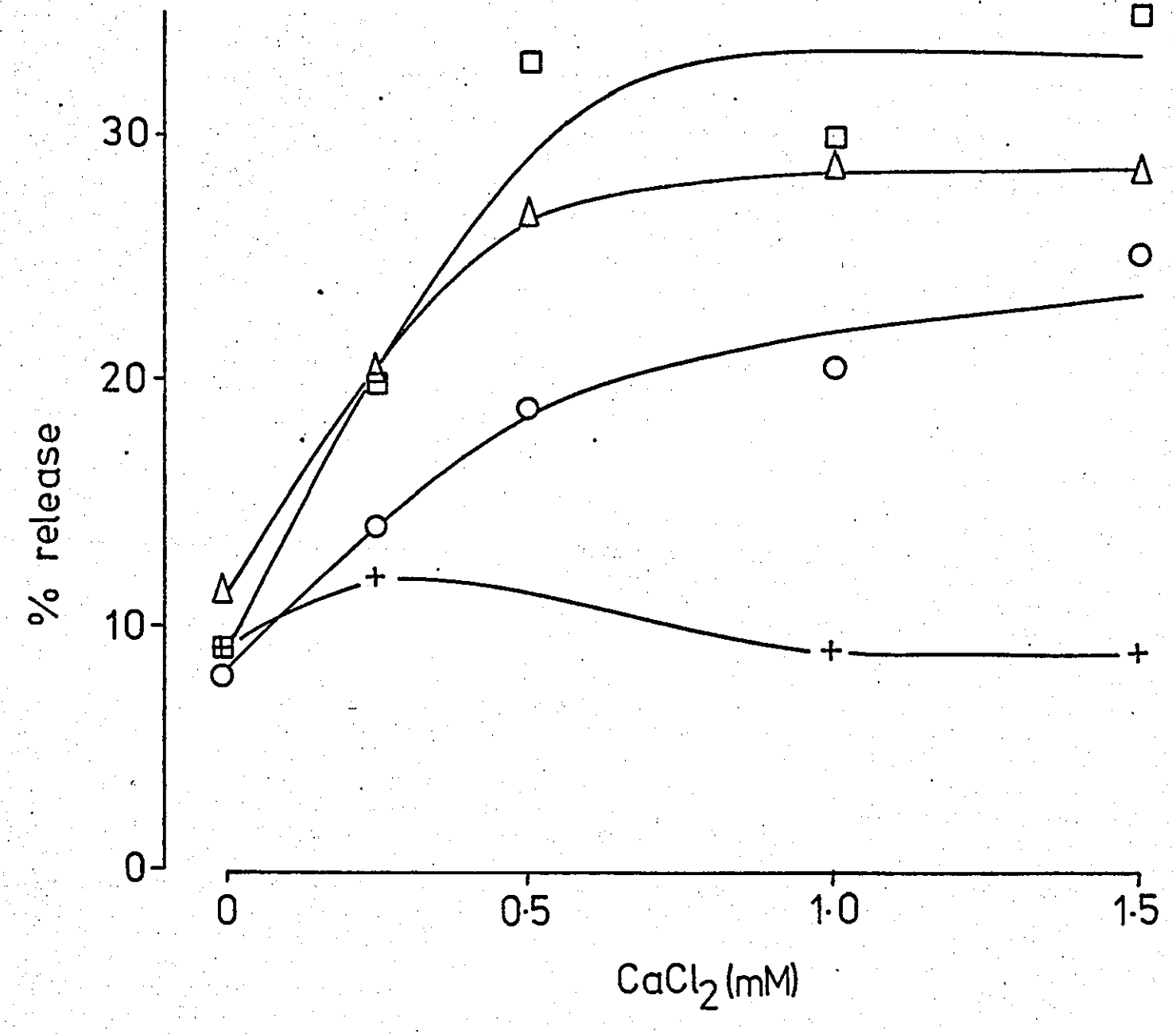

% release
30
20
10
0
0
0·5
1·0
1·5
CaCl2 (mM)

FIGURE 3.5.

Time dependancy of neutrophil enzyme release with Ca^{2+}.

Neutrophils were incubated in HEPES buffered medium containing 2.5mM Ca^{2+} at 37°C. Aliquots of the suspending medium were taken for enzyme assays (see text) at times shown.

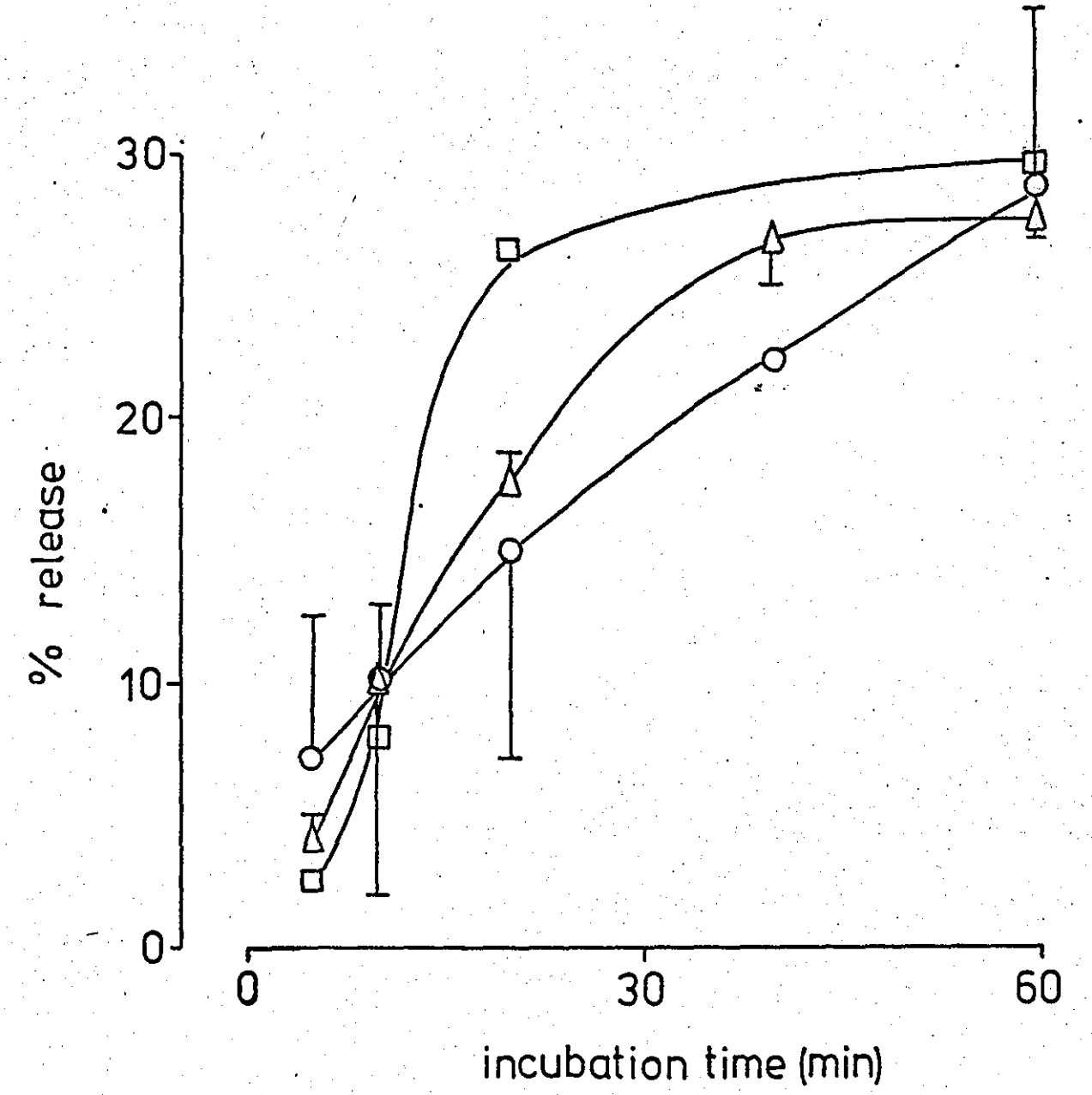
circles = β - glucuronidase

triangles = phospholipase A

squares = lysozyme.

Vertical bars represent 1 S.E. ($n = 3$).

Other points are means of duplicate experiments.


30
20
10
0
% release
0
30
60
incubation time (min)

The secretion of lysosomal constituents was also time dependant. Release of enzymes was rapid during the first 10 mins and then rose slowly reaching approximately 30% by 1 hour (figure 3.3). Lysozyme and β - glucuronidase showed similar release patterns to phos - pholipase A.

The degree of phagocytosis (table 3.5) with time again showed a similar response demonstrating that release of phospholipase activity occured during phagocytosis.

2.2 Release of phospholipase by calcium ions

Addition of calcium ions to neutrophil leucocytes (which were previously washed in a calcium free suspending medium) and incubation at 37°C for 1 hour also caused the release of phospholipase A_2 from the cells. The release of this enzyme, (i) followed the release of β - glucuronidase and lysozyme but not of lactate dehydrogenase, (ii) was concentration dependant, 2.5×10^{-3}M Ca^{2+} being sufficient to cause maximal release of over 30% (figure 3.4) and (iii) time dependant as shown in figure 3.5.

2.3 Properties of the zymosan - complement released phospholipase A_2 of neutrophil leucocytes

The properties of the enzyme obtained from zymosan - complement release experiments were examined.

(i) Positional specificity

The enzyme caused release of (1 - ^{14}C) oleic acid from the 2 - position of membrane phospholipids of E.coli. All of the released radioactivity was recovered in the fatty acid fraction and nonein the lysophospholipids (figure 3.6). This suggested that the enzyme was of A_2 specificity. These determinations were carried out at pH 9.0.

Furthur evidence for the A_2 specificity of neutrophil phospholipase was obtained using sonicated neutrophils and (1 - ^{14}C) palmitic acid labelled E.coli. 77% of the radiolabel in this case is present in position 1 of E.coli phospholipids as determined by hydolysis with Crotalus adamenteus venom phospholipase A_2 (page 66). The results obtained are shown in table 3.6. The blank showed a non - enzymic hydr - olysis of 2.4% over 1 hour. The sonicated enzyme preparation showed 52.9% hydrolysis of which 20% of the radioactivity appeared in the fatty acid fraction and 80% in the lysophospholipid fraction. As 77% of the palmitic acid label was present in position 1 of E.coli phos - pholipids then it appears that the neutrophil phospholipase activity is exclusively A_2.

(ii) pH profile

A pH profile suggested the presence of two pH optima as shown by figure 3.7. The enzyme had a broad acidic pH optimum covering the range pH 6 -6.5 and an alkaline peak at pH 9.0. The activity at pH 7.5 was reproducibly lower than that obtained at pH 6.0 or 9.0.

(iii) Calcium requirement

The released phospholipase activity had an absolute

requirement for calcium ions at both pH optima, reaching maximal activity at 5mM (figures 3.8. and 3.9.). In the absence of added ca^{2+} less than half of the maximal activity was attained. This residual activity sugg - ested the presence of endogenous calcium. Indeed addition of 5mM ethylene diamine tetra acetic acid (EDTA) totally abolished all activity (figure 3.9).

(iv) Kinetics

Figure 3.10 and 3.6 examine the effects of protein and time respectively on the enzymic hydrolysis of E.coli phospholipids. Enzymic activity was linear up to 64 μg protein and up to 40 minutes.

The effect of substrate was examined at concentrations varying from 0 -20 μM ... E.coli phospholipid. Using 96 μg enzyme protein (figure 3.11) the hydrolysis reached a plateau at 20 μM ... of phos - pholipid. Lower amounts of protein were generally used to allow substrate concentrations to be at saturating levels. Figure 3.12 shows the double reciprocal plot of data expressed in figure 3.11. The results show an apparent Km value of 25×10^{-6} M phospholipid.

(v) Stability

Stability of the enzyme activity in HEPES buffered medium was examined by storage at 4°C over 20 days (table 3.7). The activity at pH 9.0 was relatively stable and lost approximately 20% of its original activity over 20 days. Activity at pH 6.0 however was much more unstable and lost between 75 - 80% of its original activity over the same time period.

FIGURE 3.6.

Hydrolysis of E.coli phospholipids with the zymosan - complement released PLA of neutrophils. Reaction mixtures contained 40 mM Tris pH 9.0, 96 μg enzyme protein, 5mM Ca^{2+}, 5×10^8 autoclaved E.coli labelled with (1 - ^{14}C) oleic acid representing 10 nmoles phospholipid (PL).
Each point represents mean of duplicate determinations.

Figure 3.6

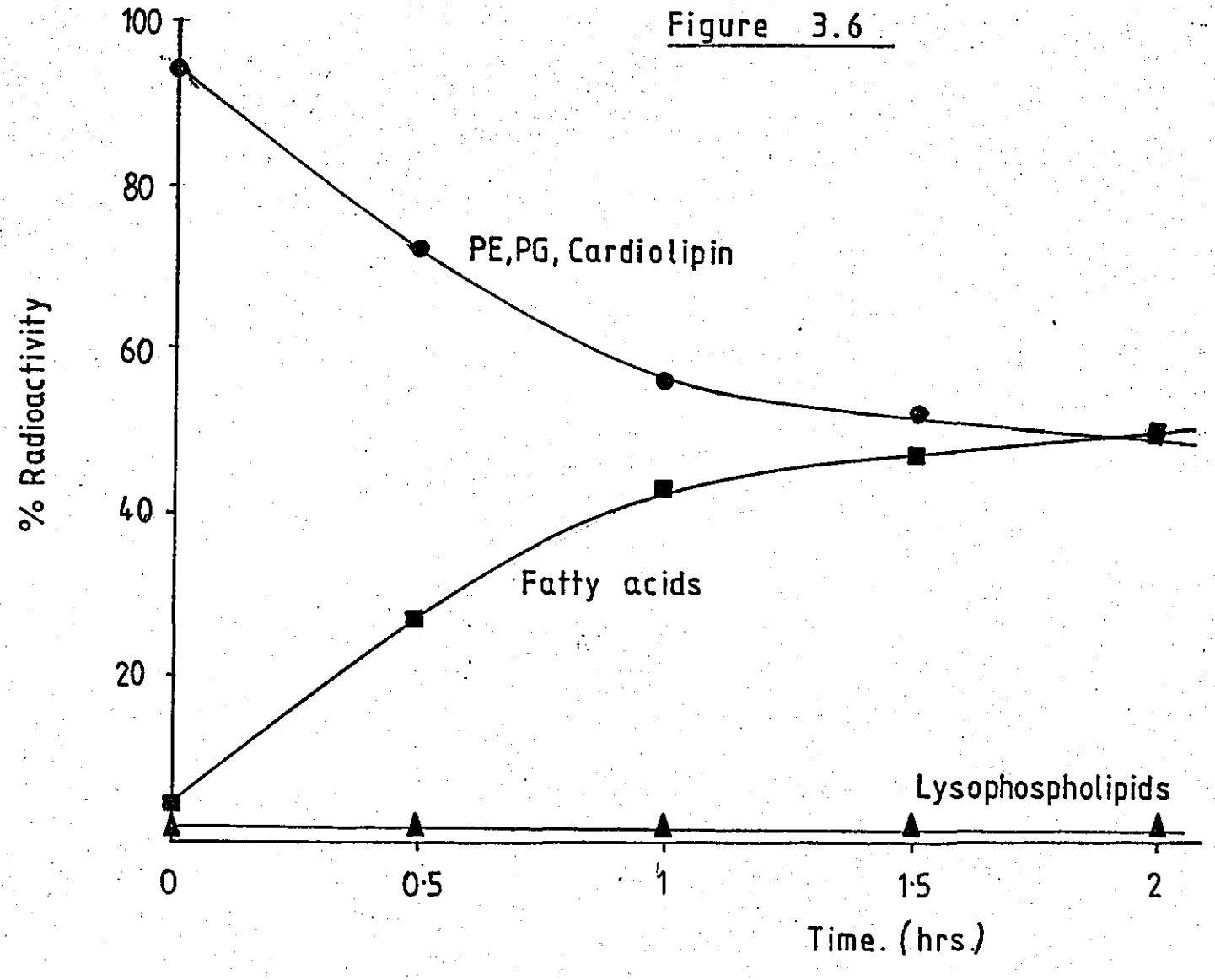

100
80
60
40
20
% Radioactivity
PE,PG, Cardiolipin
Fatty acids
Lysophospholipids
0
0·5
1
1·5
2
Time. (hrs.)

TABLE 3.6

HYDROLYSIS OF (1-^{14}C) PALMITATE LABELLED E.COLI PHOSPHOLIPIDS BY PMN SONICATES AT pH 6.0

FRACTION	% RADIOACTIVITY			
	E.COLI WITHOUT INCUBATION	E.COLI BLANK INCUBATION	E.COLI + PMN SONICATE ENZYME	HYDROLYSIS (%)
FATTY ACID	16.5	16.8	27.2	+10.4
PHOSPHOLIPIDS	73.0	70.6	17.7	-52.9
LYSOPHOSPHOLIPIDS	9.5	12.6	55.0	+42.4

52.9% Hydrolysis was observed. 20% of this activity appeared in the fatty acid fraction and 80% in the lysophospholipid fraction.

As palmitate label is only 77% in position 1 of phospholipids, hydrolysis at pH 6.0 is exclusively A_2.

Reaction mixtures contained 20nmoles phospholipid, 5mM Ca^{2+}, 0.1mg PMN sonicate enzyme, 40mM tris - HCl buffer pH 6.0. Incubations were carried out for 1hr at 37°C.

FIGURE 3.7.

The effect of pH on the activity of ZC - released phospholipase A_2 from neutrophil leucocytes. Reaction mixtures contained 60 μg protein, 5mM Ca^{2+}, 10 nmoles phospholipid and 40mM appropriate buffer pH 4 - 6.0 sodium acetate, pH 5.5 - 7.5 Tris - malic ; pH 7.5 - 9.0 Tris - HCl. Incubations were for 1hr at 37°C.

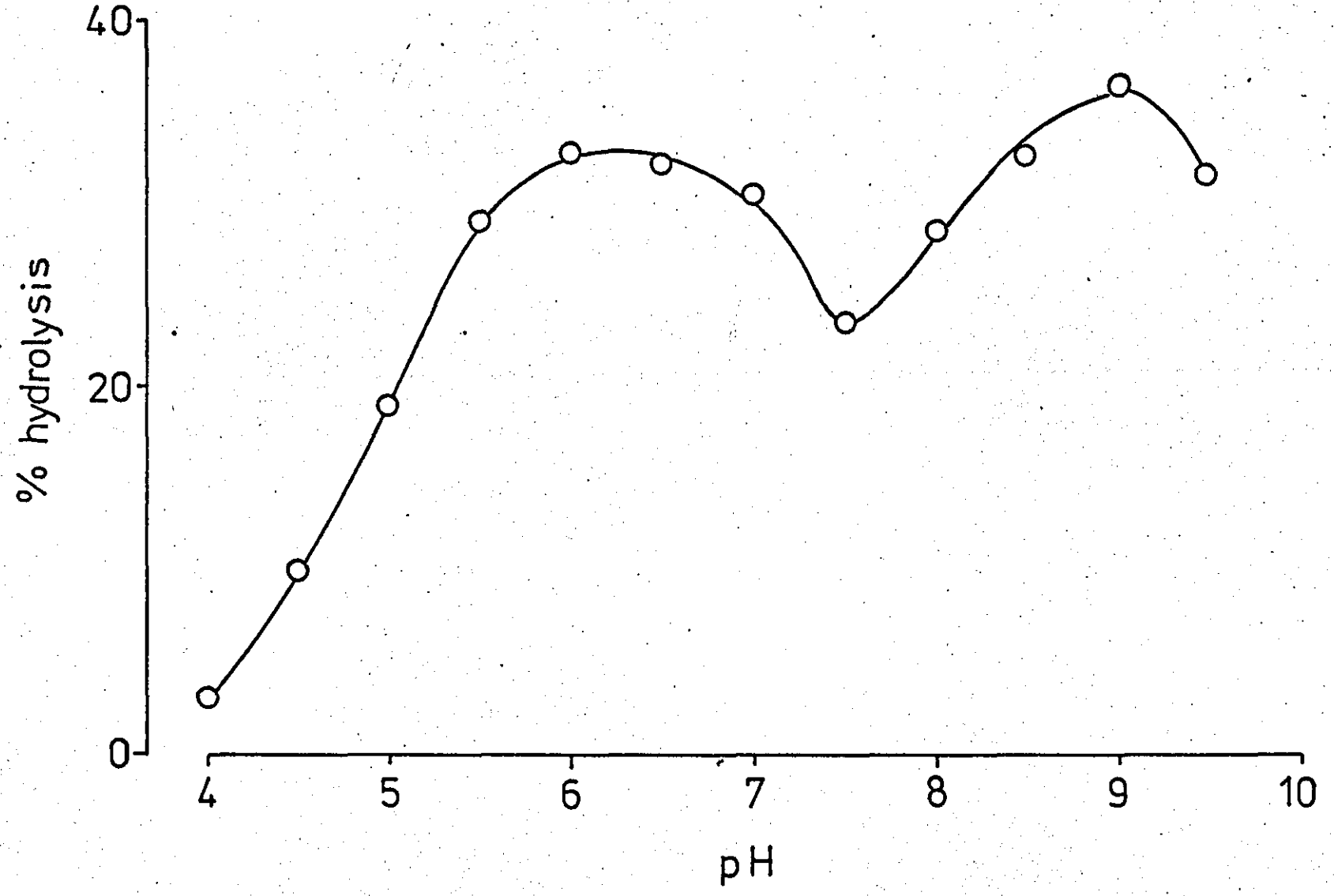

40
20
0
% hydrolysis
4
5
6
7
8
9
10
pH

FIGURE 3.8.

Effect of Ca^{2+} on the neutrophil released phospholipase A_2 at pH 9.0.
Reaction mixtures contain 10nmoles PL, 128 μg enzyme protein, 40mM Tris - HCl buffer, and varying amounts of calcium.

FIGURE 3.9.

Effect of Ca^{2+} on the neutrophil released PLA_2 at pH 6.0.
Reaction mixtures contain 10nmoles PL, either 128 μg (circles) or 64 μg (triangles) enzyme protein, 40mM Tris - malic acid buffer, and varying amounts of calcium.

All points represent mean of duplicate determinations.
Incubations were carried out for 1hr at 37°C.

Figure 3.9

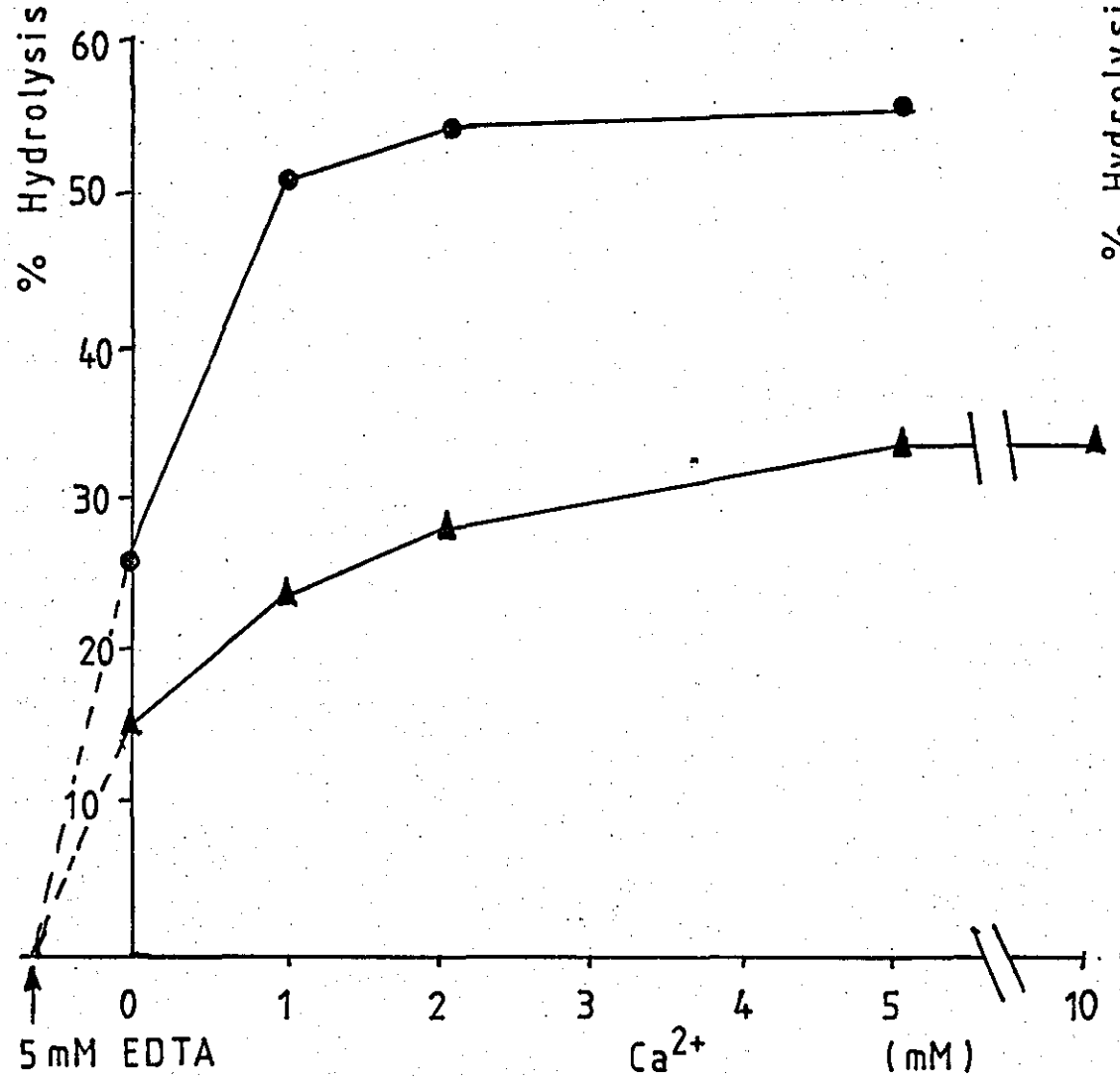


Figure 3.8

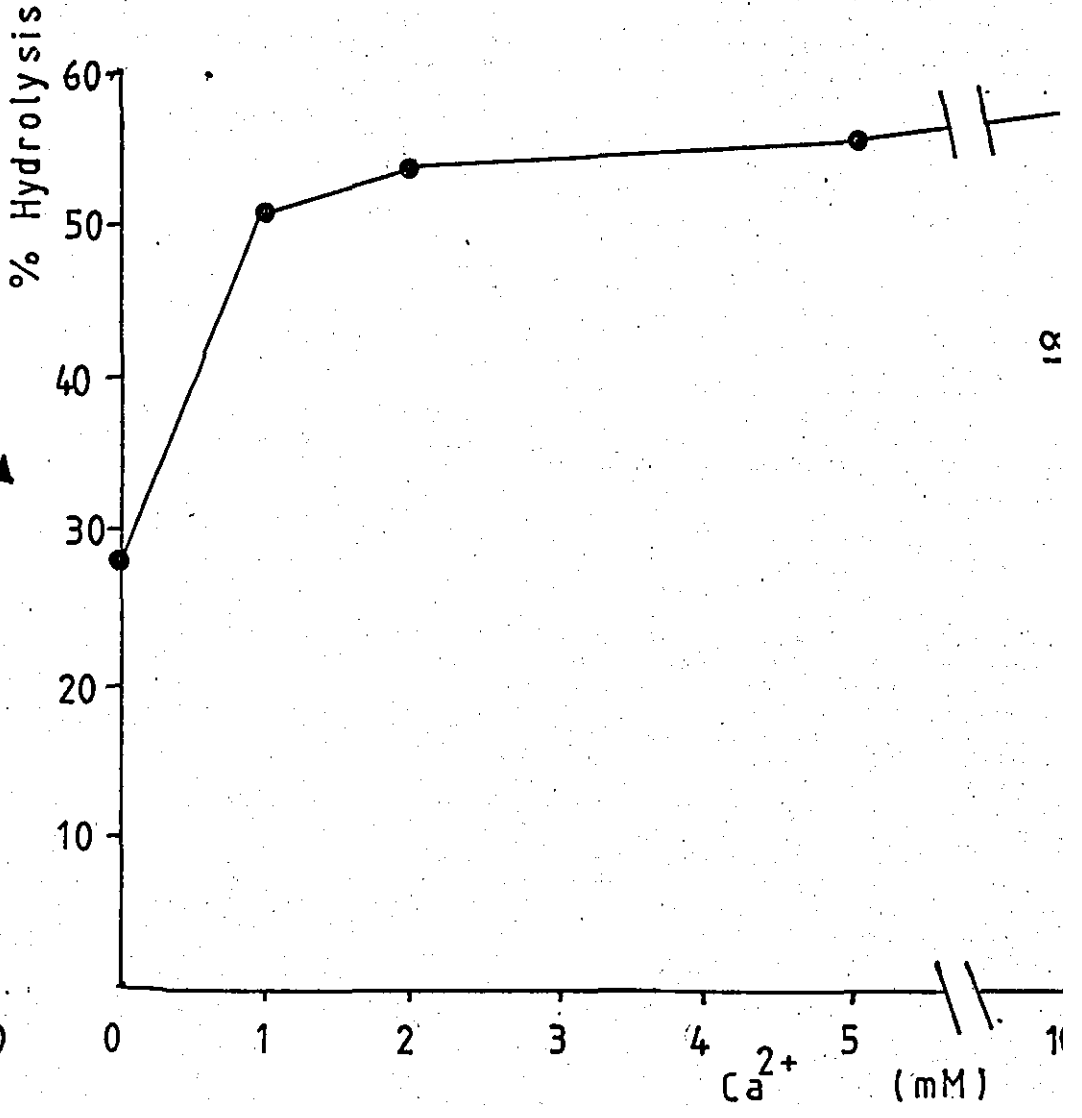


FIGURE 3.10.

Effect of protein concentration on enzyme activity of the neutrophil released PLA_2.
Reaction mixtures contain 10 nmoles PL, 5mM Ca^{2+}, 40mM Tris - malic acid buffer and varying amounts of enzyme protein.
Each point represents mean of duplicate determinations.
Incubations were for 1 hr. at 37°C.

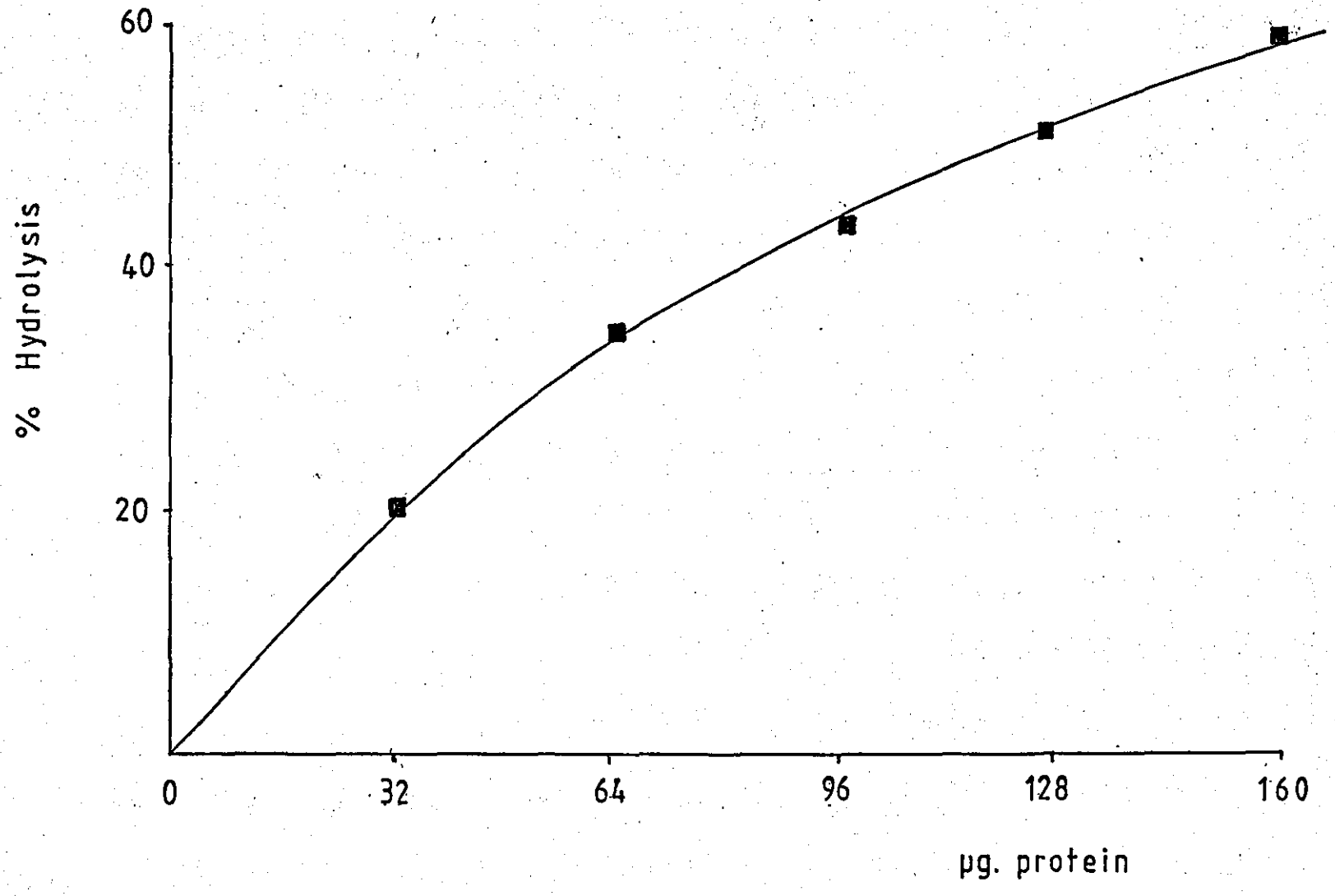
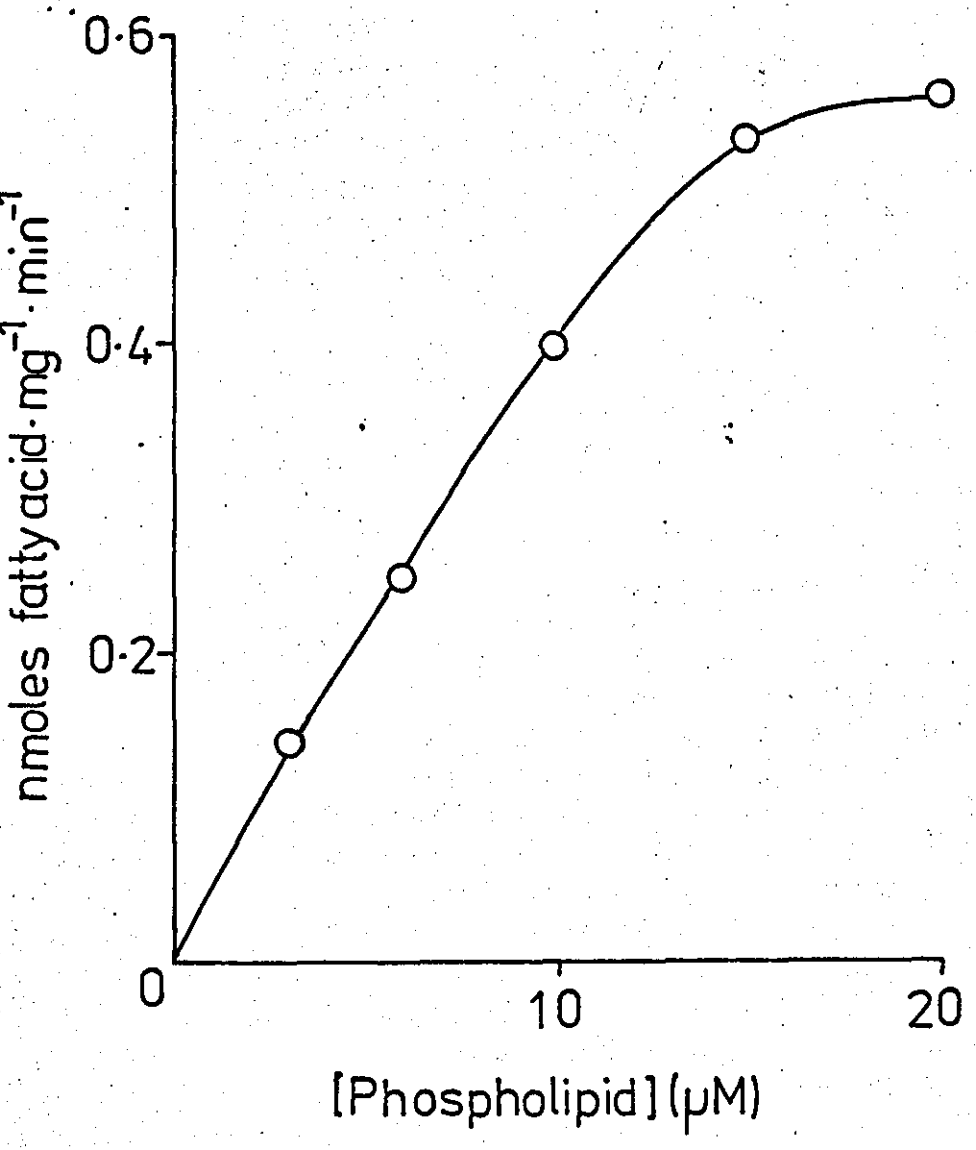

60
40
20
% Hydrolysis
0
32
64
96
128
160
µg. protein

FIGURE 3.12.

Double reciprocal plot of substrate variation.

FIGURE 3.11.

Effect of substrate variation on the neutrophil released PLA_2 activity.
Reaction mixtures contained 40mM Tris - HCl buffer pH 9.0, 96 μg enzyme protein, 5mM Ca^{2+} and 3 - 20 μM phospholipid (0.75 - 5 $\times 10^8$ E.coli).
Incubations were carried out at 37°C. for 1 hr.
All points are mean of duplicate determinations.


0·6
0·4
0·2
0
10
20
nmoles fatty acid·mg⁻¹·min⁻¹
[Phospholipid] (μM)

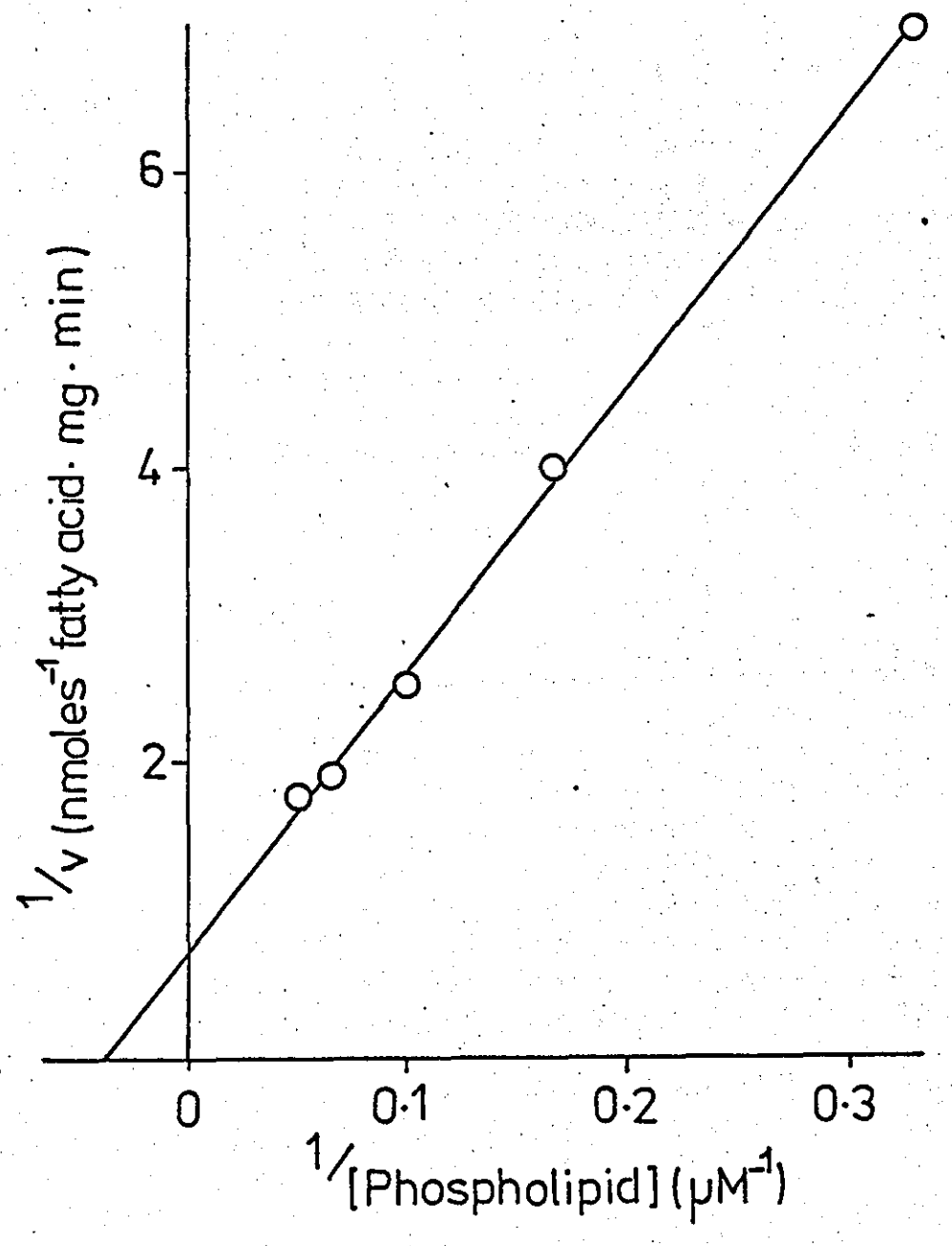

6
4
2
0
0·1
0·2
0·3
1/v (nmoles⁻¹ fatty acid· mg · min)
1/[Phospholipid] (μM⁻¹)

TABLE 3.7

STABILITY OF PHOSPHOLIPASE A_2 OF SONICATED NEUTROPHIL LEUCOCYTES, STORAGE AT 4°C

TIME (DAYS)	ACTIVITY NANOMOLES PL HYDROLYSED/HR/mg PROTEIN pH 6.0	pH 9.0
3	40.9	45.8
11	26.8	42.2
17	16.0	42.9
20	8.2	36.6
LOSS OF ACTIVITY AT 20 DAYS	78%	20%

Each value represents mean of duplicate determinations.

Reaction mixtures contained 40mM tris - malic acid buffer pH 6.0 or tris - HCl buffer pH 9.0, 5mM Ca^{2+}, 0.13mg protein (PMN sonicates) and incubations were carried out for 1hr at 37°C.

2.4 Properties of a phospholipase present in the rabbit inflammatory PERITONEAL EXUDATE (PE)

A very active phospholipase activity was found in the rabbit inflammatory peritoneal fluid supernatant after the invading cells had been removed by centrifugation. The properties of this enzyme were characterised.

(i) Effect of time of incubation on enzyme activity

The hydrolysis with time of 10 nmoles of (1 -^{14}C) oleate labelled E.coli phospholipid with 0.5mg enzyme protein is shown in fig. 3.13. Enzyme activity was linear up to 10 minutes when 40% hydrolysis had occured, after which the activity levelled off reaching 53% by 30 minutes. In subsequent experiments a 5 minute incubation period was used.

(ii) Effect of protein concentration on enzymic activity

Figure 3.14 shows the enzyme activity as a function of protein concentration and as can be seen enzyme activity was linear up to 0.5mg enzyme protein under the conditions used.

(iii) Specificity

The enzymic hydrolysis again appeared to be of A_2 specificity as shown by figure 3.15. A decrease of radioactivity in the phospholipid fraction was simultaneously recovered by an increase in the fatty acid fractions but not in the lysophospholipid products.

FIGURE 3.13.

Variation of time on the peritoneal exudate PLA_2 activity.
Reaction mixtures contain 40mM Tris - HCl buffer pH.8.0, 5×10^8 autoclaved labelled E.coli (10 nmoles PL), 0.5 mg enzyme protein, 5mM Ca^{2+}. Incubations were at 37°C for 5 mins.

FIGURE 3.14.

Effect of protein concentration on the peritoneal exudate PLA_2.
Reaction conditions as above except protein conce - ntration was varied from 0 - .75 mg.
All points are mean of duplicate determinations.

Fig. 3.14

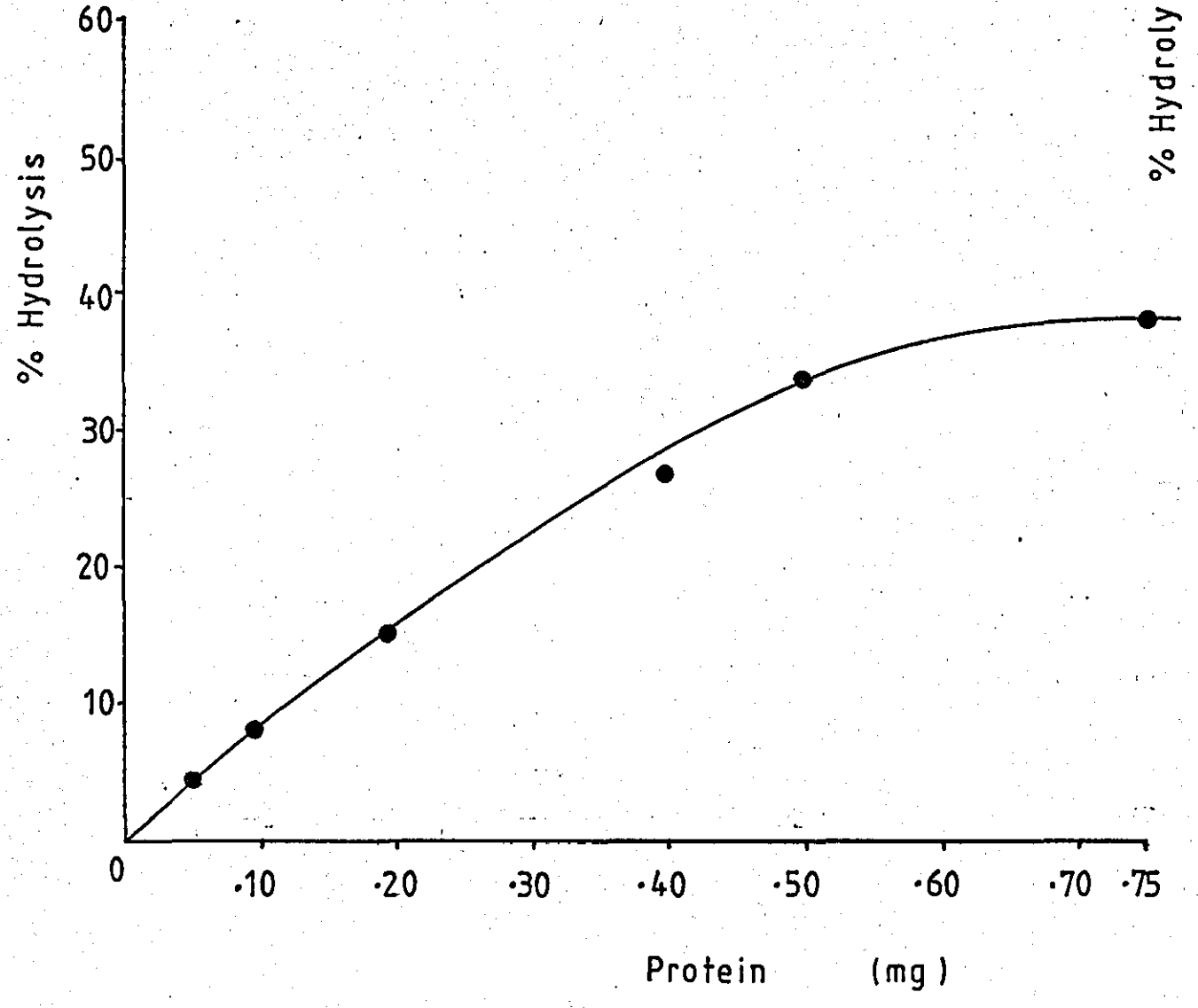


Fig. 3.13

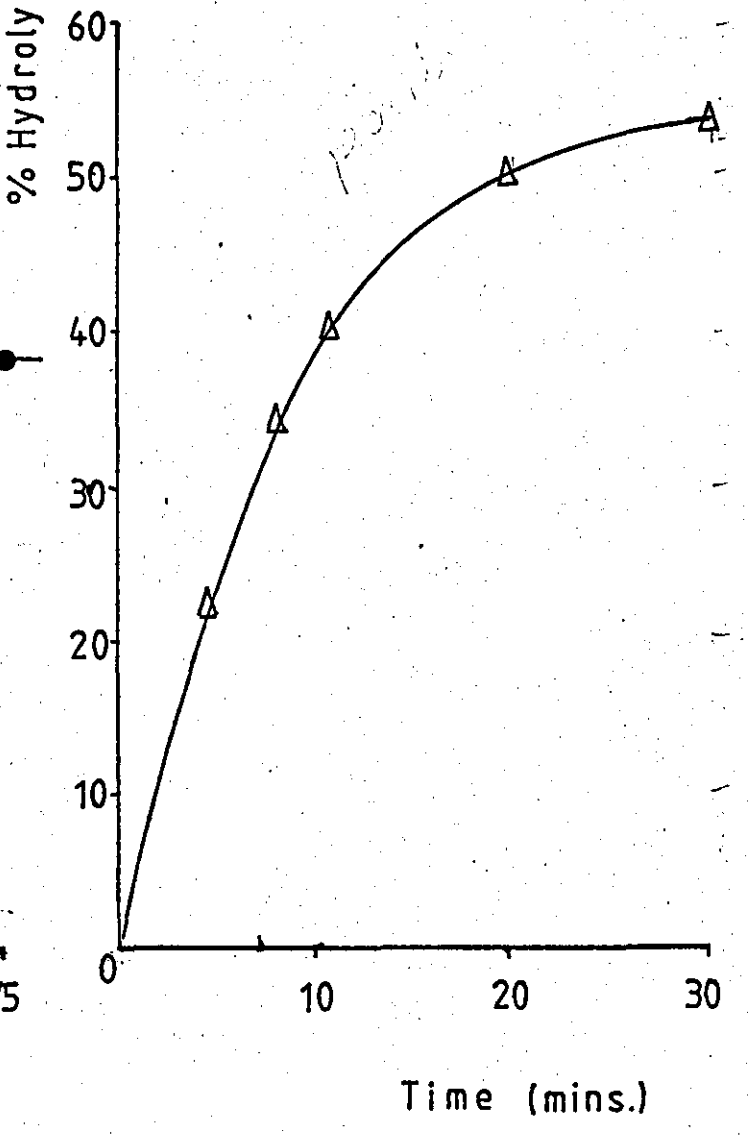
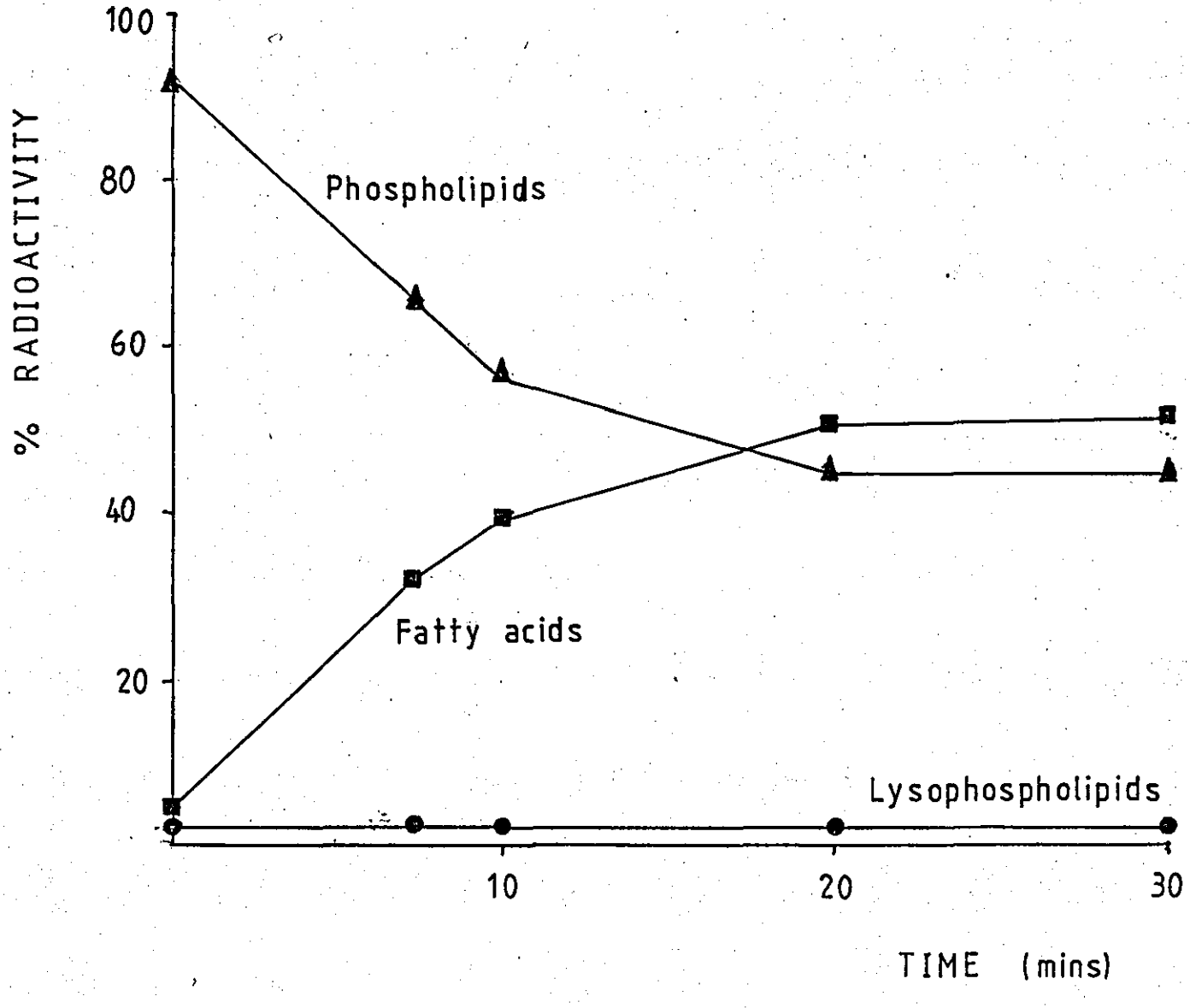


FIGURE 3.15.

Hydrolysis of E.coli phospholipids by the peritoneal exudate PLA_2.
Reaction conditions are identical to figure 3.13.


100
80
60
40
20
% RADIOACTIVITY
Phospholipids
Fatty acids
Lysophospholipids
10
20
30
TIME (mins)

(iv) pH Profile

A pH profile of the enzymic hydrolysis showed two pH optima at pH 8.0 and at pH 6.0 (figure 3.16). The acid peak was broad. This pH profile was remarkably similar to that obtained with the neutr - ophil released enzyme (figure 3.7) although the exact position of maxima and minima of activity varied slightly.

(v) Calcium requirement

Figures 3.17 (a) and 3.17 (b) represent the calcium requirement of the peritoneal fluid phospholipase A_2. The enzymic activity has an absolute requirement of Ca^{2+} as addition of greater than 150 μM EDTA resulted in a total loss of activity. Maximum hydrolysis occured when 1mM Ca^{2+} was added to the reaction mixture. Larger amounts of added Ca^{2+} caused slight inhibition of activity. The figure also shows that on the absence of added calcium about 95% of the activity is still retained. This suggests the presence of sufficient endogenous Ca^{2+} in the peritoneal fluid to give almost total activity. This was supported by the studies with EDTA which showed that increasing amounts of EDTA caused phospholipase activity to decrease proportionately (figure 3.17 (b)).

As one EDTA molecule is able to bind to two divalent cations, this suggests that the concentration of the latter was 300 μM in the reaction mixture. Flame photometer studies showed the calcium concentration to be 200 μM. This indicated that the peritoneal fluid phospholipase activity rose very sharply with an increase of calcium ions from 0 - 0.2 mM, reached a maximum at 1.2 mM and thereafter the activity slightly decreased with large increases of calcium. These studies

FIGURE 3.16.

Effect of pH variation on the peritoneal PLA_2.

Reaction mixtures contain 20 nmoles, 0.3mg enzyme protein, 5mM ca^{2+} and 40 mM appropriate buffer.

pH 5.0 - 7.5 Tris - malic acid.

pH 7.5 - 9.0 Tris - HCl.

Incubations were at $37^{b}C$ for 5 mins.

All points are mean of duplicate observations.

fig 3.16

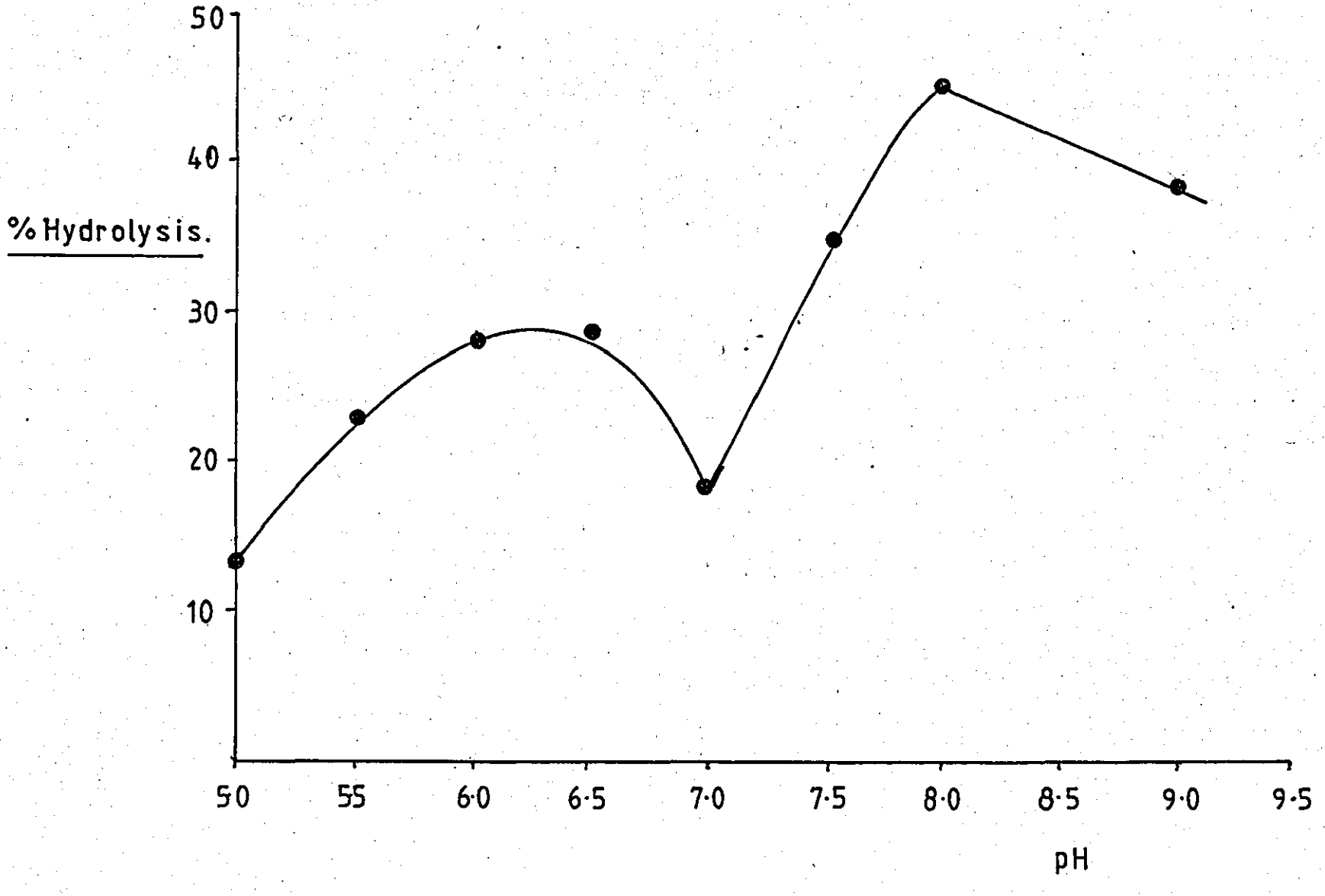


FIGURE 3.17 (a) and (b).

The effect of Ca^{2+} on the peritoneal exudate PLA_2.

Reaction mixtures contain 23 nmoles PL, 40 mM Tris - HCl buffer pH 6.0, 0.5 mg protein and either varying amounts of Ca^{2+} or EDTA.

Incubations were carried out for 5 mins at 37°C.

All points are mean of duplicate determinations.

(b)

Figure 3.17

(a)

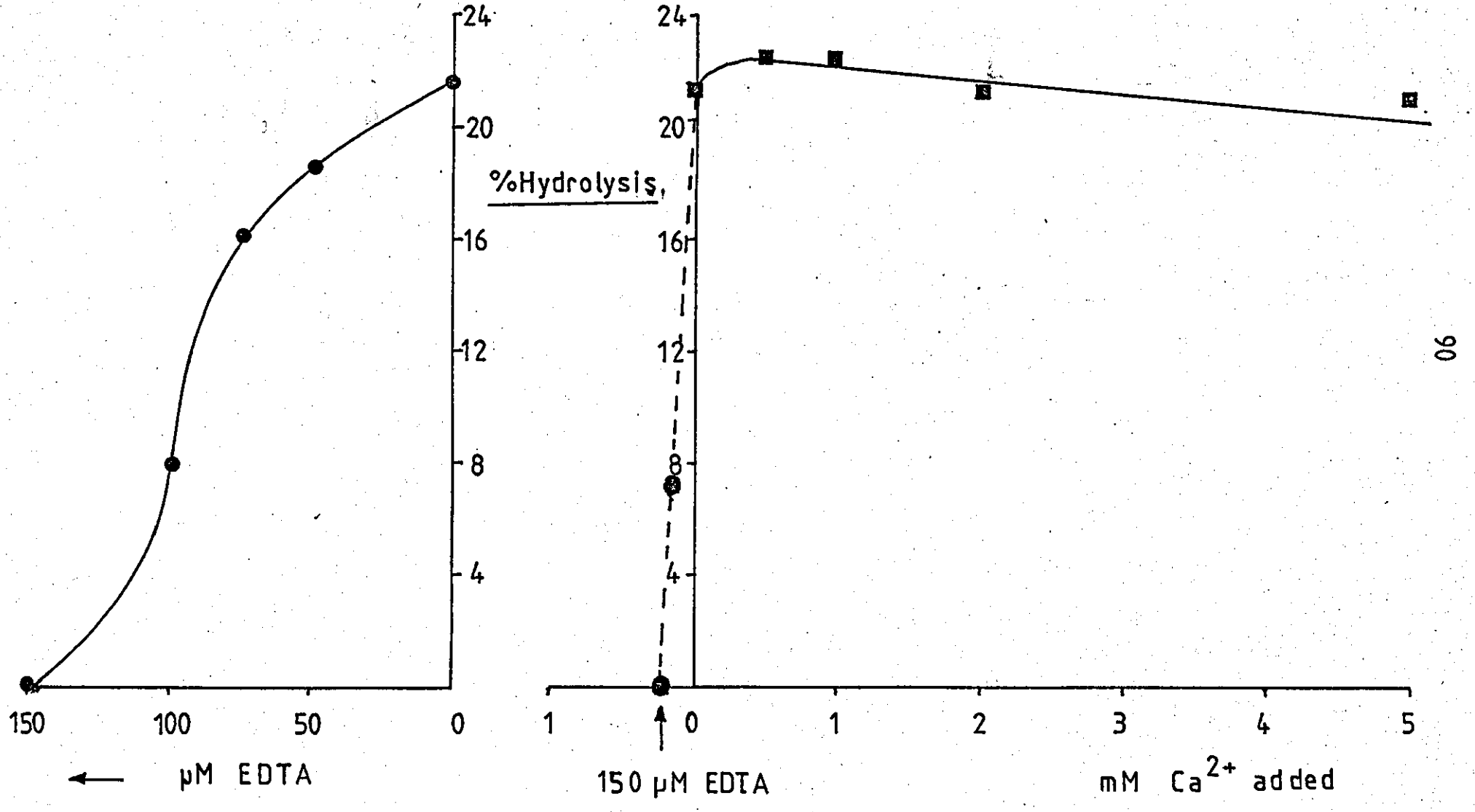


TABLE 3.8

STORAGE OF PERITONEAL EXUDATE PLA_2 ACTIVITY

DAYS OLD	ACTIVITY AT PH 6.0		
	% HYDROLYSIS OF 20 NMOLES PL	SPECIFIC ACTIVITY NMOLES PL HYDROLYSED / mg PROTEIN/MIN	% CHANGE IN ACTIVITY
0	19.4	1.11	0
1	25.8	1.47	+32
3	34.9	1.99	+79
9	31.0	1.77	+59
22	24.5	1.40	+26
37	22.1	1.20	+8
80	17.1	0.98	−12

Each value represents mean of duplicate determinations which did not vary by more than 3.0% hydrolysis.

Reaction mixtures contained 10^9 autocleaved radiolabelled E.coli (20 nmoles phospholipid), 5mM Ca^{2+}, 40mM tris - malic acid buffer and incubated for 5 min at 37°C.

were carried out at pH 6.0.

(vi) Stability

The effect of storage of the enzyme at 4°C is shown in table 3.8. The enzyme activity at pH 6.0 was relatively stable over a few months. However in the first few days an increase of specific activity by as much as 79%, was observed at day 3. After this the enzyme activity slowly decreased with time.

PART C EFFECTS OF CHLOROQUINE - LIKE AGENTS ON INFLAMMATORY AND OTHER PHOSPHOLIPASES

3.1 Studies of effects of chloroquine - like agents on phospholipases

A_2 from Crotalus adamenteus venom and porcine pancreas

(i) Optimal conditions for hydrolysis with Crotalus adamenteus venom phospholipase A_2

a) Calcium concentrations

Figures 3.18 and 3.19 show the patterns of hydrolysis obtained using this system and the optimal concentration of calcium required for maximum enzymic activity. Reaction rates were linear in the first 4 - 6 minutes and thereafter slowly levelled off. Initial rates were taken and converted to micromoles fatty acid released per minute per milligramme protein. Calcium ions were essential for enzyme activity. Activity rose with an increase of Ca^{2+} concentration up to 6mM after which there was only a very slight increase of activity as shown in figure 3.19. Subsequently 8mM Ca^{2+} was used in all reaction mixtures.

b) Sodium deoxycholate

Approximately half of the total enzyme activity was present in the absence of sodium deoxycholate. Optimal activity was obtained at a concentration of 1mM (figure 3.20). Higher concentrations

FIGURE 3.18.

Hydrolysis of egg yolk phospholipids by Crotalus adamenteus venom. Progress of reaction in the presence of varying amounts of Ca^{2+}.

A = 4.9 mM Ca^{2+}
B = 3.9 mM "
C = 2.8 mM "
D = 1.97mM "
E = 0.98mM "
F = NO Ca^{2+}

Reaction conditions as described in Materials and Methods.

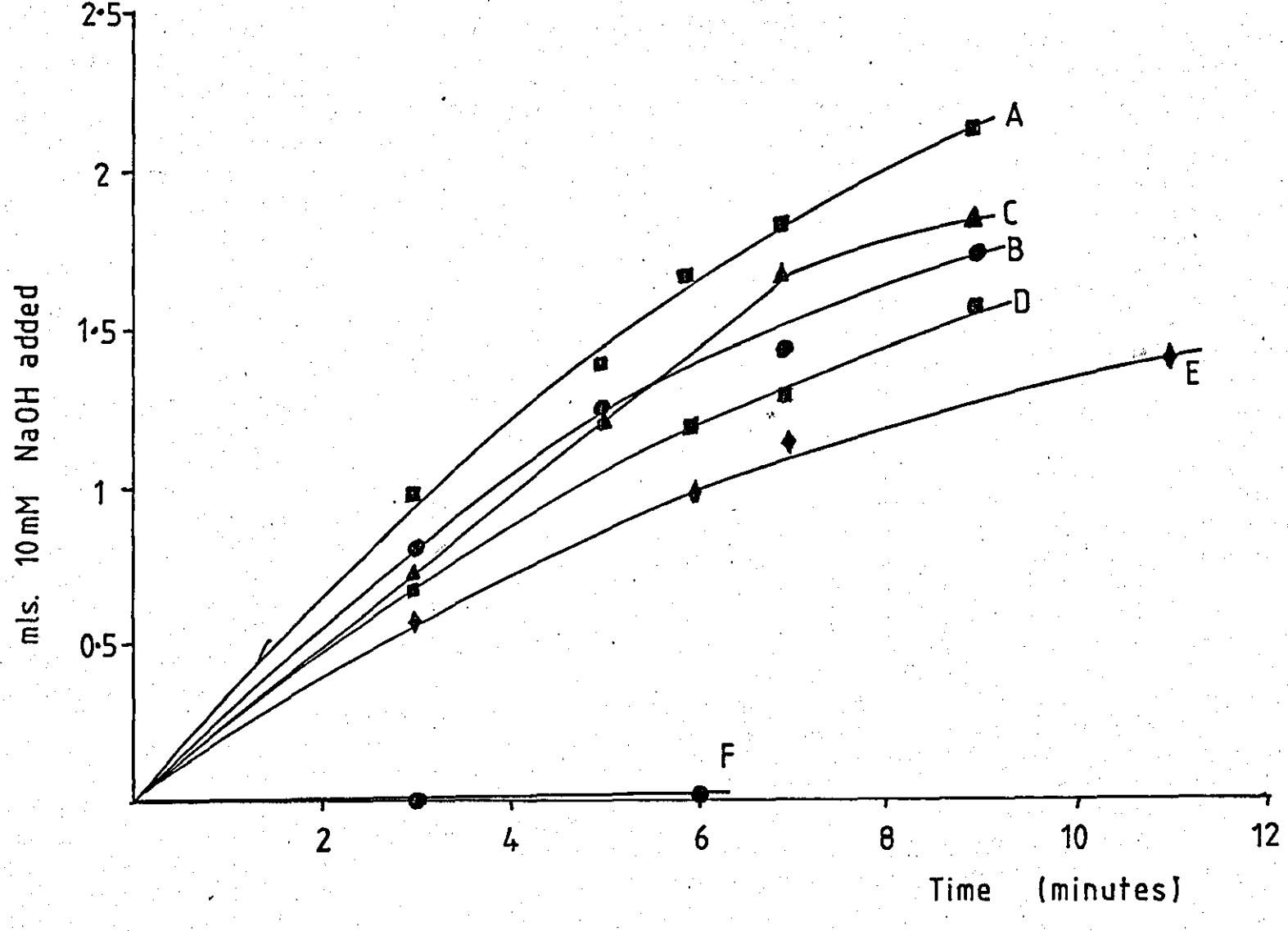

2·5
2
1·5
1
0·5
mls. 10mM NaOH added
A
C
B
D
E
F
2
4
6
8
10
12
Time (minutes)

FIGURE 3.19.

Effect of Ca^{2+} variation on the hydrolysis of egg yolk phospholipids by Crotalus adamenteus venom.

FIGURE 3.20.

Variation of deoxycholate on the hydrolysis of egg yolk phospholipids by Crotalus adamenteus venom.

All points are means of duplicate determinations.

Fig. 3.20

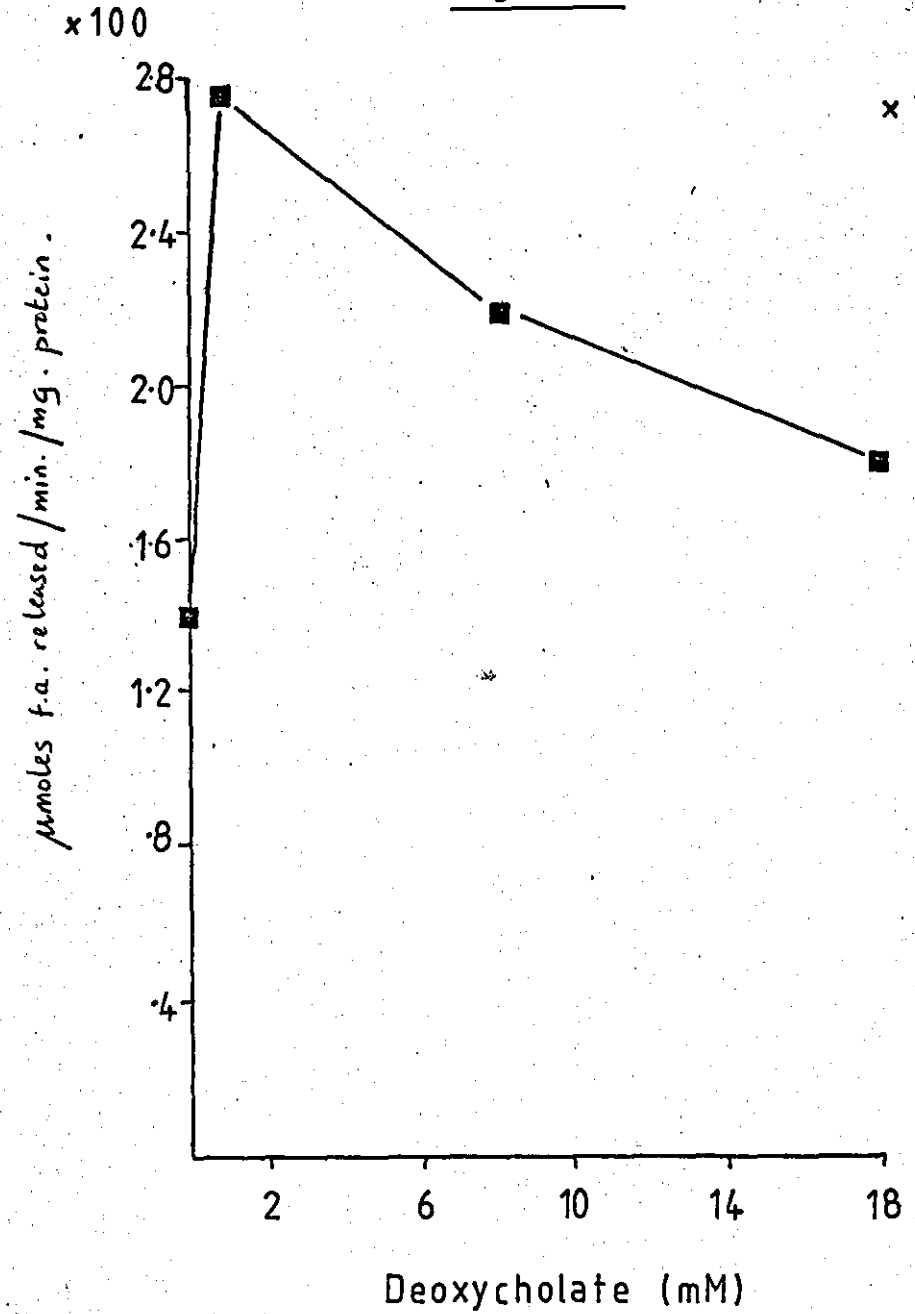


Fig. 3.19

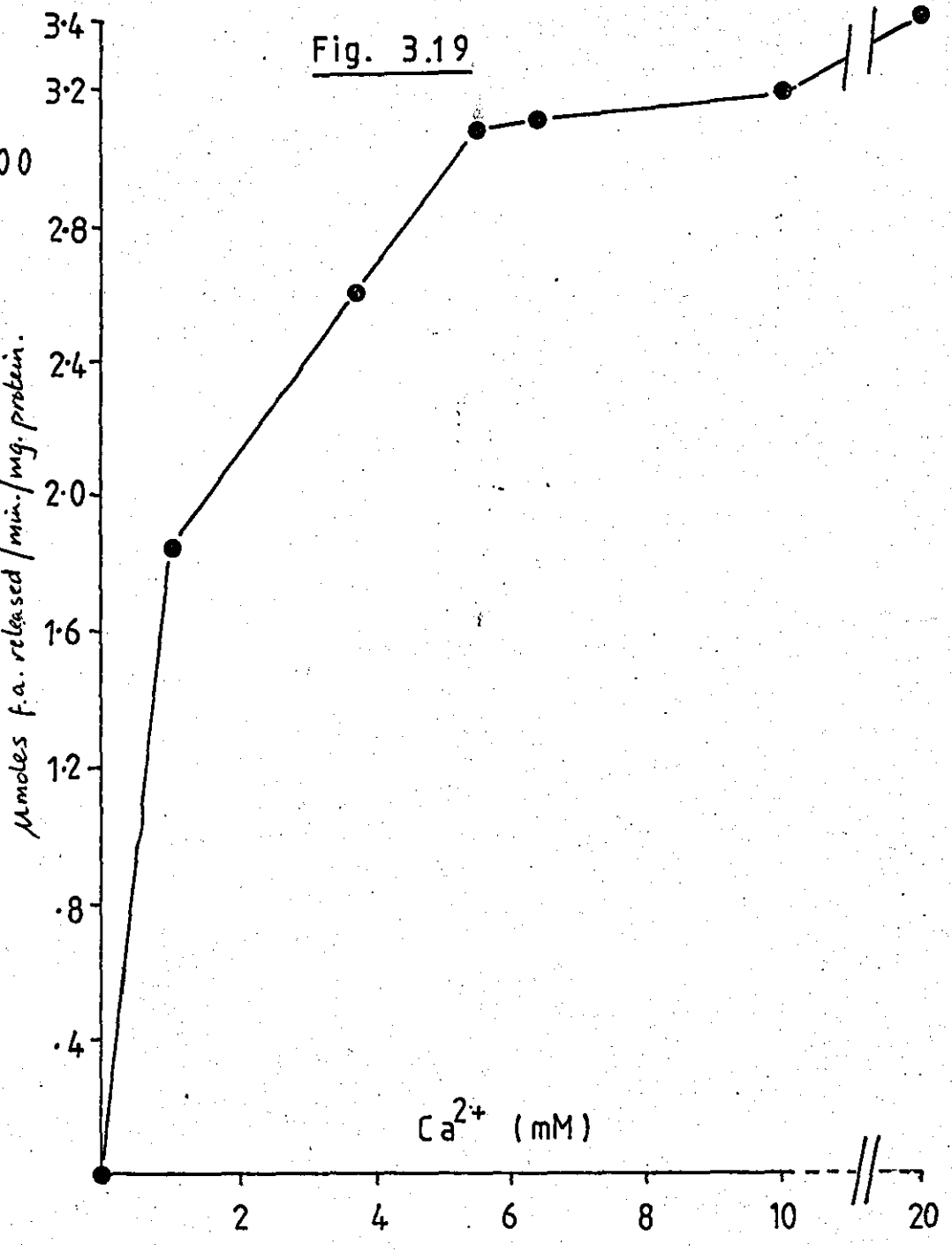


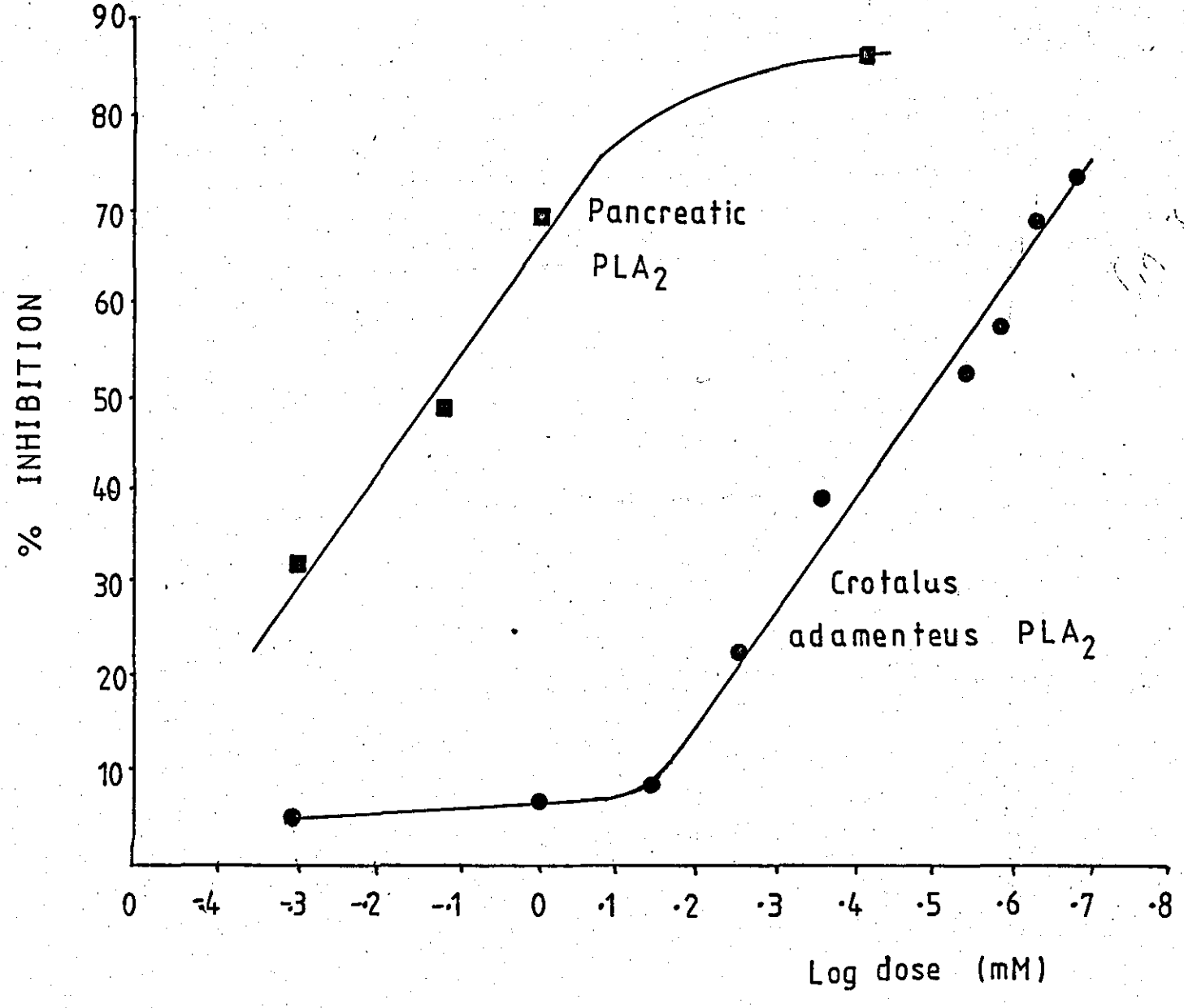
FIGURE 3.21.

Variation of Ca^{2+} with enzyme activity of pig pancreas PLA_2.
All points are mean of duplicate determinations.

µmoles f.a. released · /min./ mg. protein.
x 10^3
1·8
1·6
1·4
1·2
1·0
·8
·6
·4
·2
0
2
4
6
8
10
12
14
16
18
20
mM Ca^2+

FIGURE 3.22.

Effect of chloroquine diphosphate on PLA_2 activity of pig pancreas and Crotalas adamenteus venom. Best fit lines are calculated by least squares analysis. All points are means of duplicate determinations.


90
80
70
60
50
40
30
20
10
% INHIBITION
Pancreatic
PLA2
Crotalus
adamenteus PLA2
0
-4
-3
-2
-1
0
·1
·2
·3
·4
·5
·6
·7
·8
Log dose (mM)

actually caused a decrease in enzymic hydrolysis and therefore a concen - tration of 1mM was used in subsequent experiments.

(ii) Optimal conditions for hydrolysis with pig pancreas phospholipase A_2

Phospholipase activity from pig pancreas was analysed at 37°C according to the method of Nieuwenhuizen, Kunze and Haas (1974). Optimum calcium concentration was again 8mM (figure 3.21) and 1mM for sodium deoxycholate.

(iii) Drug experiments

a) Inhibition of Crotalus adamenteus venom phospholipase A_2 by chloroquine diphosphate.

Chloroquine diphosphate showed a dose - related inhibition of phospholipase A_2 activity of Crotalus adamenteus venom over the range 0 - 4mM as shown in figure 3.22. 50% Inhibition of enzyme activity was achieved at 3.0 mM chloroquine.

b) Inhibition of pig pancreas phospholipase A_2 by chloroquine

Inhibition of the purified pig pancreas enzyme was also observed by chloroquine but at four fold lower doses (figure 3.22). 50% Inhibition of activity as calculated from the best fit curve was obtained at 0.7 mM.

c) Inhibition of pig pancreas phospholipase A_2 by other agents

These were cinchonine sulphate and dibucaine hydrochloride

TABLE 3.9

EFFECT OF INDOMETHACIN, SALICYLIC ACID, DIBUCAINE AND CINCHONINE SULPHATE ON THE PHOSPHOLIPASE FROM PIG PANCREAS TOWARDS EGG YOLK EMULSIONS

	INITIAL RATES (μMOLES F.A. RELEASED / MIN./mg. PROTEIN)		% INHIBITION
	ACTUAL VALUES	MEAN	
CONTROL	6.06, 5.80	5.93	
CINCHONINE SULPHATE (0.5mM)	2.77, 2.63	2.70	54%
DIBUCAINE (1mM)	4.23, 4.07	4.15	30%
CONTROL	6.17, 6.37	6.27	
INDOMETHACIN (1mM)	5.36, 5.5	5.43	13%
(5mM)	5.23, 5.07	5.13	18%
SALICYLIC ACID (1mM)	5.83, 5.57	5.7	10%
(5mM)	4.5, 4.23	4.37	30%

Phospholipase activities using egg yolk emulsions were determined as described in Materials and Methods.

(closely related structures) and two anti - inflammatory agents; indo - methacin and salicylic acid. All four agents inhibited phospholipid hydrolysis (table 3.9). Cinchonine sulphate and dibucaine were more effective than indomethacin and salicylic acid. Cinchonine sulphate and dibucaine also caused a lag period of 3 minutes before maximum enzyme activity was reached and this initial rates recorded were after this period. The order of potency of drugs acting on pancreatic phospholipase A_2 was as follows:

Cinchonine sulphate $\succ$ chloroquine diphosphate $\succ$ dibucaine hydrochloride

$\succ$ salicylic acid $\succ$ indomethacin.

(d) Kinetics of inhibition of pig pancreas PLA_2 by chloroquine

The nature of inhibition caused by chloroquine was further investigated

(i) The variation of substrate and its effects on the inhibition of phospholipase A_2 activity by chloroquine is shown in figure 3.23 . The double reciprocal plot shows the two curves run parallel to each other indicating an uncompetitive inhibition with respect to phospholipid substrate.

(ii) It has been previously reported that calcium antagonises the inhibitory effects of local anaesthetics on phospholipase A_2 of human seminal plasma (Kunze et al, 1976). This finding was also invest - igated using the present system. Figure 3.24 shows the double reciprocal plot of the variation of calcium and its effect on the inhibition of pancreatic phospholipase A_2 by 0.75mM chloroquine and 1mM dibucaine .

FIGURE 3.23.

Double reciprocal plot of $\frac{1}{\text{enzyme activity}}$ in the presence and absence of 0.75mM chloroquine.
Best fit lines are calculated by least squares analysis.
Points represent mean of duplicate determinations.

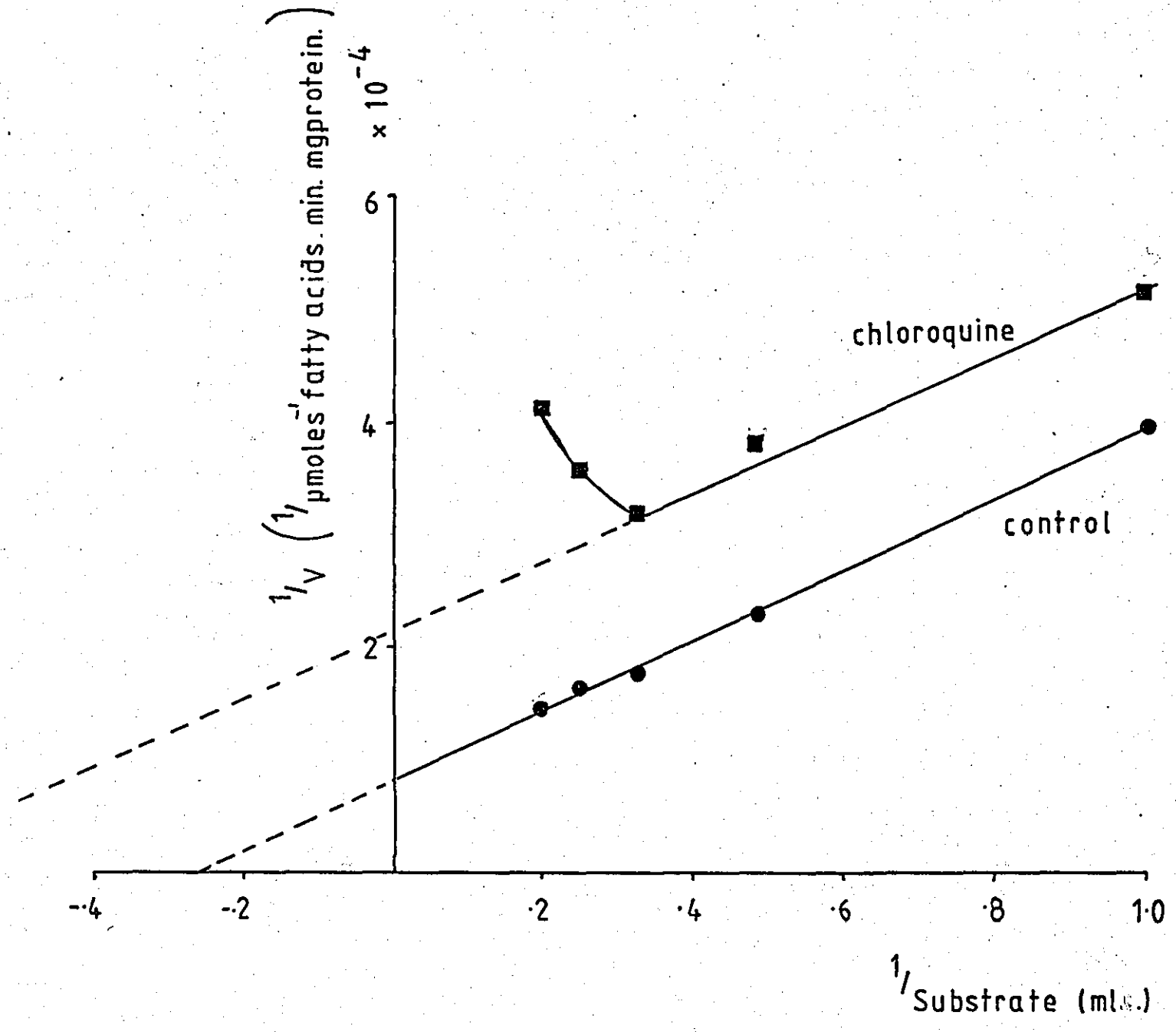
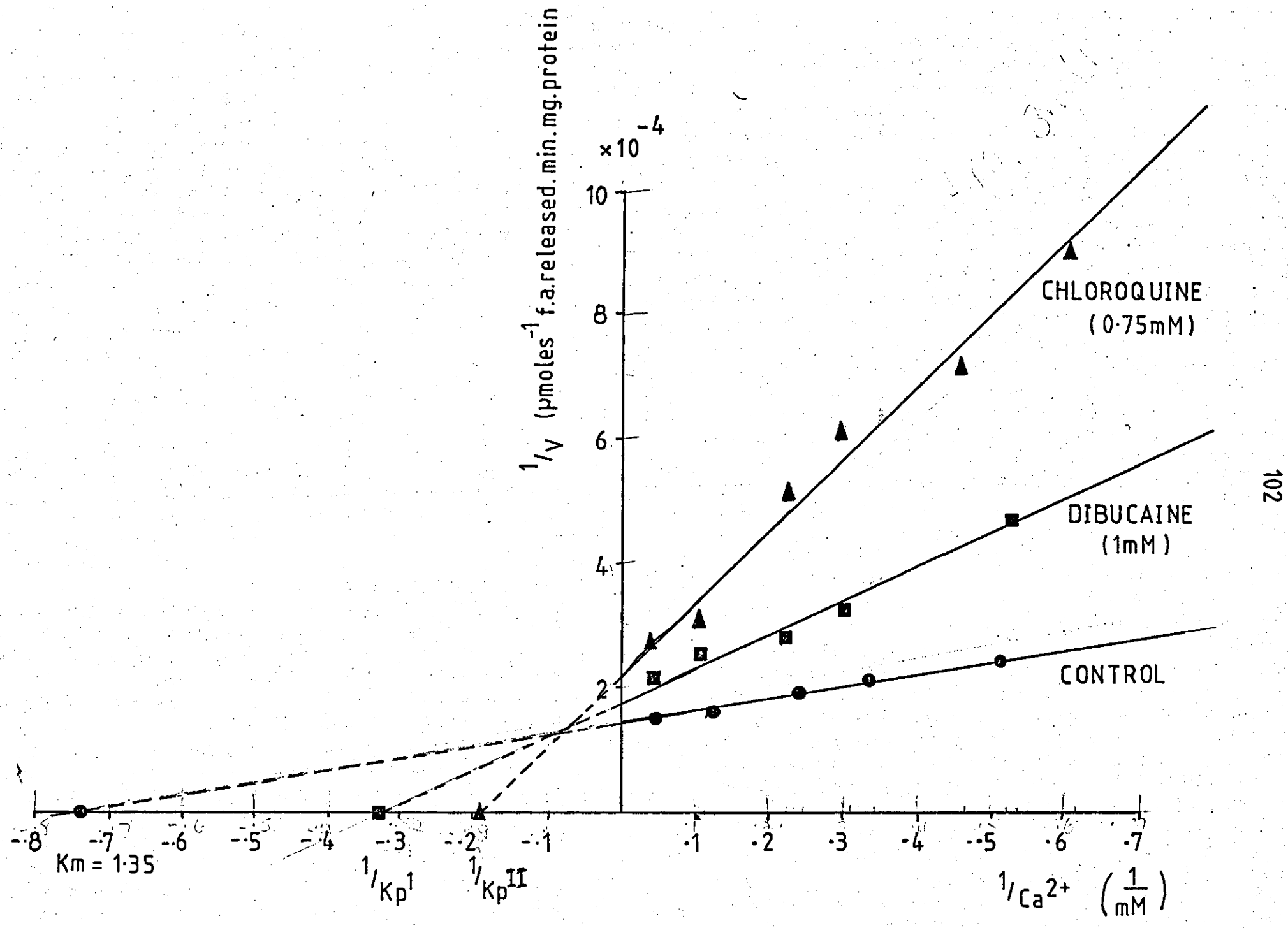

$1/V$ ($1/\mu moles^{-1}$ fatty acids. min. mgprotein.) $\times 10^{-4}$
6
4
2
chloroquine
control
-·4
-·2
·2
·4
·6
·8
1·0
$1/$Substrate (mls.)

FIGURE 3.24.

Double reciprocal plot of Ca^{2+} variation in the presence and absence of chloroquine and dibucaine using pancratic PLA_2.
Best fit lines are calculated by least square analysis.
Points represent mean of duplicate determinations.


$^1/_V$ (μmoles^{-1} f.a.released.min.mg.protein
×10^{-4}
10
8
6
4
2
CHLOROQUINE
(0·75mM)
DIBUCAINE
(1mM)
CONTROL
-·8 -·7 -·6 -·5 -·4 -·3 -·2 -·1 ·1 ·2 ·3 ·4 ·5 ·6 ·7
Km = 1·35
$^1/_{Kp^I}$
$^1/_{Kp^{II}}$
$^1/_{Ca^{2+}}$ $\left(\frac{1}{mM}\right)$

The data suggests a competitive mode of inhibition with calcium by both chloroquine and dibucaine. The changes in Km values for Ca^{2+} illustrated below.

Drug	Km	Ki*
CONTROL	1.35×10^{-3}M	-
DIBUCAINE (1×10^{-3}M)	-	0.76×10^{-3}M
CHLOROQUINE (0.75×10^{-3}M)	-	0.25×10^{-3}M

$$Ki = \frac{(I)}{\frac{Kp}{Km} - 1}$$

Where I= Inhibitor concentration

Kp= inverse of intercept on abcissa (in presence of inhibitor)

Km= Michaelis Menten constant = inverse of intercept on abcissa (in the absence of inhibitor)

3.2 Effects of chloroquine - like agents on the peritoneal fluid

phospholipase A_2 activity

a) Dose - response curves

The anti - malarial drugs chloroquine, mepacrine and primaquine all inhibited phospholipase activity (at pH 6.0) of the peritoneal fluid towards *E.coli* phospholipids in a dose related manner as shown in figure 3.25a. The order of inhibitory potency was

FIGURE 3.25 a.

Effect of antimalarial drugs on the peritoneal fluid PLA_2.

Chloroquine ▲

Mepacrine ●

Primaquine ■

Reaction mixtures contained 40mM Tris – malic acid pH 6.0, 5mM Ca^{2+}, 0.5mg exudate protein, 5×10^8 autoclaved *E.coli*, and varying amounts of antimalarials.

Incubations were for 5 mins at 37°C.

Points represent means of duplicate determinations.

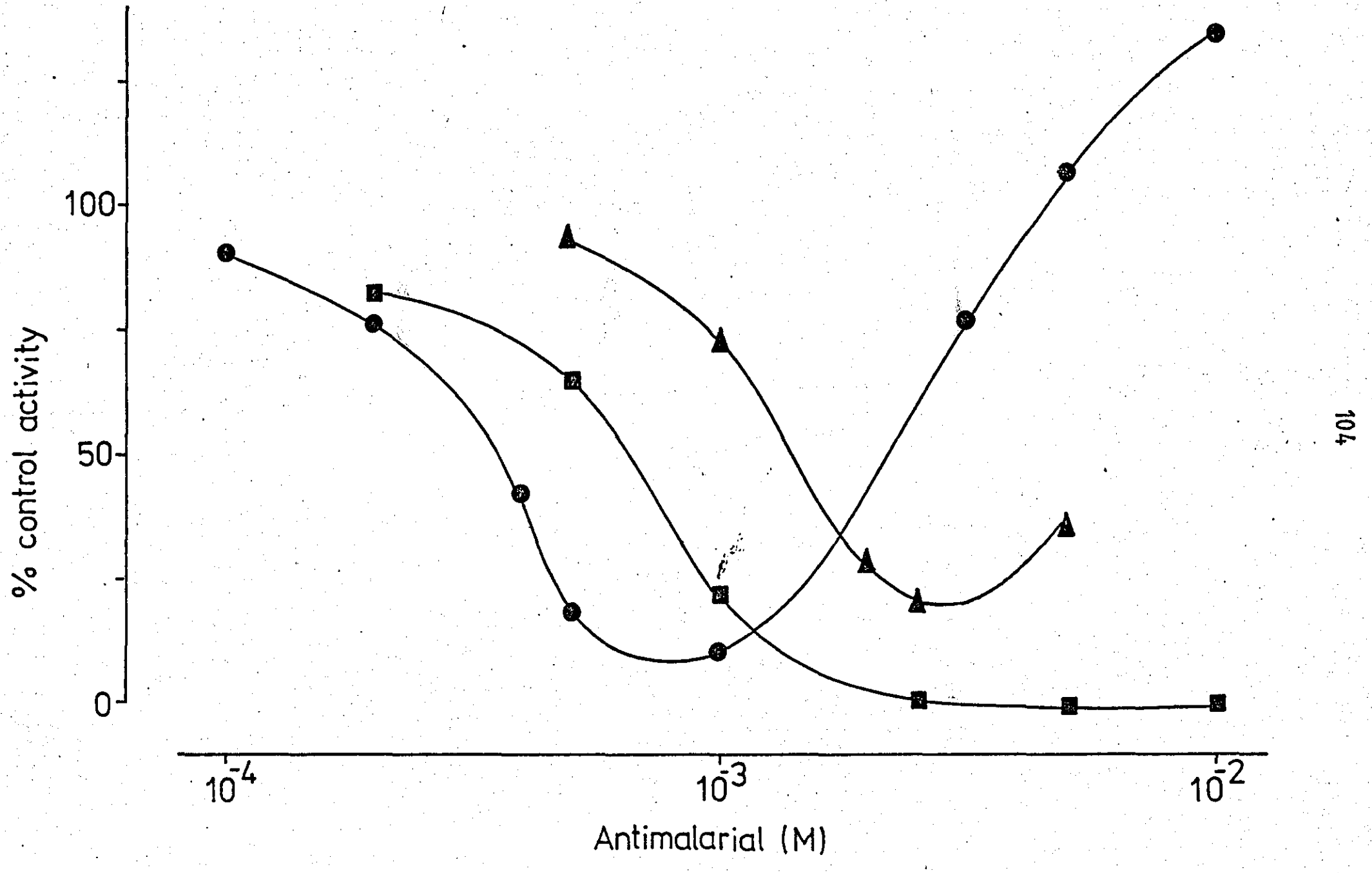
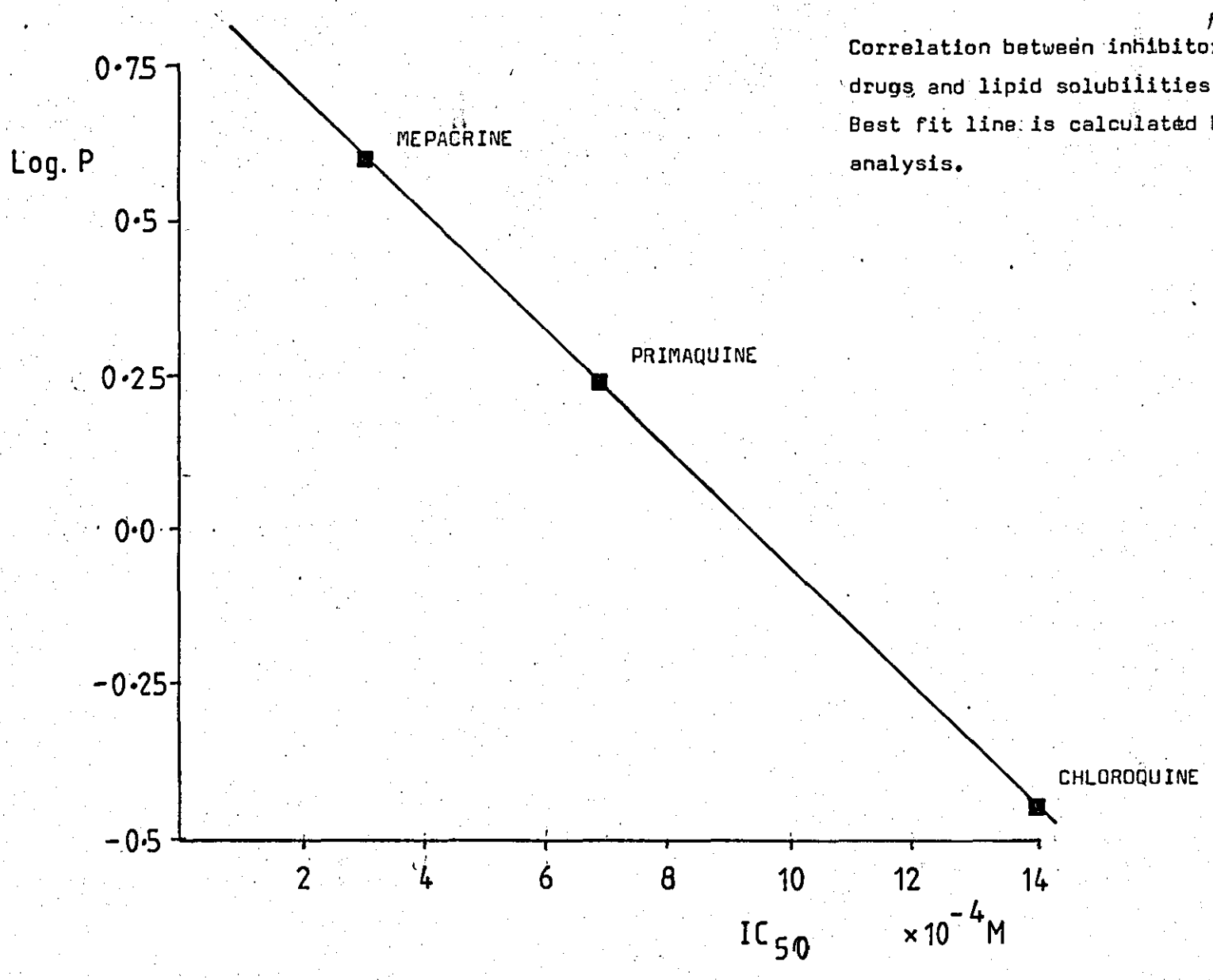

100
50
0
% control activity
10^{-4}
10^{-3}
10^{-2}
Antimalarial (M)

FIGURE 3.25 b.

Correlation between inhibitory potency of antimalarial drugs and lipid solubilities.
Best fit line is calculated by least square analysis.



mepacrine ≥ primaquine ≥ chloroquine, and correlated well with the lipid solubilities of the drugs (figure 3.25b).

Inhibition of peritoneal fluid phospholpase A_2 by antimalarial drugs

DRUG	IC_{50}	Log P
MEPACRINE	3.3×10^{-4}M	0.59
PRIMAQUINE	5×10^{-4}M	0.23
CHLOROQUINE	1.5×10^{-3}M	-0.45

Log P value represent the partition of 10μM antimalarials between chloroform and tris - malic acid buffer at pH 6.0.

With mepacrine and chloroquine at doses larger than those causing maximum inhibition, a reversal of the effect was seen. This even resulted in stimulation of enzyme activity if high doses were used (+ 31% with 10mM mepacrine). This reversal of inhibition at higher than inhibitory doses was not observed with primaquine.

b) Effect of pH on the inhibition of phospholipase A_2 activity by chloroquine

The degree of inhibition by chloroquine was dependant on the pH of the reaction medium. Table 3.10 gives the results obtained using 1mM chloroquine with the pH varied between pH 6.0 to pH 8.5. As can be seen inhibition was found to increase with increasing pH. This least inhibition (16%) caused by 1mM chloroquine was found at pH 6.5

TABLE 3.10

INHIBITION OF PERITONEAL EXUDATE PLA_2 BY CHLOROQUINE AND THE VARIATION OF pH

pH	% INHIBITION CAUSED BY 1mM CHLOROQUINE
6.0	27%
6.5	16%
7.5	36%
8.0	46%
8.5	53%

Values indicate mean of duplicate observations.

Phospholipase activity was assayed as described in Materials and Methods. Tris - malic acid buffer was used for pH between 6.0 and 7.5, and tris - HCl for pH between 7.5 and 8.5.

TABLE 3.11

INHIBITION OF PERITONEAL EXUDATE PLA_2 BY MEPACRINE AT pH 6.0 AND LONG TERM STORAGE OF THE ENZYME PREPARATION AT 4°C

AGE (WEEKS)	% INHIBITION CAUSED BY 0.2mM MEPACRINE
1	33%
12	50%
24	70%

Values represent mean of duplicate determinations, and phospholipase activity was assayed as described earlier.

FIGURE 3.26 (a) and (b).

Variation of Ca^{2+} and inhibition of peritoneal exudate PLA_2 by mepacrine.
Reaction mixtures contained 40mM Tris - malic acid buffer pH 6.0, 0 - 0.3 mM EDTA or 0 - 1 mM $CaCl_2$, 0.5mg exudate protein and 1.15×10^9 autoclaved (^{14}C) E.coli without (circles) or with 90 μM (squares), or 200 μM (triangles) mepacrine.
Incubations were for 5 mins at 37°C.

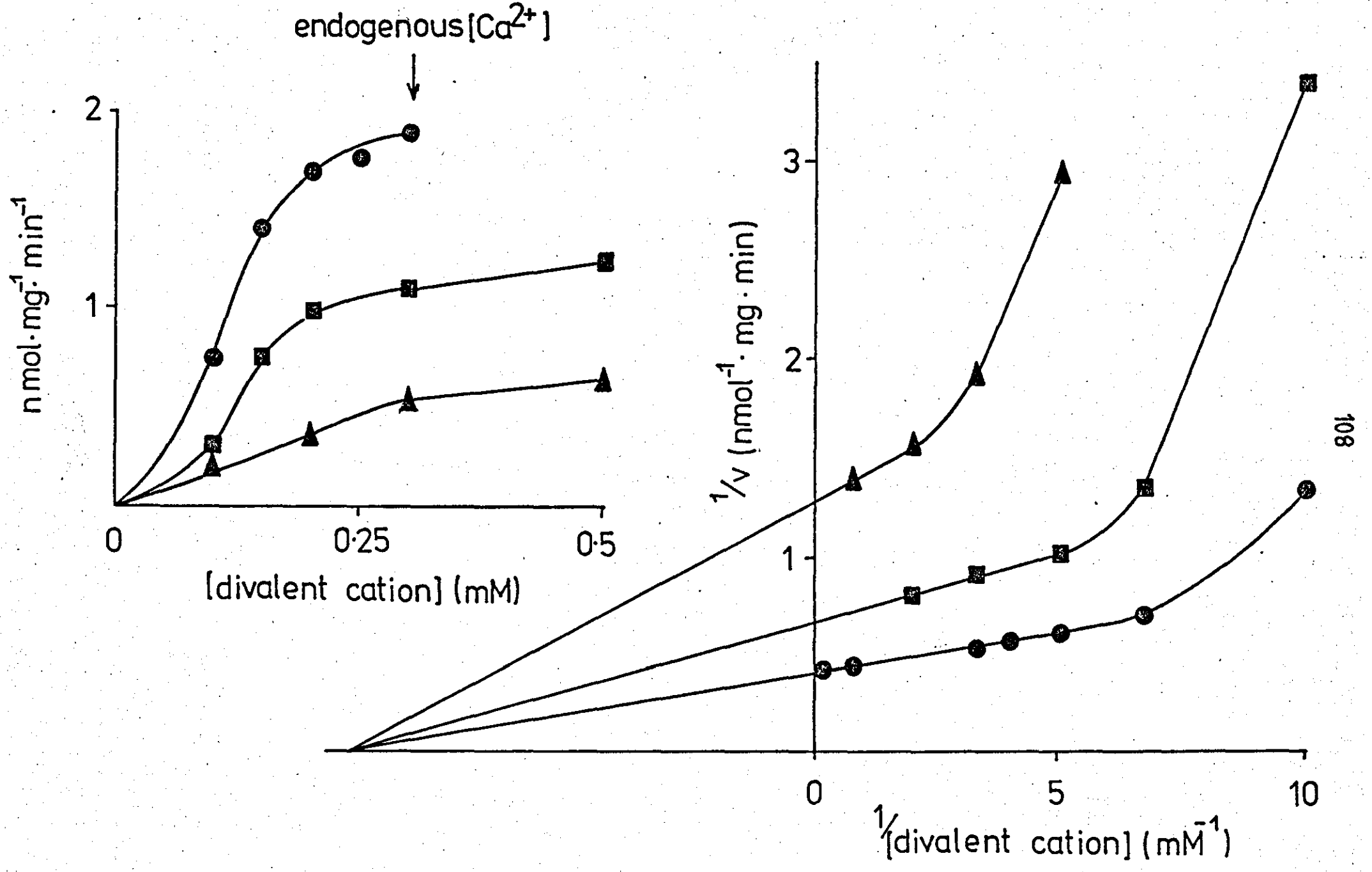

endogenous [Ca2+]
2
1
nmol·mg-1·min-1
0
0·25
0·5
[divalent cation] (mM)
3
2
1
1/v (nmol-1·mg·min)
0
5
10
1/[divalent cation] (mM-1)
108

FIGURE 3.27.

Low dose effects of chloroquine and mepacrine one ONE peritoneal exudate enzyme preparation.
Reaction mixtures contained 40 mM Tris - malic acid buffer pH 6.0, 5×10^8 autoclaved (^{14}C) *E.coli* (10 nmoles PL), 0.5 mg exudate protein, 5mM $CaCl_2$, and varying amounts of antimalarial drug.
All points are means of duplicate determinations.

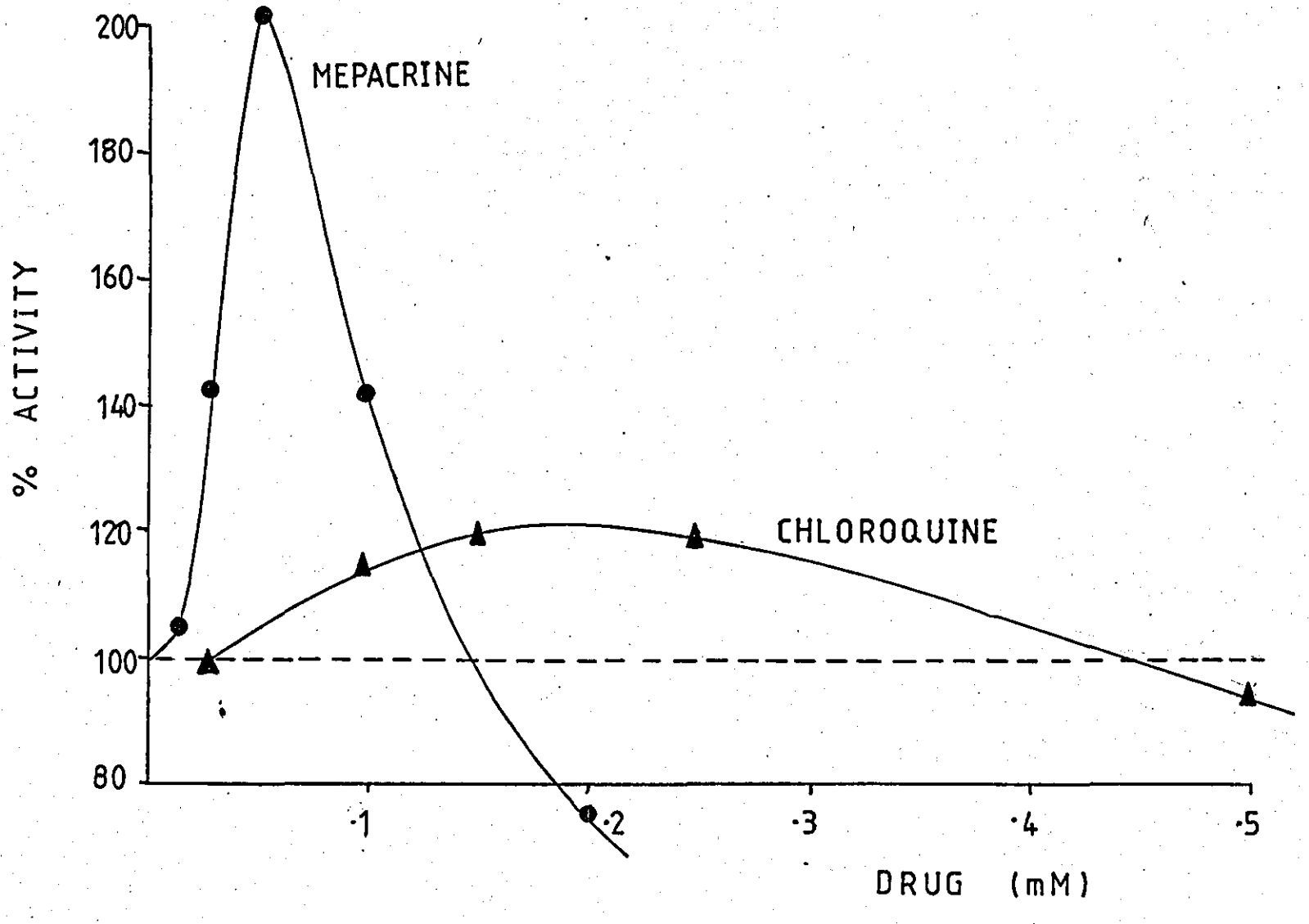

200
180
160
140
120
100
80
% ACTIVITY
MEPACRINE
CHLOROQUINE
·1
·2
·3
·4
·5
DRUG (mM)

TABLE 3.12

EFFECTS OF MEPACRINE (AT LOW DOSES) ON THE PERITONEAL EXUDATE PLA_2 USING ISOLATED, SONICATED E.COLI PHOSPHOLIPIDS

	CONTROL	μM MEPACRINE 30	50	75
% HYDROLYSIS OF	3.0	2.6	2.6	2.5
20 NMOLES PL	3.0	2.8	3.0	2.8
AVERAGE OF DUPLICATES	3.0	2.7	2.8	2.65

LEGEND

Isolated, sonicated E.coli phospholipids were prepared as follows: Phospholipids of 10^{10} E.coli (200 nmoles) were extracted by the method of Bligh and Dyer, and the organic solvents were evaporated to dryness.

1ml 0.9% NaCl was added and sonication carried out for 2 min., 2 min. and 1 min with 2 minutes between each sonication period.

20 nmoles PL or 100 μl had radioactivity of 7609 dpm. and used for each assay along with 5mM Ca^{2+}, 40mM buffer pH.6.0, 0.7mg enzyme protein, and incubated at 37°C for 10 mins.

After enzyme assays the total extracted radioactivity was 6446 ± 337 dpm (9determinations).

0.7mg enzyme protein assayed against 20 nmoles intact E.coli phospholipids showed 24% hydrolysis (2 determinations 22.4%, 25.5%).

whilst at pH 8.5 the same level of drug caused 53% inhibition.

c) Age of enzyme preparation

The age of the enzyme preparation was also found to have a measurable effect on the inhibitory activity of mepacrine (table 3.11). Initially enzyme activity in the presence of 0.2mM mepacrine was 67% of control values and fell to 30% after 24 weeks.

d) The effect of variation of calcium on the inhibition of peritoneal fluid phospholipase A_2 by mepacrine

The Kinetics of calcium variation on the inhibitory property of mepacrine on this system are shown in figures 3.26(a) and (b). Sigmoidal curves are obtained both in the presence and absence of mepacrine (figure 3.26a). The data in the presence of less than 0.3mM divalent cation concentration was obtained by adding varying concentrations of EDTA. With the data expressed as a double reciprocal plot, it appeared that calcium interfered with the inhibition by mepacrine in a non - competitive manner, and contrasted with the results obtained using pancreatic phospholipase A_2 and egg - yolk substrates.

e) Low - dose stimulatory effects by chloroquine and mepacrine

On one peritoneal fluid enzyme preparation, at doses lower than those causing inhibition, chloroquine (250 μM) and mepacrine (50 μM) stimulated enzyme activity at pH 6.0 (figure 3.27). The greatest effect was seen with mepacrine at 50 μM where a 100% stimulation of enzyme activity was observed. Chloroquine produced a 20% stimulation

at 250μM. This effect was reproducible only on this particular enzyme preparation and could not be observed on other preparations. These effects were reproducible up to 2 months storage of the enzyme prep - aration but were lost after this period.

Previous workers (Sherphof and Westenberg, 1975) have observed similar inconsistences when working with pancreatic phospholi - pase A_2 and labelled mitochondrial lipids. Such effects were explained by the nature of the interfacial substrate and were not obtained with purified lipids. In our system using isolated E.coli phospholipids as sonicated, micellar dispersions failed to reproduce the low dose stimulatory effects by mepacrine and chloroquine that was seen on one peritoneal fluid enzyme preparation. Table 3.12 shows the data obtained using the isolated E.coli phospholipids, and as can be seen low doses of mepacrine does not have much effect. The results also show an 8 - fold reduction of hydrolytic activity when micellar dispersions of phospholipids are used as compared with intact membrane phospholipids.

f) Effects of other anti - inflammatory and anti - arthritic agents on the peritoneal fluid phospholipase A_2

Table 3.13 shows the data obtained with a variety of drugs using 10^{-3}M as the drug concentration. The anti - arthritic drugs sodium aurothiomalate and D - penicillamine were without effect but the anti - inflammatory agents did show inhibitory effects. Indomethacin (93%) and benzydamine (81%) were particularly effective.

TABLE 3.13

SCREENING OF ANTI - RHEUMATIC AND ANTI - INFLAMMATORY AGENTS ON THE PERITONEAL FLUID PLA_2 ACTING AT pH 6.0

DRUG (10^{-3}M)	ACTIVITY NANOMOLES PL HYDROLYSED/MIN/mg PROTEIN	cf. CONTROL
CONTROL	5.6	100%
SODIUM AUROTHIOMALATE	5.75	103%
D-PENICILLAMINE	5.84	104%
CONTROL	1.73	100%
INDOMETHACIN	0.12	7%
BENZYDAMINE	0.322	18.6%
ASPIRIN	1.37	78%
SALICYLIC ACID	0.782	45%

Values represent average of duplicates which did not vary by more than 0.2 nmoles PL hydrolysed/min/mg protein. Phospholipase assays were determined as described in Materials and Methods.

3.3 Effects of chloroquine - like agents on the phospholipase activity of polymorphonuclear leucocytes

a) Studies on sonicated neutrophil suspensions

The experiments were first carried out on neutrophil suspensions that had been sonicated to release all the intracellular enzymes. The effects of chloroquine, mepacrine and primaquine on the phospholipase A_2 activity at pH 6.5 towards E.coli phospholipids are shown in figure 3.28. Both chloroquine (0.25 - 10mM) and mepacrine (0.025 - 5mM) showed a dose - related stimulation of phospholipase activity. Mepacrine was 10 times more active than chloroquine. No inhib - ition of phospholipase activity was observed at any time of the conce - ntrations studied.

Primaquine on the other hand showed a different res - ponse. At doses between 0.1 - 1.0mM a slight stimulatory effect was recorded but at higher levels a strong inhibition was observed.

Phospholipase A_2 activity released from neutrophils suspended in water also showed stimulation by 0.5mM mepacrine (table 3.14).

b) Effect of mepacrine on the phospholipase activity of neutrophil 8200g granules

A purer preparation of neutrophil phospholipase A_2 was obtained by centrifugation of a neutrophil homogenate in sucrose

FIGURE 3.28.

Effects of antimalarial drugs on the PLA_2 activity of sonicated neutrophils.
Reaction mixtures contained 0.1 mg enzyme protein, 10^9 autoclaved labelled *E.coli* (20 nmoles PL), 5mM Ca^{2+}, 40mM Tris buffer pH 6.5, and varying amounts of antimalarial drugs. Incubations were for 30 mins at 37°C.
All points are means of duplicate determinations.

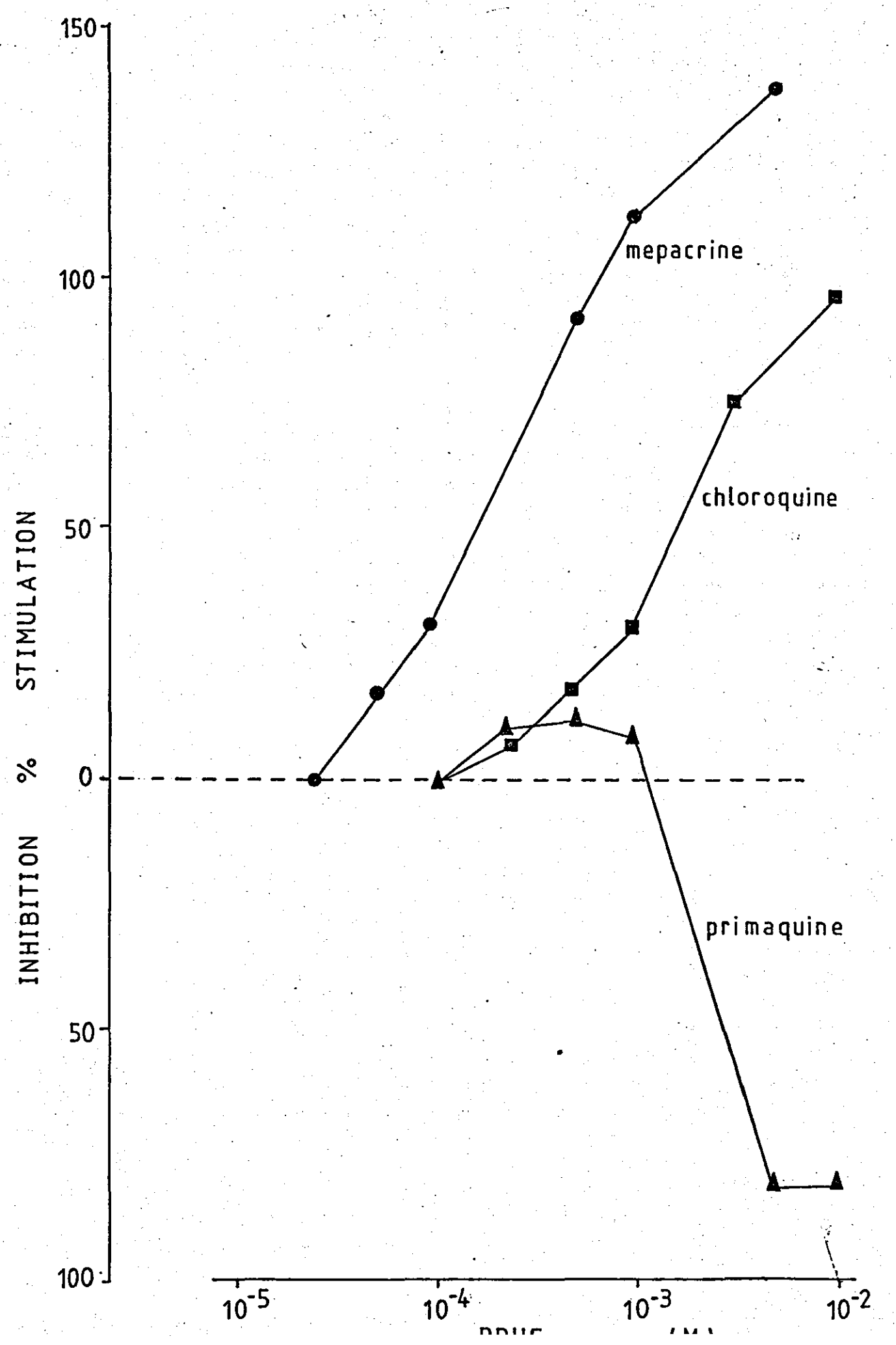

150
100
50
0
50
100
INHIBITION % STIMULATION
mepacrine
chloroquine
primaquine
10^{-5}
10^{-4}
10^{-3}
10^{-2}

TABLE 3.14

EFFECT OF 0.5mM MEPACRINE ON PLA_2 FROM NEUTROPHILS SUSPENDED IN WATER (5×10^7 CELLS/ml)

	CONTROL	+0.5mM MEPACRINE	STIMULATION OF ACTIVITY
% HYDROLYSIS OF 20NMOLES PL	10.5, 6.7	16.1, 13.5	
MEAN	8.6	14.8	72%

Reaction mixtures contained 0.24mg protein (neutrophils lysed in water), 40mM buffer pH 6.0, 5mM Ca^{2+}, 10^9 autoclaved labelled E.coli, with or without drug, and incubations were carried out for 30 min at 37°C.

TABLE 3.15 AND 3.16

STIMULATION OF NEUTROPHIL GRANULE (8,200g PELLET) PHOSPHOLIPASE A_2 BY MEPACRINE

	% HYDROLYSIS OF 20 NMOLES PL	NMOLES PL HYDROLYSED/HR /mg PROTEIN	% STIMULATION
pH 6.5	(AVERAGE)		
CONTROL	14.9 11.5 ⟩ 13.2	21.1	0
MEPACRINE +50 μM	17.3 19.6 ⟩ 18.5	29.6	40%
+100 μM	22.0 23.3 ⟩ 22.7	36.3	72%
pH 9.0			
CONTROL	17.5 14.2 ⟩ 15.9%	25.4	0
MEPACRINE +50 μM	19.7 16.4 ⟩ 18.1%	28.8	14%
+100 μM	20.0 21.0 ⟩ 20.5%	32.8	29%

Reaction mixtures contained 0.5mg protein (8,200g pellet), 40mM buffer (tris - malic acid pH 6.5, tris - HCl pH 9.0), 10^9 autoclaved *E.coli*, 5mM Ca^{2+}, with or without drugs and incubated for 15 min at 37°C.

TABLE 3.17

STIMULATION OF ZC RELEASED PLA_2 FROM NEUTROPHIL LEUCOCYTES, BY MEPACRINE AT pH 6.0

	% HYDROLYSIS OF 20 NMOLES PL	NMOLES PL HYDROLYSED/HR /mg PROTEIN	% STIMULATION
CONTROL	11.0 9.9 > 10.5%	17.5	0
+ 0.5mM MEPACRINE	16.9 17.0 > 17.0	28.3	62%

Reaction mixtures contained 40mM tris – malic buffer pH 6.0, 5mM Ca^{2+}, 0.12mg protein, 10^9 autoclaved radiolabelled E.coli, with or without 0.5mM mepacrine, and incubated for 1hr at 37°C.

TABLE 3.18

EFFECT OF CHLOROQUINE SULPHATE (1mM) ON THE ZYMOSAN - COMPLEMENT RELEASED PLA_2 ACTIVITY OF PMN NEUTROPHILS

	% HYDROLYSIS OF 20 NMOLES PL	NMOLES PL HYDROLYSED/HR /mg PROTEIN	% CHANGE
pH 6.0	AVERAGE		
CONTROL	9.2, 9.8 > 9.5	20.4	
+ CHLOROQUINE	14.5, 13.5 > 14.0	30.1	+47%
pH 7.5			
CONTROL	9.0	19.4	
+ CHLOROQUINE	15.0	32.3	+66%
pH 9.0			
CONTROL	19.7, 20.5 > 20.1	43.2	
+ CHLOROQUINE	14.7, 14.7 > 14.7	31.6	-27%

Reaction mixtures contained 5mM Ca^{2+}, 40mM buffer, 10^9 autoclaved radiolabelled *E.coli*, 93μg protein, with or without 1mM chloroquine and incubated for 1hr at 37°C.

at 8,200g. The 8,200g pellet consists of nuclei, and lysosomal granules and contained over 90% of the total phospholipase activity of the homogenate. 100 μM mepacrine tested against this granule preparation at pH. 6.5 showed a 72% stimulation of phospholipase activity (table 3.15). When the same determinations were carried out at pH 9.0, the stimulation of activity was only 29% (table 3.16) showing that stimulation of activity was greatest at acid pH.

c) Effect of mepacrine and chloroquine on Z - C released phospholipase A_2

The phospholipase A_2 released by neutrophils exposed to zymosan - complement particles also showed stimulation of enzyme activity in the presence of mepacrine comparable to the effects seen above (table 3.17). Chloroquine at 1mM concentrations also stimulated the Z - C released phospholipase A_2 activity at pH 6.0 and 7.5, but the same level at pH 9.0 caused an inhibition (table 3.18).

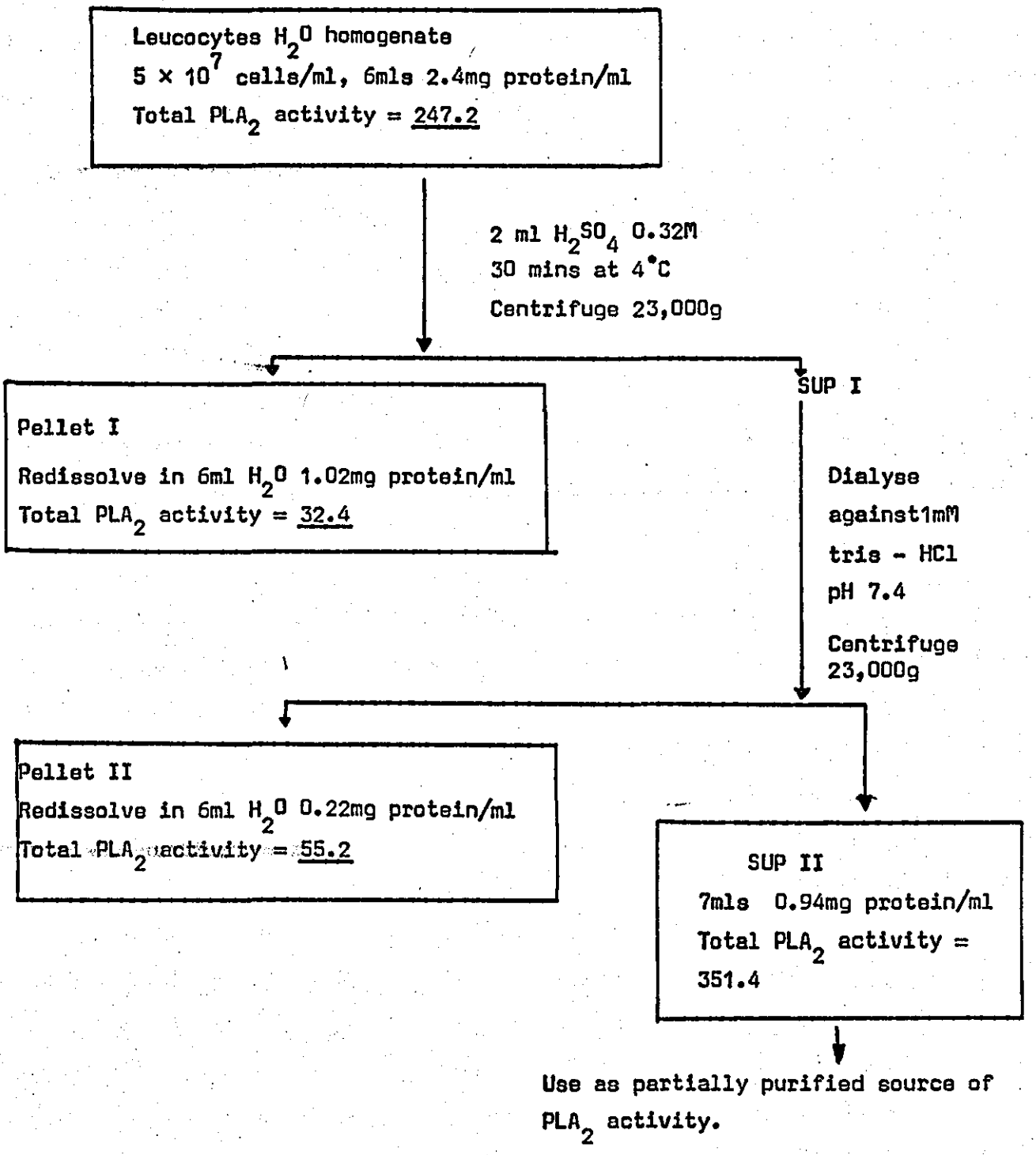
d) Studies on a partially purified phospholipase A obtained by sulphuric acid extraction

The effects of the antimalarial drugs were further investigated by obtaining a partially purified preparation of phospholi - pase activity from neutrophil leucocytes by sulphuric acid extraction. This procedure yielded a preparation (Sup II, figure 3.29) which cont - ained slightly more enzyme activity than the water homogenate with less than 40% of the original protein. There was a 3 fold increase of specific activity.

The effects of chloroquine, mepacrine and primaquine

FIGURE 3.29.

Partial purification of neutrophil leucocyte PLA_2 by sulphuric acid extraction



* PLA_2 activity is expressed as nonomoles *E.coli* phospholipids hydrolysed per hour.

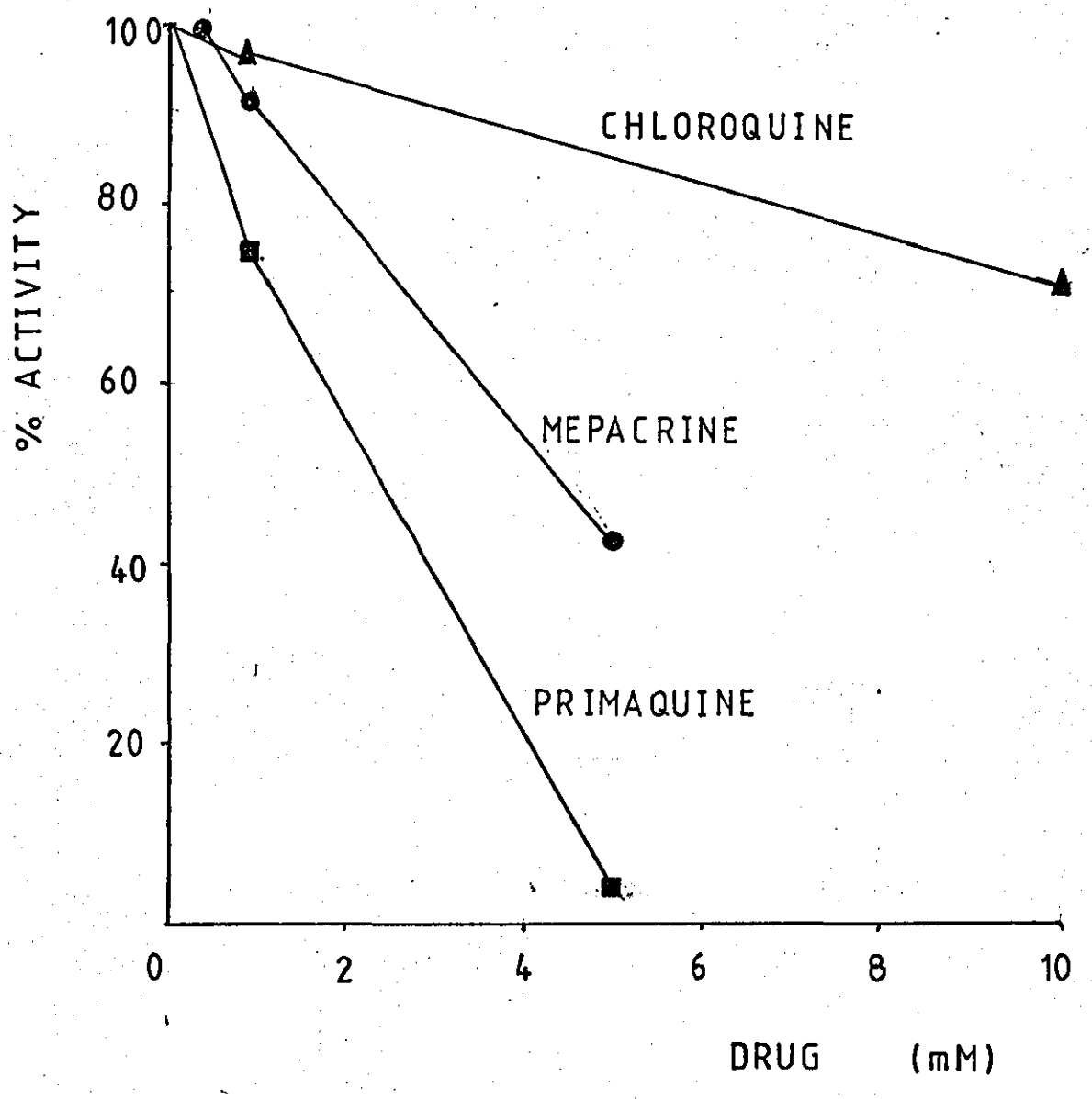


FIGURE 3.30.

Effects of antimalarial drugs on the acid purified preparation of neutrophils.

Reaction mixtures contained 40 mM Tris buffer pH 6.0, 0.047 mg enzyme protein, 5×10^8 autoclaved E.coli, 5mM Ca^{2+} and varying amounts of drugs.

Incubations were for 15 mins at 37°C.

100% Activity ie. control = 3.45. nmoles fatty acid released per min per mg protein.

All points represent mean of duplicate determinations.

on the partially purified enzyme preparation are expressed in figure 3.30. All these drugs showed inhibition of the purified phospholipase activity with no evidence of stimulatory activity. Mepacrine and chloroquine at doses which showed strong stimulation of the enzyme from sonicated neutrophils, 8,200g granules and Ž - C released phospholipase activities now showed inhibition of the acid purified enzyme from the same source. The inhibitory response with primaquine was of the same order and magnitude as that obtained with the homogenate enzyme preparation.

3.4 Interactions of heparin with inflammatory phospholipases and with antimalarial drugs

The antimalarial drugs are cationic amphiphilic agents. Thus the contrasting effects observed above may be due to alteration of phospholipase A_2 activities by interference with endogenous anionic agents. Such as the glycosaminoglycans heparin or chondroitin sulphate. These have been shown to be present in neutrophils in measurable quantities (Olsson and Gardell, 1967., Olsson et al, 1968., Avila and Convit, 1976).

a) Interaction of heparin with antimalarial drugs

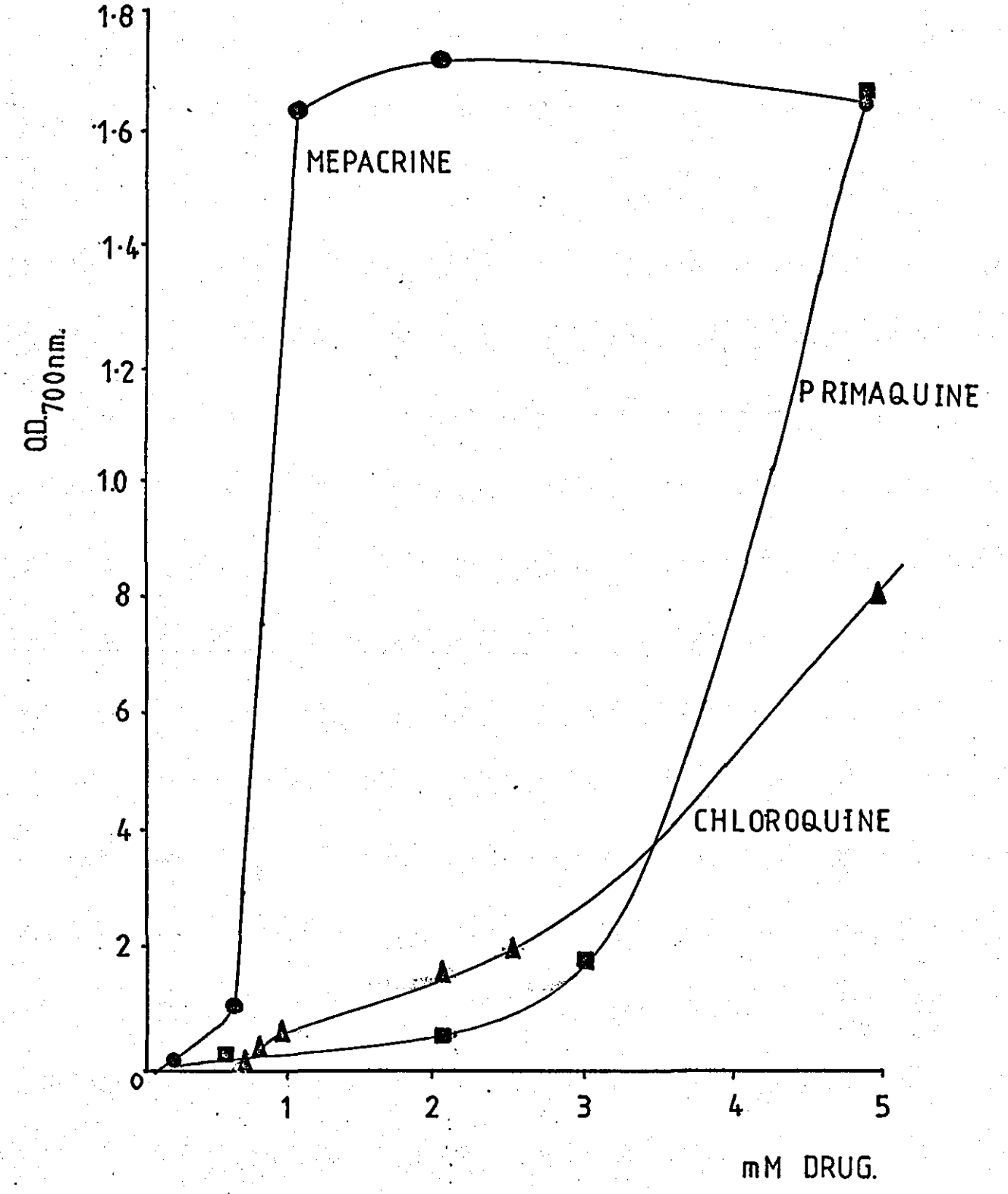
An interaction between chloroquine and heparin was immediately apparent when two solutions containing these compounds are mixed together. A visible complex was formed which could be monitored by optical density measurements at 700nm. The results obtained when 50 units/ml heparin was added to varying amounts of antimalarial drugs, are expressed in figure 3.31. All three drugs alone or heparin alone

FIGURE 3.31.

Interactions of antimalarial drugs with heparin

Heparin (50 units / ml final concentration) was mixed with varying amounts of antimalarial drugs and the resulting optical density changes were measured at 700nm.

Antimalarial drugs or heparin alone exhibited negligble O.D. at 700nm.


1·8
1·6
1·4
1·2
1.0
8
6
4
2
0
O.D.700nm.
MEPACRINE
PRIMAQUINE
CHLOROQUINE
1
2
3
4
5
mM DRUG.

showed less than 0.02 optical density units at 700nm. The complexes retained the original colours of the drug solutions; with mepacrine - yellow, primaquine - orange, and chloroquine - white. On the basis of optical density measurements mepacrine was the most active drug, followed by primaquine and then chloroquine.

b) Effects of heparin on inflammatory phospholipase activities

Heparin affected the phospholipase activities of both the peritoneal fluid and neutrophil leucocytes, although in opposite ways.

Stimulation of the peritoneal fluid phospholipase A_2 occured at physiological levels and was dose - dependent (figure 3.32). The stimulatory effect was greater at pH 6.0 then at 7.5 (table 3.19).

The effect of heparin on the acid purified neutrophil phospholipase was inhibitory. Table 3.20 shows the contrasting data obtained using the acid purified neutrophil enzyme and indicating that 6 units/ml heparin was sufficient to cause almost total inhibition of phospholipase activity.

Addition of mepacrine (5mM, which itself caused 52% inhibition) and varying amounts of heparin (1 - 40 units/ml) to the acid purified enzyme showed an additive inhibitory effect by the two agents (table 3.21). No antagonistic effect between the cationic and anionic agents was observed.

FIGURE 3.32.

Effect of heparin on the peritoneal exudate PLA_2

Reaction mixtures contained 40mM Tris - malic acid buffer pH 6.0, 0.3mg enzyme protein, 15×10^8 autoclaved E.coli, 5mM Ca^{2+} and varying amounts of heparin.

Incubations were for 10 mins at 37 C.

Control activities = 1.65 nmoles f.a. released / min / mg protein

= 100%.

Vertical bars represent 1 S.E.

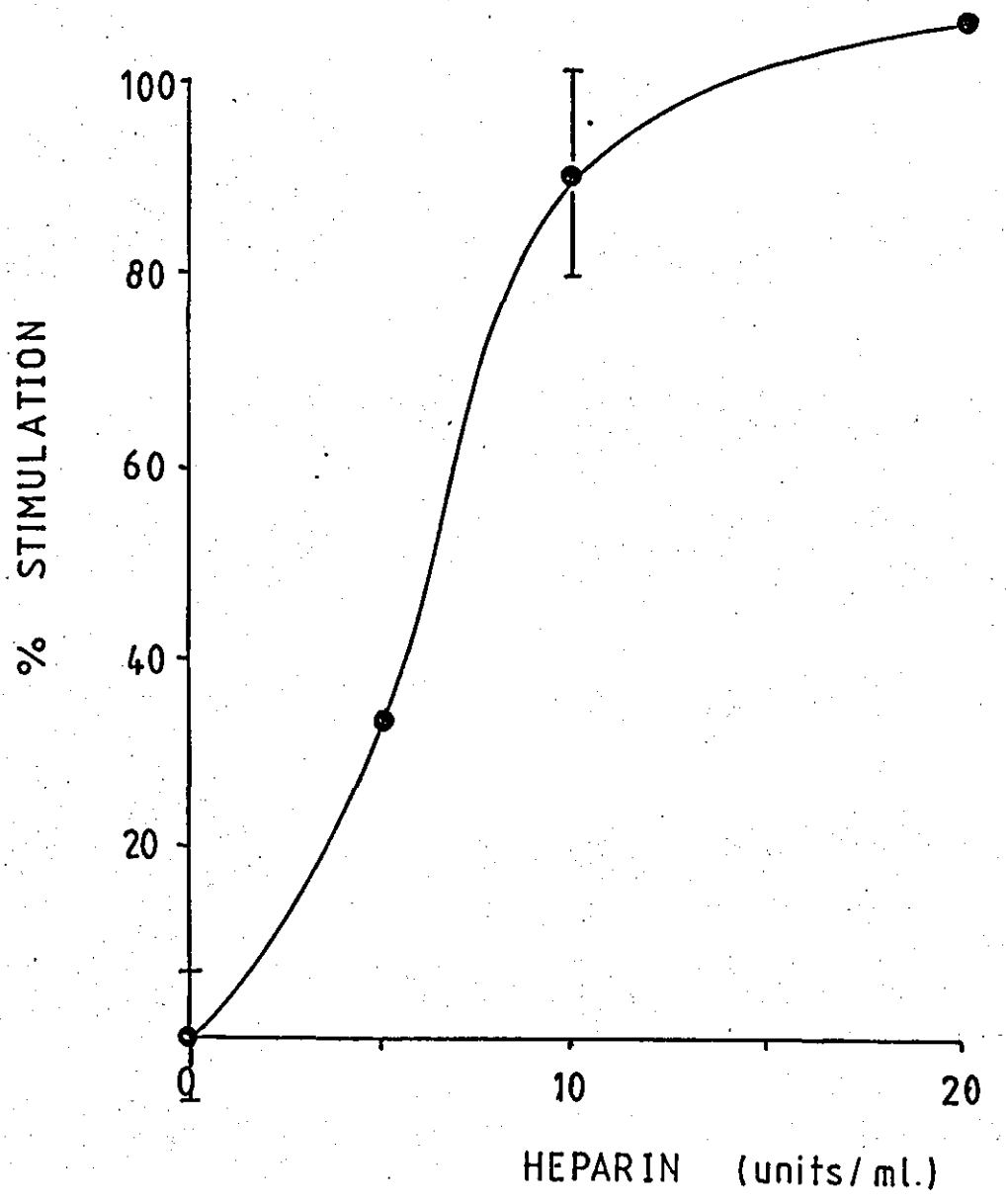

100
80
60
40
20
% STIMULATION
0
10
20
HEPARIN (units/ml.)

TABLE 3.19

ACTION OF HEPARIN ON THE PERITONEAL EXUDATE

PLA_2 AND VARIATION OF pH

pH	CONTROL	ENZYME ACTIVITIES* + 10 UNITS/ml HEPARIN	% INCREASE OF ACTIVITY
6.0	18.1, 18.1 MEAN = 18.1	36.3, 31.6 MEAN = 34.0	+88%
7.5	21.9, 17.0 MEAN = 19.5	28.0, 25.3 MEAN = 26.7	+37%

* Enzyme activities are expressed as % hydrolysis of 30 nmoles *E.coli* phospholipid.

Reaction mixtures contained 40mM tris – malic acid buffer 30 nmoles PL (15×10^8 autoclaved *E.coli*), 5mM Ca^{2+}, 0.3mg protein (peritoneal exudate), with and without 10 units/ml heparin, and incubated for 10 mins at 37°C.

TABLE 3.20

EFFECT OF HEPARIN ON THE ACID PURIFIED PLA_2 FROM NEUTROPHIL LEUCOCYTES

	CONTROL	HEPARIN UNITS/ml		
		2	4	6
ACTIVITY NMOLES PL HYDROLYSED/ MIN/mg PROTEIN	13.1, 14.4 mean = 13.75	11.3, 9.3 mean = 10.3	2.3, 1.9 mean = 2.1	0.69, 0.32 mean = 0.505
cf. CONTROL	100%	75%	15%	3.5%

Reaction mixtures contained 10 nmoles PL (5×10^8 E.coli), 40mM tris - malic buffer pH 6.0, 0.047mg protein, 5mM Ca^{2+}, with or without heparin, and incubated for 15 min at 37°C.

TABLE 3.21

EFFECT OF MEPACRINE AND VARYING AMOUNTS OF HEPARIN TOGETHER ON THE ACID PURIFIED PLA_2 FROM NEUTROPHIL LEUCOCYTES

	ENZYME CONTROL	+ 5mM MEPACRINE	5mM MEPACRINE + HEPARIN UNITS/ml					HEPARIN (1 U/ml) WITHOUT MEPACRINE
			1	5	10.	20	40	
% HYDROLYSIS OF 5×10^8 E.COLI	17.45	8.3	6.05	2.2	0.35	0.3	0.9	13.5
NANOMOLES PL HYDROLYSED/ MIN/mg PROTEIN	0.372	0.177	0.13	0.047	0.007	0.006	0.019	0.287
cf. CONTROL	100%	48%	35%	13%	2%	2%	5%	77%

% Hydrolysis values represent mean of duplicate determinations.

Reaction mixtures contained 5×10^8 autoclaved radiolabelled E.coli, 5mM Ca^{2+}, 40mM tris - malic buffer pH 6.0, 0.047mg protein, with or without drug mixtures, and incubated for 10 mins at 37°C.

3.5 Membrane actions of antimalarial drugs on guinea - pig erythrocytes

Cationic amphiphilic drugs (eg antimalarial drugs) being surface - active in nature are also considered to have an effect on membranes. In an attempt to help explain the paradoxical inhibitory/ stimulatory effects on phospholipases, the membrane actions of the three antimalarials on the hypotonic haemolysis of guinea - pig red blood cells were examined.

The results obtained are expressed in figure 3.33. All three of the antimalarial compounds did indeed show paradoxical dose - response curves.

At low doses all three drugs show stabilization of the erythrocyte membrane to osmotic shock. With mepacrine and primaquine maximum stabilization as measured by a reduction in the relative haemolysis was shown at 2×10^{-4}M. Chloroquine showed maximum stabili - zation at 10^{-3}M and was five times less active.

At higher doses all three drugs showed labilization of erythrocyte membranes (as measured by an increase in the relative haemolysis) to osmotic shock. Primaquine showed maximum disruption at 2.5×10^{-3}M, mepacrine at 5×10^{-3}M and chloroquine at 10^{-2}M. At even higher doses ie above 10^{-2}M the destabilizing effect was again reversed to stabilization again indicating the membranes having a tri - phasic response to the drugs.

FIGURE 3.33.

Effects of antimalarial drugs on the hyptonic haemolysis of guinea - pig erythrocytes.

Reaction mixtures contained 50 µl fresh heparinised blood in 0.425% NaCl and indicated amounts of antimalarial drugs.

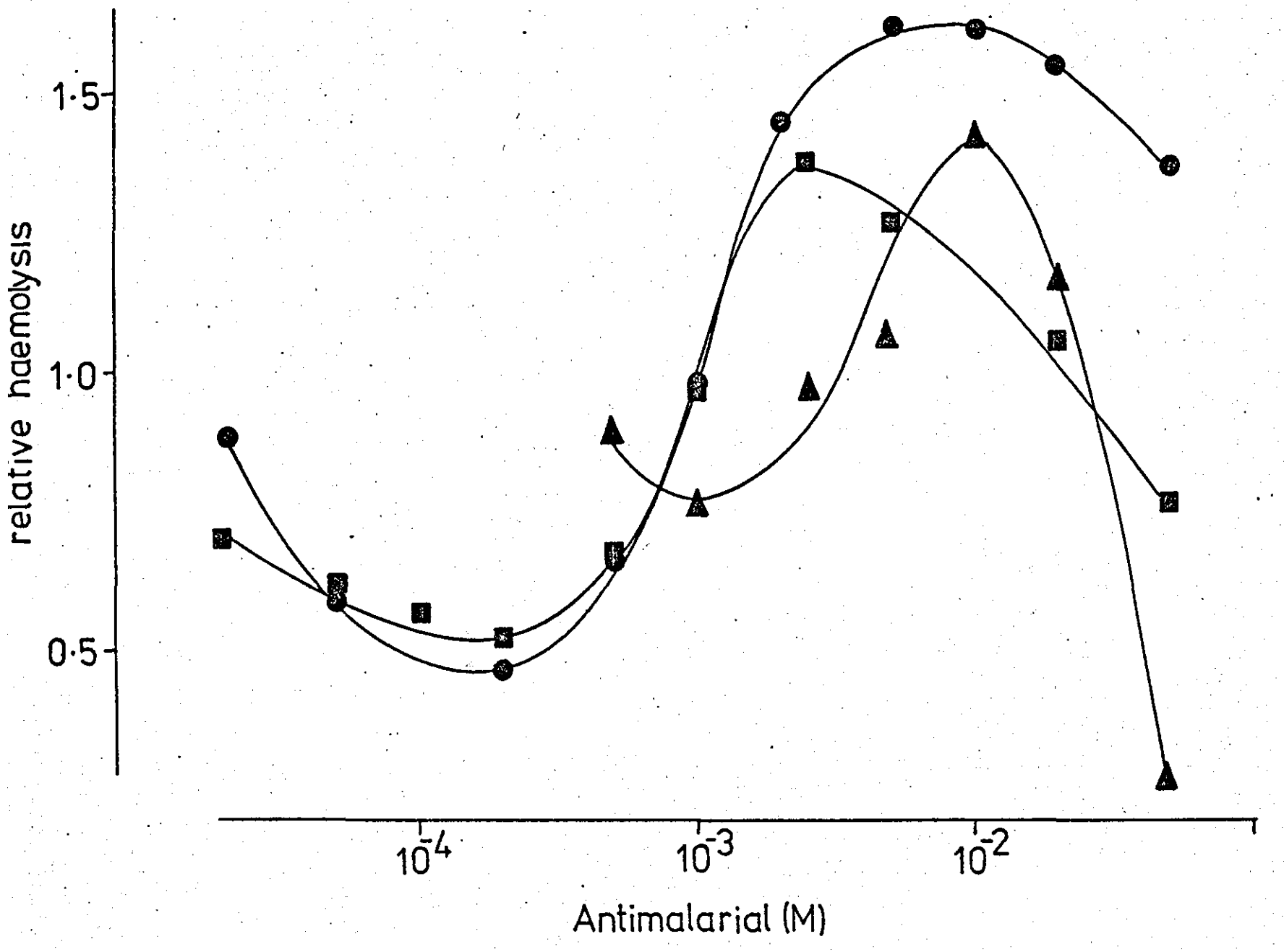
mepacrine = ●

primaquine = ■

chloroquine = ▲

Incubations were performed for 30 mins at room temperature

0.425% NaCl alone caused 50% haemolysis of g - p erythrocytes.


1·5
1·0
0·5
relative haemolysis
10^{-4}
10^{-3}
10^{-2}
Antimalarial (M)

PART D STUDIES OF AN ENDOGENOUS INHIBITOR OF PHOSPHOLIPASE A_2 IN THE 8,200g SUPERNATANT OF NEUTROPHIL LEUCOCYTES

4.1 Effect of the neutrophil 8,200g supernatant on the peritoneal fluid phospholipase A_2 activity

Figure 3.34 shows the effects of adding different amounts of 8,200g supernatant protein to reaction mixtures containing sufficient peritoneal fluid phospholipase A_2 activity to cause approx - imately 20% hydrolysis at pH 9.0. Enzyme activity was inhibited in a dose - dependent manner and maximal inhibition of 64% was obtained with 230 μg supernatant protein. Similar findings were obtained at pH 6.0, and indeed as shown in figure 3.35, the degree of inhibition caused by the neutrophil 8,200g supernatant on the peritoneal fluid enzyme appeared to be independent of pH (between pH 5.5 and 9.0).

However inhibition of phospholipase activity by the 8,200g supernatant was not independent of pH if the neutrophil granule (8,200g pellet) phospholipase activity was used. Indeed as shown by table 3.22 the 8,200g supernatant does not have very much effect on the granular enzyme activity at pH 6.5, although inhibition of enzyme activity has been observed at pH7.5 (Franson et al, 1974).

FIGURE 3.34.

Effect of 8,200g neutrophil leucocyte supernatant on the hydrolytic activity of peritoneal exudate PLA_2.
Reaction mixtures contained 40mM Tris - HCl buffer pH 9.0, 5mM Ca^{2+}, 15×10^8 autoclaved E.coli (^{14}C), either 0.3mg enzyme protein and varying amounts of neutrophil 8,200g supernatant protein (circles) or just varying amounts of 8,200g supernatant protein (triangles). All points are mean of duplicate determinations.

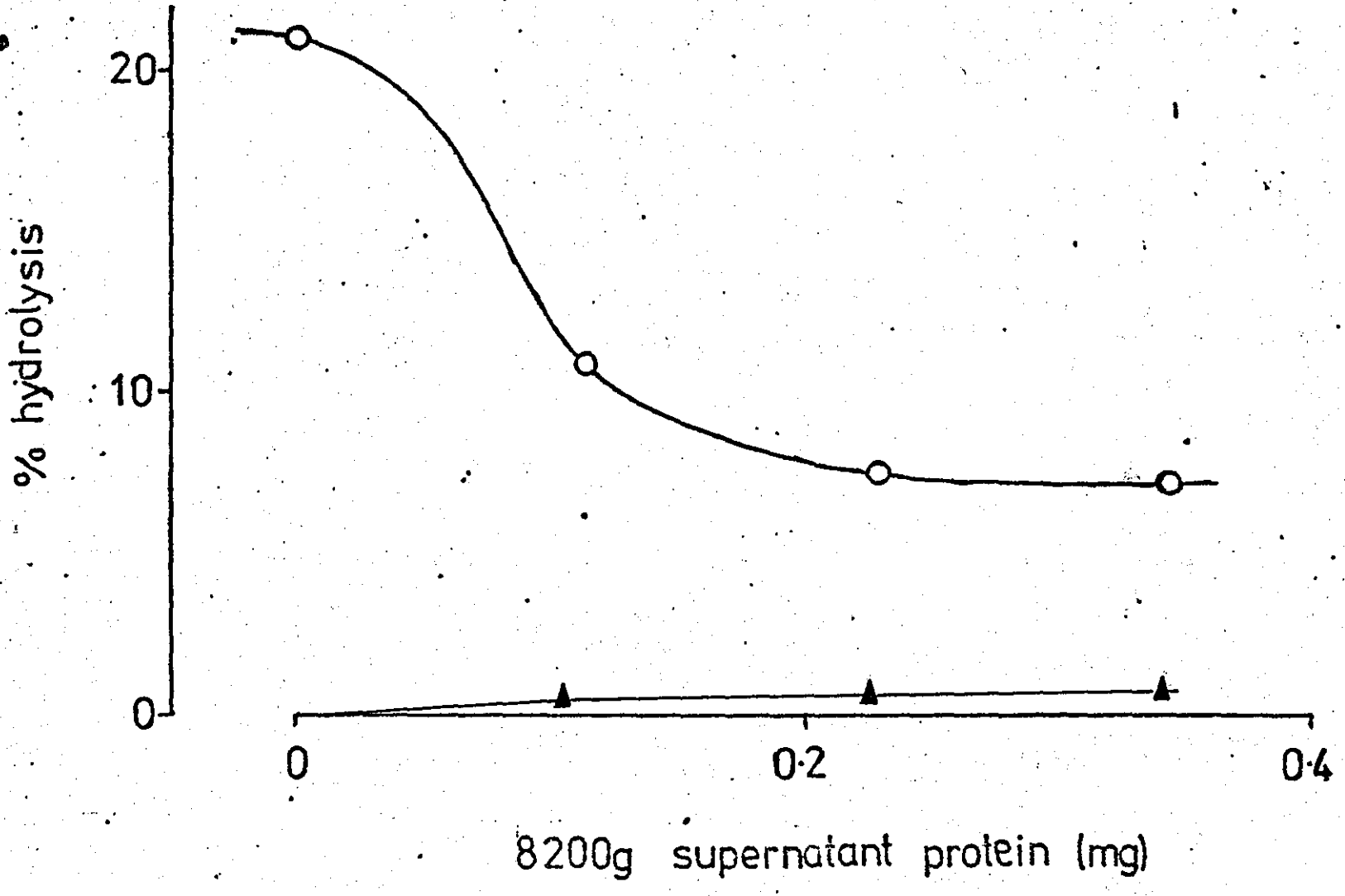

% hydrolysis
20
10
0
0
0·2
0·4
8200g supernatant protein (mg)

FIGURE 3.35.

Effect of pH on the activity of the peritoneal exudate PLA_2 in the presence and absence of neutrophil 8,200g supernatant protein (0.42mg).

Incubations were as described in Materials and Methods with 0.28mg peritoneal exudate protein.

All points are means of duplicate determinations.

— O control

■ + 8,200g supernatent.

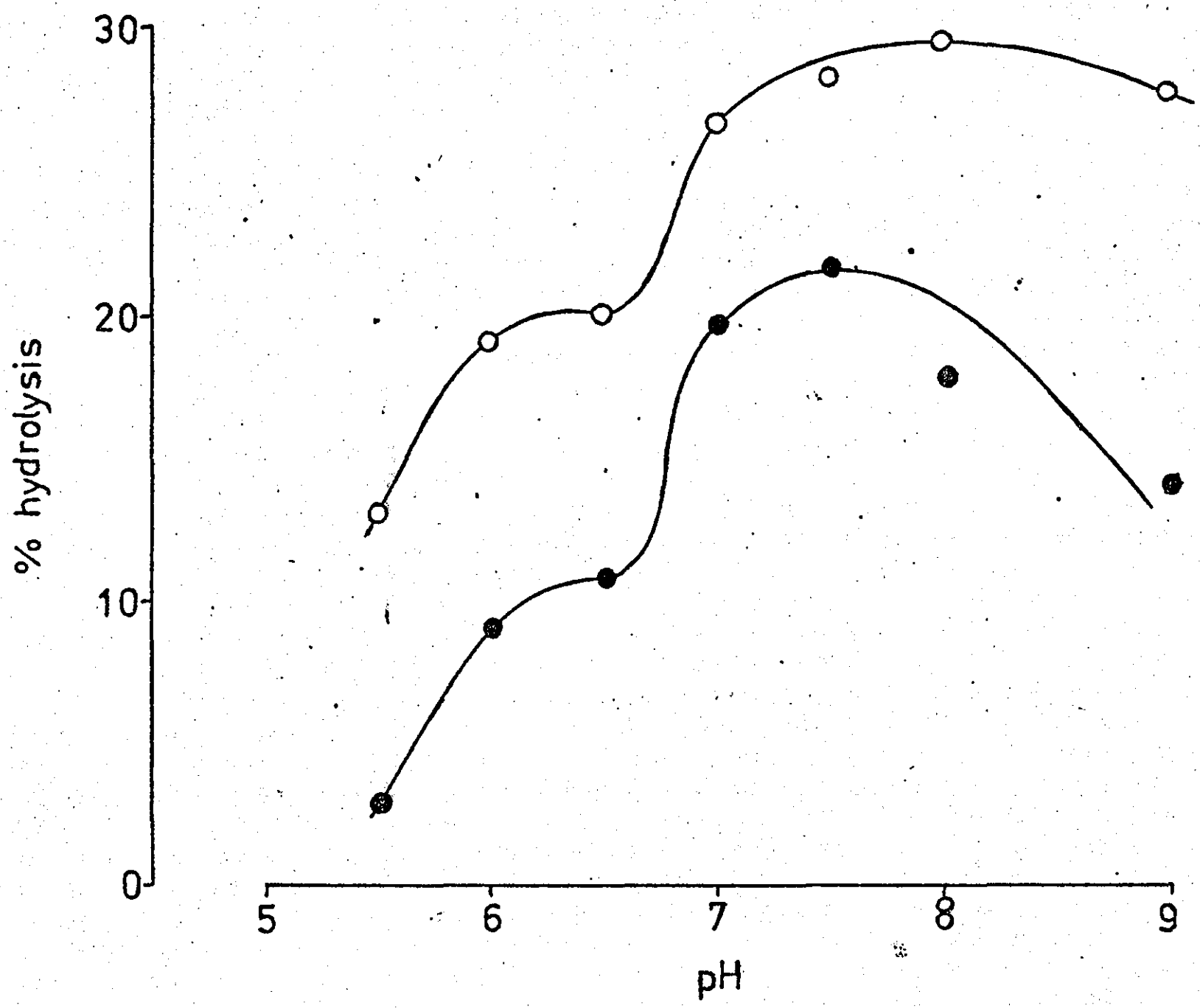

30
20
10
0
% hydrolysis
5
6
7
8
9
pH

TABLE 3.22

EFFECT OF 8,200g SUPERNATANT ON THE NEUTROPHIL GRANULE PHOSPHOLIPASE ACTIVITY AT pH 6.5

	CONTROL GRANULES ALONE	+ 8,200g SUPERNATANT 105μg	230μg	355μg
ACTIVITY % HYDROLYSIS OF 20 NMOLES PL	9.8, 9.6 mean = 9.7	9.1, 9.6 mean = 9.35	8.0, 9.9 mean = 9.0	8.5, 7.5 mean = 8.9
cf. CONTROL	100%	96%	93%	92%

Reaction mixtures contained 40mM tris - maleate buffer pH 6.5, 5mM Ca^{2+}, 10^9 auto - claved radiolabelled E.coli, 0.5mg enzyme protein (8,200g pellet containing granules) and with or without varying amounts of 8,200g supernatant protein. Incubations were carried out at 37°C for 15 mins.

4.2 Effect of the PMN 8,200g supernatant on the phospholipases from pig pancreas and Crotalus adamenteus and Naja naja venoms

In an attempt to further investigate its selectivity of action, the endogenous phospholipase inhibitor present in the 8,200g supernatant of neutrophil leucocytes was tested against phosp - holipases from pig pancreas and venoms of Crotalus adamenteus and Naja naja towards E.coli phbspholipids at pH 7.5. Table 3.23 shows the results obtained.The 8,200g supernatant displayed inhibitory effects of a similar potency with the enzymes from rabbit peritoneal exudate, the highly purified phospholipase A_2 from pig pancreas and the crude enzyme from Crotalus adamenteus venom (39%, 45% and 26% respectively with 420μg 8,200g supernatant protein). However with Naja naja venom a marked stimulation of activity was observed (+58%).

To check whether these effects were due to non - specific protein actions, the experiments were repeated with a similar concentration of BSA (420μg) that itself exhibited no phos - pholipase activity. In all cases a marked stimulation of phospholipase activity was observed ranging from +36% with Crotalus adamenteus venom to +63% with the enzyme from pig pancreas.

All the phospholipases examined were calcium dependant as addition of 1mM EDTA resulted in total loss of enzyme activities (table 3.24). The inhibitory activity of the 8,200g supernatant was not very stable to storage, and lost approximately one - half of its inhibitory potency over 3 weeks storage at +4°C, as tested against the rabbit peritoneal fluid phospholipase A_2 (table 3.25).

TABLE 3.23

EFFECT OF 8,200g NEUTROPHIL LEUCOCYTE SUPERNATANT, AND BOVINE SERUM ALBUMIN (BSA) ON THE HYDROLYTIC ACTIVITY OF SOLUBLE PHOSPHOLIPASES A_2 TOWARDS (1-^{14}C) OLEATE LABELLED ESCHERICHIA COLI PHOSPHOLIPIDS

ENZYME SOURCE	PHOSPHOLIPASE A_2 ACTIVITY (% CONTROL VALUES) IN THE PRESENCE OF 8,200g SUPERNATANT	BSA(% CONTROL VALUES)
PERITONEAL EXUDATE	61	141
PIG PANCREAS	55	163
CROTALUS ADAMENTEUS VENOM	74	136
NAJA NAJA VENOM	158	138

Incubations were carried out as described in Materials and Methods at pH 7.5 in the presence of 420 μg, 8,200g supernatant, or BSA protein. Enzyme protein (peritoneal exudate 0.35mg; pig pancreas 0.05ng; *Crotelus adamenteus* 1 μg and *Naja naja* 0.5 μg) was sufficient to produce approximately 20% hydrolysis of labelled bacterial phospholipids.

TABLE 3.24

EFFECT OF EDTA ON PHOSPHOLIPASES. CALCIUM DEPENDANCY

ENZYME SOURCE	CONTROL ACTIVITIES*	+ 1mM EDTA
PERITONEAL EXUDATE	19.6	0
PIG PANCREAS	20.7	0
CROTAL S ADAMENTEUS VENOM	19.3	0
NAJA NAJA VENOM	32.4	0

* Control activities expressed as % hydrolysis of 30 nmoles PL, and in the presence of 5mM Ca^{2+}.

Reaction mixtures contained same amounts of enzyme proteins as described in table 3.23, and 15×10^8 autoclaved E.coli, 40mM tris - malic buffer pH 7.5 and either 5mM Ca^{2+} or 1mM EDTA. Incubations were carried out for 15 mins at 37°C.

TABLE 3.25

INHIBITORY POTENCY OF 8,200g SUPERNATANT AGAINST RABBIT PERITONEAL PLA_2 AND STORAGE AT 4°C

TIME (DAYS)	% INHIBITION SHOWN BY 420μg 8,200g SUPERNATANT ON 280g ENZYME PROTEIN (PERITONEAL EXUDATE)
1	42
5	39
15	33
22	26

% Inhibition values are calculated from duplicate control and test (ie + 8,200g supernatant) determinations. All assays were carried out at pH 7.5.

Chapter Four

DISCUSSION

DISCUSSION

PART A ESCHERICHIA COLI SUBSTRATE

The substrate used in this work for the determination of phospholipase A activities was E.coli radiolabelled with (1 - ^{14}C) oleic acid during growth, then autoclaved to destroy bacterial phospholipases and to render the lipids more accessible to hydrolytic attack. Use of bacterial membranes as subtrates for phospholipases was first described by Patriarca et al (1972) and subsequently by many authors. It has the following advantages:

(a) The presentation of the lipids as part of a biological structure, although altered by the autoclave procedure, probably provides conditions for the action of PLA_2 that are more physiological than those that prevail when the substrate is in micellar form.

(b) This substrate is readily broken down under conditions where micellar dispersions of isolated phospholipids are not (see later).

Uptake and distribution of (1 - ^{14}C) oleic acid in E.coli

Adequate uptake of (1 - ^{14}C) oleic acid by the bacterium during growth did require specific conditions. These included complexing of the fatty acid to albumin prior to contact with E.coli; a suitable number of organisms in log - phase of growth; and an optimal exposure time of the E.coli with the radiolabelled fatty acid of 3hrs. A furth r incubation of 2hr to allow maximum incorporation of the fatty acid into cellular phospholipids was also necessary.

Analysis of radiolabelled E.coli revealed that almost all of the (1 - ^{14}C) oleic acid appeared in the phospholipids. Indeed the distribution of radioactivity closely resembled the lipid composition. The most heavily labelled compounds were phosphatidyl ethanolamine (60%), phosphatidyl glycerol (20%) and cardiolipin (10%) with very little radioactivity appearing in lysophospholipids and neutral lipids. The presence of a small amount of radioactivity (approx. 4%) associated with free fatty acid may be due to adherence of fatty acid molecules to the organism, as this could be reduced to less than 1% by further washings in saline. Therefore the substrate used for most of the work was membrane - associated phosphatidyl ethanolamine.

The (1 - ^{14}C) oleic acid in E.coli phospholipids was exclusively in the 2 - position as determined using the specific phospholipase A_2 present in Crotalus adamenteus venom. This was to be expected as oleic acid is an unsaturated long chain fatty acid and such acids generally occupy the 2 - position of phospholipids (Fransen et al, 1973). This was confirmed using (1 - ^{14}C) linoleic acid which was incorporated in the 2 - position to 87%, and the unsaturated (1 - ^{14}C) palmitic acid which was incorporated preferentially (77%) in the 1 - position. Attempts to increase this selectively exclusive to the 1 - position failed even if cold oleic acid was included in the procedures.

PART B STUDY OF INFLAMMATORY PHOSPHOLIPASES

2.1 Release of phospholipase A from stimulated neutrophils

The results demonstrate that stimulation of rabbit peritoneal PMN leucocytes by zymosan - complement or exposure of these cells to extracellular calcium ions led to a release of phospholipase activity.

The release of the enzyme activity with ZC follows phagocytosis of the particles in a concentration dependant manner, and was not associated with cell lysis as only small amounts of the cytoplasmic enzyme, lactate dehydrogenase, were released. The source of the released enzyme activity would appear to be the lysosomes as β- glucuronidase and lysozyme were released to similar extents and in similar manner. The release of phospholipase activity was also time dependant and follows the time dependancy of phagocytosis ind - icating a link between the two.

The active principle in causing selective enzyme release is the complement component, C3b, which becomes bound to zymosan particles when incubated in serum. Zymosan particles alone have been shown not to cause release of lysosomal enzymes from neutrophils (Henson, 1971). Indeed the serum treated with zymosan has also been shown to contain a factor, C5a, which is able to induce release of lysosomal enzymes from cytochalasin B treated neutrophils (Goldstein et al, 1975). Thus the release process is initiated by the combination of specific receptors on the leucocyte plasma membrane with activated components of the complement pathway. In this system release of lysosomal constituents occurs by a process called "regurgitation during feeding" resulting from fusion of the lysosomes with incompletely

closed phagosomes (figure 1.3). The pattern of selective release occured in the absence of added calcium. Previous work by Henson (1971) using peripheral leucocytes and ZC was carried out in the presence of added calcium.

The present results also demonstrate that addition of calcium ions to rabbit peritoneal neutrophils suspensions is a suitable stimulus for lysosomal enzyme release as measured by release of β- glucuronidase and lysozyme. Under these conditions release to the suspending media of approximately 30% of the total cellular phospholipase A_2 activity, as assayed at pH 6.0 towards E.coli membrane phospholipids, occured in a concentration and time dependant manner.

The release of β- glucuronidase and lysozyme from rabbit peritoneal PMN leucocytes has been previously reported by Northover (1977). Approximately 15% selective release of both enzymes was recorded with 2.0mM Ca^{2+} together with a very low, 2%, release of LDH. In this study the amounts of enzymes released, both lysosomal and cytoplasmic, were greater and is perhaps a reflection of the state of the cells at the end of the experiment. The present observed release of LDH (10%) also reflects 90% cell viability as compared with 2% release of LDH and 98% cell viability, obtained by Northover. However the release of LDH was still much lower than that of the lysosomal enzymes β- glucuronidase and lysozyme and of phospholipase A_2.

Previous work on peripheral human PMN leucocytes has shown that Ca^{2+} alone only induces release of lysozyme and not β- glucuronidase (Goldstein et al, 1975) thus implying its ability to cause release of enzymes from the specific granules. The present

work on peritoneal polymorphs and in agreement with the findings of Northover (1977) suggests that both types of granule enzymes may be released. Thus peritoneal PMN leucocytes appear to show a greater capability of lysosomal enzyme release than the peripheral counterparts. However direct evidence, for or against this suggestion, requires further work on rabbit peripheral PMN's since the observed discrepan - cies may be species dependant. This difference in capability to lysoso - mal enzyme release by the calcium may be due to the fact that peritoneal PMN's are already in a "stimulated" state as they have migrated to the site of inflammation.

2.2 Properties of the released phospholipase A

These studies were carried out on the released enzyme obtained from zymosan - complement treated neutrophils. As calcium ions also caused a selective release of phospholipase A, the properties were assumed to be the same.

The released phospholipase activity has an unusual pH profile, having two pH optima, a broad peak in the acid region and a sharper one at alkaline pH with a consistent drop of activity between the two peaks at pH 7.5. This may indicate the presence of two phospholipid splitting activities , perhaps two isozymes, or a single protein may be responsible. Both activities show absolute requirements for Ca^{2+}, and maximal activity at 5×10^{-3}M Ca^{2+}. This property is in contrast to the lysosomal phospholipases of alveolar macrophages which are inhibited by Ca^{2+}, but also have a lower pH optimum of 4.0. From studies in the literature so far, there appears to be two basic types of lysosomal phospholipase activities:

1) Enzyme activity inhibited by Ca^{2+} and : active in the pH range 3.3 - 4.5, for example, macrophages.

2) Enzyme activity requiring Ca^{2+} and active in the pH range 5.0 - 7.5 (eg. leucocytes).

The activity is considered to be A_2 specific as all of the enzymically hydrolysed radioactivity, at both pH 9.0 and pH 6.0, from (1 - ^{14}C) oleic acid labelled E.coli, appeared in the fatty acid fraction and mone in the lysophospholipid fraction. Furth r use of (1 - ^{14}C) palmitate labelled E.coli, which is predominantly incorpor - ated in the 1 position, revealed the phospholipase activity at pH 6.0 of neutrophil homogenates to be A_2 specific as the radioactive product of hydrolysis was lysophospholipid and not fatty acid.

Kinetic studies examining the effects of time and protein concentrations indicated that phospholipase activity was linear for 40 minutes using up to 64 μg protein if 10 nmoles of substrate were used (5×10^8 autoclaved E.coli). The deviation from linearity after these values may be due to inhibition by products. Certainly free fatty acids and lysophospholipids have been demonstrated to inhibit rabbit PMN leucocyte granule phospholipases (Franson et al, 1974). Kinetic studies involving substrate variation revealed an apparent Km value for the enzyme of 25×10^{-6}M phospholipid.

Storage of the phospholipase A_2 activities of both sonicated neutrophil suspension and the released enzyme in HEPES buffered medium exhibited a difference in the stability of the acid and alkaline activities. The activity at pH 6.0 being approximately four times less stable than the activity at pH 9.0. This together with

the fact that the phospholipase activity has two pH optima, is perhaps a further indication that two proteins may be responsible.

2.3 Subcellular localisation of phospholipase activities

Selective release of phospholipase A activity without cell death and in conjunction with β- glucuronidase and lysozyme suggests its association with the granules of neutrophil leucocytes.

Three types of granules have been identified in rabbit PMN leucocytes by sucrose density gradient centrifugation and electron microscopy (Baggiolini et al, 1969., Wetzel et al, 1967).

(1) The primary or azurophil granules which contain most of the β- glucuronidase, myeloperoxidase, approximately 33% of the lysozyme and are the largest of the three types with a diameter of 0.5 - 0.8 μm.

(2) The secondary or specific granules which are present in the largest numbers, contain most of the alkaline phosphatase activity, 67% of the lysozyme activity and range in size from 0.3 - 0.5 μm in diameter.

(3) Tertiary granules which are the smallest (·05 - 0·2 μm), contain the remainder of the acid hydrolases, are often rod shaped and tend to occur in small clusters in the peripheral cytoplasm (Wetzel et al, 1967).

From our findings it is not possible to say with

which granule (s) the released phospholipase A_2 is associated. The phospholipase A_2 described in this study has however marked similarities to a membrane - bound phospholipase A_2 associated mostly with the azurophil and specific granules with some activity present in the tertiary ill - defined granules (Fransen et al, 1974).This latter enzyme also has two pH optima, at pH 5.5 and 7.5 with a drop in activity at pH 7.0. It also requires Ca^{2+}, shows similar kinetics with E.coli substrate, but differ in that the activity at both acid and alkaline pH is stable.

A similar study on the cellular localisation of phospholipase activities in human peripheral PMN leucocytes has also been reported (Fransen et al, 1977). Human PMN PLA_2 is also membrane - bound, Ca^{2+} dependant, has one optimum peak at pH 7.0, and is associ - ated in part with the azurophilic and specific granules but a major portion was found with an " empty vesicular fraction" containing 85% of the total alkaline phospholipase activity of whole homogenates. The empty vesicular fraction are probably tertiary granules as they sediment after specific granules from sucrose density zonal centrifu - gation procedures. Another difference is that phospholipases from rabbit PMNs and serum show 20 fold more activity than phospholipases from the corresponding human sources (Weiss et al, 1979; Kaplan - Harris et al, 1980). Whether this has any evolutionary significance is not known.

2.4 The role of PMN lysosomal phospholipase A_2 in the inflammatory Response

PMN leucocytes play an extremely important role in the infammatory process. In reponse to chemotactic stimuli they are able to migrate into tissues in substantial numbers and perform

the task of phagocytosis and degradation of biological material. Neutrophils are particularly important in defence against microorganisms and lysosomal phospholipases participate in the intracellular killing and breakdown of the organisms. Indeed when live E. coli labelled with (1 - ^{14}C) palmitic acid are incubated with rabbit neutrophils, within 15 minutes 99% of the E. coli are killed (non - viable) and 30% E. coli phospholipid breakdown occurs (Elsbach et al, 1972).

During phagocytosis or stimulation of PMN leucocytes by non - phagocytosable stimuli such as immune complexes bound to basement membranes, there occurs release of hydrolytic enzymes which are able to impart considerable damage to the surrounding tissues. Indeed in chronic inflammatory states such as rheumatoid arthritis much of the tissue damage is thought to result from lysosomal enzyme activity (Weissmann et al, 1972). The released phospholipase activity is able to hydrolyse phospholipids of cell membranes to yield polyenoic acids and lysophospholipids. Lysophospholipids are surface active and cyto - toxic agents and will potentiate the inflammation. Polyenoic fatty acids can be converted to prostaglandin - like products by cyclooxygenase enzymes which are ubiquitous.

PMN leucocytes themselves have been shown to release a variety of prostaglandin - like products during phagocytosis, for example PGE_2, PGF_2, (Higgs & Youlten, 1972), PGE_1 (Higgs et al, 1975), TXA_2 (Higgs et al, 1976), TXB_2 (Goldstein et al,1978), hydroxy acids (Borgeat et al,1976) and leucotrienes (Samuelsson et al, 1979).

The generation of all the prostaglandin - like products is dependant upon a supply of polyenoic fatty acids and as the major source of these are phospholipids then the action of

phospholipase A_2 is very important.

2.5 Phospholipase A activity of the cell - free peritoneal inflammatory exudate

The peritoneal inflammatory exudate obtained 17 hrs after thioglycollate injection, was found to be a very potent source of phospholipid splitting activity. This enzyme activity has properties some similar, some different, to the enzyme released from the neutrophil during phagocytosis of zymosan - complement particles. Using comparable concentrations of substrates (10 - 20 nmoles), substantial enzyme activity could be detected within 5 minutes incubation at 37°C. This preparation also showed an acid and alkaline optima although the position of the alkaline peak was at pH 8.0 compared with pH 9.0 for the neutrophil released activity. The phospholipase activity was also A_2 specific.

Although this enzyme showed an absolute requirement for calcium ions, kinetic studies revealed different results from those obtained using the neutrophil released enzyme. Close examination using varying amounts of EDTA showed that enzyme activity increased in a sigmoid fashion, indicating allosteric behaviour. The optimum concentration was 1.2mM Ca^{2+} [illegible]. This implied that the exudate enzyme is very sensitive to small changes in the calcium concentration. The neutrophil released enzyme showed the normal hyperbolic curve of non - regulatory enzymes. The peritoneal exudate PLA_2 did show hyperbolic characteristics with variation of autoclaved E.coli phospholipids, and an apparent Km approx. 670×10^{-6}M. This value is substantially greater than the corresponding Km value obtained with the neutrophil released enzyme.

(see opposite for data)

The data obtained on the storage of the peritoneal exudate phospholipase activity is particularly interesting and obviously contrasted with the corresponding data of the neutrophil enzyme. The former activity at acid pH was found to be fairly stable to storage at 4°C and lost only 12% activity over 2.5 months. Particularly interesting, was the finding of an actual increase of specific activity, by 79%, over the first few days (table 3.8), which then gradually returned to be the original activity after approximately 1 month. One explanation of this finding may be that some of the enzymes exist in the form of a zymogen, or proenzyme, which degrades to the active species during the first few days storage, and hence shows an increase of specific activity. Such behaviour of phospholipases has been observed by other workers. Etienne et al, (1969a, 1969b) have shown that storage of serum or addition of very high concentrations of trypsin to whole human and rat serum increases the phospholipid - splitting activity many fold, and concluded that the enzyme may exist as a zymogen. Indeed plasma may indeed be a viable source of the phospholipase activity seen in the inflamed peritonea, since serum derived factors also infiltrate inflamed areas during the acute reaction. (Fransen et al, 1978).

During the course of this work a report on the isolation and characterisation of a phospholipase A_2 from rabbit peritoneal inflammatory exudate was published (Fransen et al,1978). The inflammatory reaction was elicted by the injection of glycogen in physiological saline. The phospholipase activity described had two pH optima, at pH 6.0 and pH 7.5, was A_2 specific, and also Ca^{2+} dependant. A 300 fold purification was carried out using carboxymethylsephadex columns to yield a single protein peak of 14800MW. Polyacrylamide gel electrophoresis of this fraction revealed this to be a cationic protein more basic than lysozyme (pI = 10.5). As this phospholipase has properties similar

to the granule associated activity in PMN leucocytes, it was suggested to be derived from this cell.

PART C EFFECTS OF CHLOROQUINE - LIKE AGENTS ON INFLAMMATORY AND OTHER PHOSPHOLIPASES

3.1 Effect of chloroquine on the phospholipase A_2 activity of Crotalus Adamenteus venom and of pig pancreas

Chloroquine diphosphate showed a dose - related inhibition of both phospholipase A_2 activities as assayed against egg yolk substrates. Egg yolk substrates consist primarily of phosphatidylcholine (79%) and phosphatidylethanolamine (17%) (Raheja et al, 1973). Inhibition towards the mammalian enzyme was four fold greater than towards Crotalus adamenteus venom enzyme ($E.D_{50}$ values 0.73mM and 3.1mM respectively). However the parallel nature of the inhibitory dose- response curves suggest a similar mode of inhibition of the two enzymes.

Two chemically related structures, an alkaloid cinchonine sulphate, and the local anaesthetic dibucaine, also exhibited inhibition towards pig pancreas PLA_2. The order of decreasing potency was cinchonine sulphate > chloroquine > dibucaine.

Kinetic studies involving the variation of substrate in the presence and absence of chloroquine indicated an uncompetitive inhibition. Similar studies on the variation of Ca^{2+} in the presence and absence of either chloroquine or dibucaine indicated competition between Ca^{2+} and the drugs. Thus inhibition of pancreatic PLA_2 activity may be due to binding of the drug molecules to the substrate and thus altering its physiochemical properties and/or competition with the function

of Ca^{2+} on the enzyme.

The effects of local anaesthetics such as dibucaine on phospholipase activities has generated much interest. Indeed local anaesthetic potency parallels the ability to affect phospholipase activities (Kunze et al,1976). Studies have shown the drugs to have multiple effects depending upon the nature and source of enzymes and substrates used. Thus inhibition by local anaesthetics has been observed with phospholipase A_2 of pig pancreas using egg yolk substrates (Scherphof et al, 1972), and PLA_2 of human seminal plasma and Naja naja venom towards sonicated phosphatidylcholine (Kunze et al, 1976), although with the latter two enzymes stimulation of activity at low concentrations was also observed. However the PLA_2 from Crotalus adamenteus venom is only stimulated and shows no inhibitory effect using either sonicated or egg yolk substrates (Kunze et al, 1976).

Two hypotheses have been forwarded concerning the mode pf inhibition of phospholipase activity. Firstly the anaesthetic binds to the substrate thus modifying its physiochemical properties and its susceptibility to enzymic hydrolysis (Waite and Sisson, 1972; Scherphof and Westenberg, 1975; Stewart Hendrickson and Van Dans - Mieras, 1976). Secondly the anaesthetic binds to the enzyme and displaces calcium from the enzyme - Ca^{2+} complex (Scherphof et al, 1976, Kunze et al, 1976). The results [illegible] in this study indicate both a binding of the drug to the substrate and competition with calcium. Indeed recently cationic amphiphilic drugs have also been shown to replace $^{45}Ca^{2+}$ from phospholipid monolayers in a dose - dependant fashion (Lullmann et al,1980). The potencies of the drugs were related to their lipid solubilities, although the charged form of the drug was also necessary.

However it should be noted that one should proceed with caution when interpreting kinetic data using lipids. The substrates used are not in true solution but dispersed as liposomes and micellar dispersions. Therefore any conclusions derived using classical kinetics from lipids should be interpreted as indications and not as stern facts.

It may be of importance to mention that in experiments with cinchonine sulphate and dibucaine, but not chloroquine, there was a lag phase of 3 minutes before maximum enzymic hydrolysis was reached. The reason for this is not certain but may be a physical effect such as an effect on substrate dispersion. Lag phase have been observed in a previous study by Rosenthal and Ching - Hsien Han (1970) where inhibition of phospholipase A_2 by phosphinate analogues was reported using ether systems as the reaction medium.

3.2 Effect of chloroquine - like agents on the inflammatory peritoneal exudate PLA_2 activity

Phospholipase A_2 activity of the rabbit peritoneal (cell - free) inflammatory exudate towards *E.coli* phospholipids was inhibited by chloroquine, mepacrine and primaquine. The inhibitory log - dose response curves were parallel indicating a similar mode of inhibition. Mepacrine was almost twice as active as primaquine and five times more active than chloroquine. The inhibitory potency (mepacrine > primaquine > chloroquine) was related to the lipid solubilities of these compounds as shown by a correlation between the IC_{50} of the drugs on phospholipase activity and the log P values determined between chloroform and water.

At doses above those causing maximum inhibition,

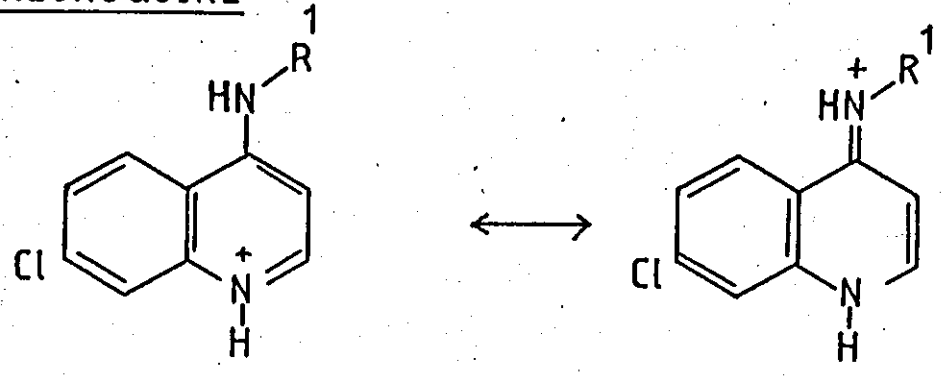
chloroquine and mepacrine showed a reversal of the effect even leading to stimulation of phospholipase activity at greater than 5mM mepacrine. Primaquine however differed and showed no reversal of the inhibitory effect at high doses. Thus chloroquine and mepacrine unlike primaquine caused biphasic effects on the peritoneal exudate phospholipase activity. This difference between the drugs may be due to structural differences of these drugs. Primaquine is an 8 - aminoquinoline whereas the other two are 4 - aminoquinoline derivatives. This difference in ring structure can relate to differences in the resonance of the charge (when protonated) between the ring N and the secondary amino - N on the side chain (see figure 4.1). Thus with chloroquine and mepacrine this resonance can occur with free movement of the proton between the nitrogens, but not with primaquine. This factor together with the difference in the terminal amino group may confer markedly different properties on the primaquine molecule. Indeed differences between the pharmacological actions of chloroquine and primaquine have been reported. They attack different phases of the life cycle of the malarial parasite, after *in vivo* administration chloroquine is avidly bound by tissues whereas primaquine is barely detectable after 24 hrs (Bowman and Rand, 1980) and primaquine, but not chloroquine, is a potent inhibitor of alcohol dehydrogenase (Li and Magnus, 1972).

The biphasic effects observed on the peritoneal exudate phospholipase A_2 also relate to paradoxical membrane active properties of these drugs. All three drugs affected the hypotonic haemolysis of guinea - pig erythrocytes in a similar manner. (figure 3.33). Stabilization was caused at low doses, followed by labilization at high doses, and a reversal again at even higher levels. Thus penetration of low amounts of drug strengthens the red cell membrane while too much leads to rupture of the ordered structure. A similar observation for-

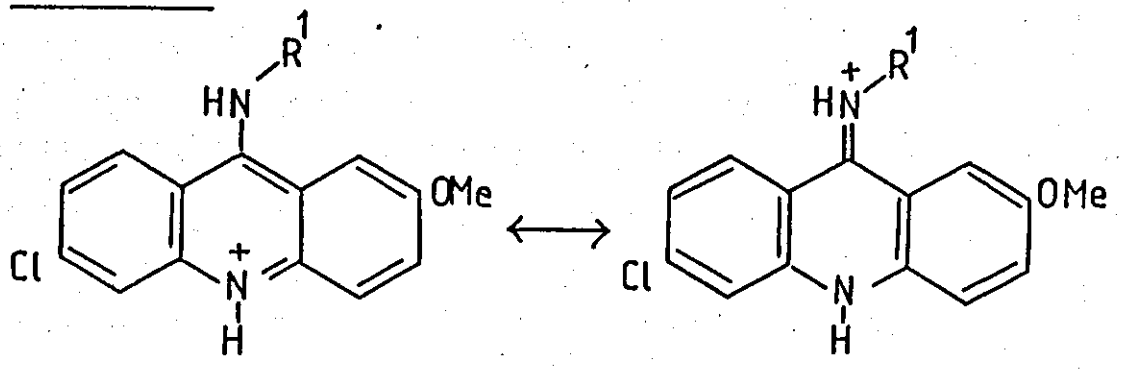
FIGURE 4.1.

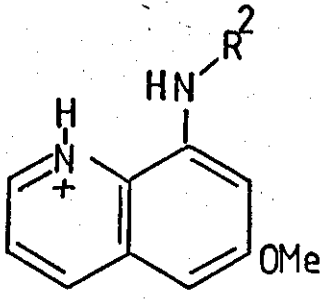
Structual differences between proton movements in the antimalarial drugs.

CHLOROQUINE

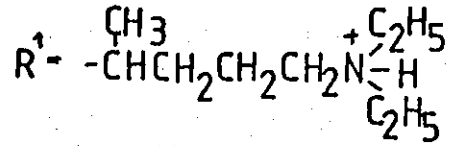


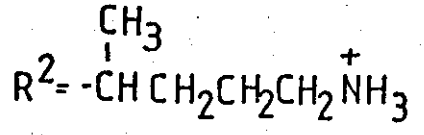
MEPACRINE





PRIMAQUINE





chloroquine has been obtained by Inglot and Wolna (1969).

The degree of inhibition of phospholipase activity also depended upon other factors. Variation of pH had a profound effect on the inhibition. Studies with 1mM chloroquine revealed the least inhibitory effect at pH 6.5, at pH 7.5 the same level of drug caused twice as much inhibition and at pH 8.5 more than three times as much. Thus inhibition increases with increasing pH, and reflects the importance of charge on the chloroquine molecule. Chloroquine has two pKa values $pKa_1 = 8.06$, $pKa_2 = 10.16$ (Rosenberg and Schulman, 1978). At pH 7.5 an equilibrium between the mono and di forms exists and 18% of the drug is in the monoprotonated form. This amount decreases at acid pH and increases at alkaline pH, suggesting that the monoprotonated form is responsible for the inhibition. This may appear to contradict with the local anaesthetics where greater inhibition of phospholipase activity is seen at acid rather than alkaline pH (Scherphof and Westenberg, 1975). On the other hand local anaesthetics such as dibucaine have essentially one ionisable group, the terminal N with pKa = 8.83 (Lullmann et al, 1980). The aromatic N will stay uncharged as its pKa is 2.2. Thus inhibition is observed at acid pH where a greater proportion of the monoprotonated species exists.

Storage of this enzyme preparation over 3 months caused a measurable increase in the inhibition caused by a fixed amount of antimalarial drug. The reasons for this are not known.

3.3 Kinetics of inhibition of peritoneal exudate PLA_2 towards E.coli phospholipids by mepacrine

Kinetic studies of the inhibition revealed results which differed from those obtained with pancreatic phospholipase A_2 activity towards egg yolk substrates. Inhibition by mepacrine was non - competitive with respect to Ca^{2+} variation whilst an experiment in which substrate was varied did not provide any conclusive results. These differences may reflect the fact that both enzyme source and phospholipid substrates are different. The fact that one substrate is present as part of a biological membrane and the other as a micellar dispersion may also be important.

It therefore appears that kinetic studies using lipid systems reveals differing results depending upon the system that is being used. Table 4.1 summarises the kinetic studies of inhibition of phospholipases by cationic amphiphilic drugs.

3.4 Important consequences of inhibition of phospholipase A_2 activities by chloroquine

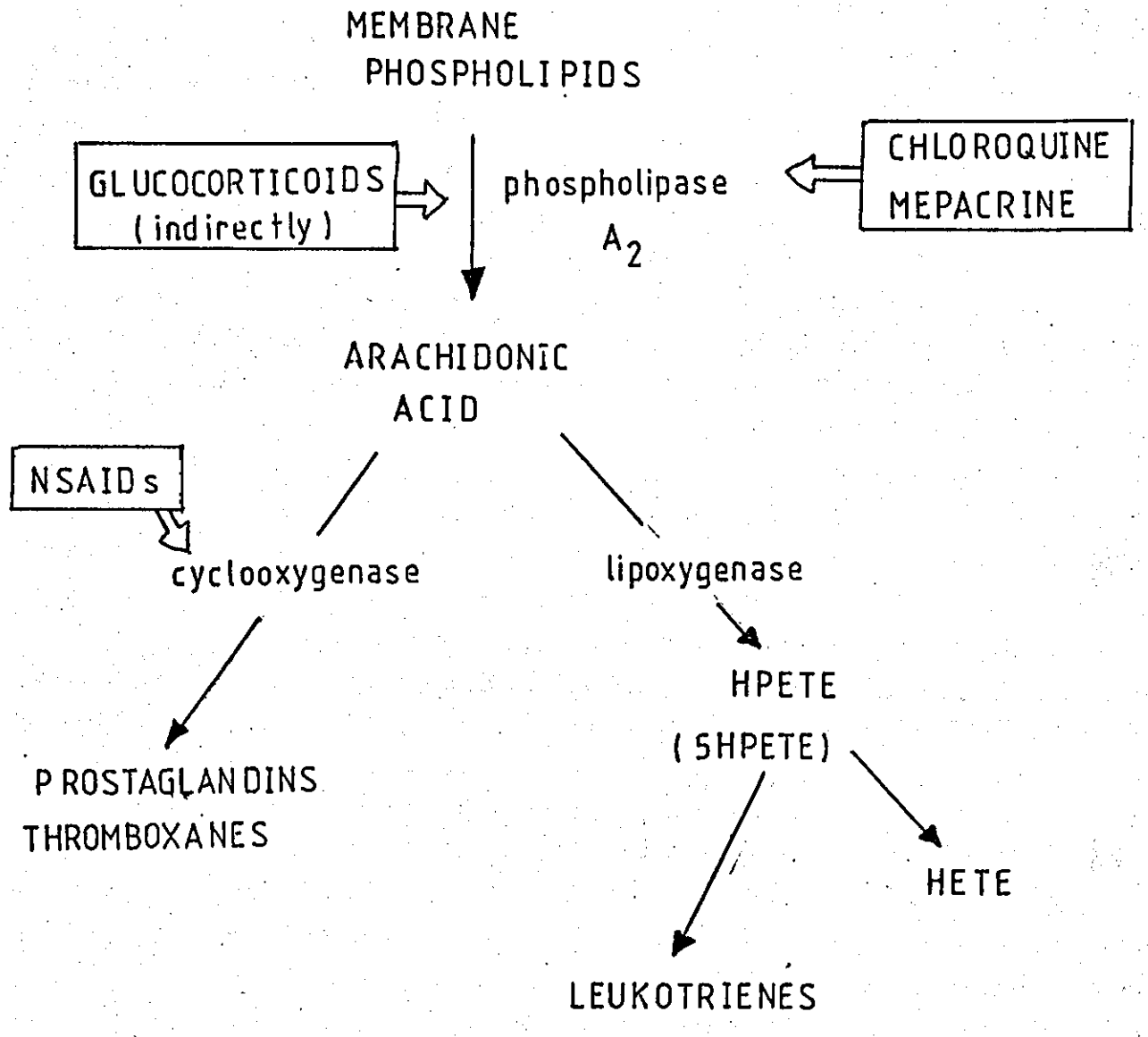
Phospholipase A_2 activities play a key role in the generation of prostaglandin - like materials by their ability to provide precursor fatty acids. Also agents that inhibit phospholipase A_2 activities have a greater potential therapeutic value in inflammatory conditions than non - steroidal anti - inflammatory drugs, as they inhibit products generated by both lipoxygenase and cyclo - oxygenase enzyme systems (figure 4.2). The NSAIDS being only active in preventing generation of cyclooxygenase products. Thus agents that inhibit phospholipase activity can block generation of prostaglandin endoperoxides, thromboxanes, HPETE

TABLE 4.1

SUMMARY OF KINETIC STUDIES OF INHIBITION OF PHOSPHOLIPASES BY LOCAL ANAESTHETICS AND ANTIMALARIAL DRUGS

ENZYME SOURCE	NATURE OF SUBSTRATE	DRUG (S)	MODE OF INHIBITION	REFERENCE
Pancreatic PLA_2	Membrane bound PL in mitochondria and egg yolk emulsions	Local Anaesthetics (L.A.'s)	Competition with Ca^{2+}	Scherphof *et al*, (1972)
PLA_2 of rat liver mitochondria	Sonicated PE	Dibucaine	Uncompetitive with substrate. Interference of Ca^{2+} with substrate and enzyme	Waite and Sisson (1972)
Pancreatic PLA_2	PC monolayers	L.A.'s	Independant of Ca^{2+} conc. Lidocaine - competitive inhibition of enzyme - lipid interface interaction	Hendrickson + Van Dam - Mieras (1976)
Human seminal plasma PLA_2	Sonicated PE and PC	L.A.'s	Competitive with Ca^{2+}	Kunze *et al*, (1976)
Naja naja venom	"	"	"	"
Pig pancreas PLA_2	Egg yolk emulsions	Chloroquine and dibucaine	Uncompetitive with substrates. Competitive with Ca^{2+}	This work
Rabbit peritoneal exudate PLA_2	*E.coli* membrane PL's	Mepacrine chloroquine primequine	Non - competitive with Ca^{2+} competitive with substrate	"

Figure 4.2. Sites of inhibition by anti-inflammatory drugs along the generation of prostaglandin - like metabolites.



BW 755C inhibits both cyclooxygenase and lipoxygenase. In leucocytes 5HPETE can be further transformed into leukotrienes which are the active components of "slow reacting substance" (Murphy et al, 1979). Leukotrienes such as LTC and LTD are extremely potent constrictors of smooth muscle (Drazen et al, 1981).

(which is pain producing), HETE which is chemotactic for polymorphs and the newly discovered leucotrienes which are very potent constrictors of smooth muscle (Samuelsson et al, 1979; Drazen et al, 1981).

The concentrations of antimalarial drugs required to inhibit phospholipase activities as presented in this study are higher than the steady state therapeutic plasma levels achieved during treatment. For mepacrine this value is approximately 10^{-7}M and for chloroquine 10^{-6}M (Wollheim et al, 1978) and for this reason the importance of inhibition of phospholipase activities by these agents may be questioned. However, because of their lysosomotrophic nature, the levels achieved in various tissues may exceed the plasma level by 400 - 700 fold (Berliner et al, 1948; De Duve et al, 1974; Ohkuma and Poole, 1978). At these levels inhibitory activity towards phospholipase A_2 is observed. The fact that chloroquine effects on inflammatory disorders are rarely seen before two months continous therapy, is a reflection of the time required to build up sufficient quantities of the drug.

The IC_{50} of these agents on the rabbit inflamed peritoneal exudate were 3.3×10^{-4}M for mepacrine and 1.5×10^{-3}M for chloroquine. On the guinea - pig isolated perfused lung preparation mepacrine has been shown to exhibit an IC_{50} = 20 μg/ml or approximately 5×10^{-5}M (Blackwell et al, 1978), and thus showing a difference of approximately 7 fold. Similarly chloroquine has been reported to inhibit PLA_2 activity of cultured inflamed rat synovium at concentrations as low as 10 μg/ml or 2×10^{-5}M (Floman, 1978), and a difference of approx - imately 75 fold. Thus phospholipases of different tissues show different susceptibilities to inhibition by antimalarial drugs. These differences may be partly related to the differing ability of the tissues to concentrate these compounds.

The inhibition of the generation of prostaglandin - like materials may in part explain some of the anti - rheumatic effects of the drug. However inhibition of this enzyme may also explain the phenomenon of lipidosis which is a build up of polar lipids in lysosomes. (Drenchahn and Lullman - Rauch, 1978). The accumulation of polar lipids can have grave consequences in tissues such as the retina, where a specially balanced lipid metabolism is essential for normal function. Lipidosis is one of the factors which leads to the drug - side effect, retinopathy.

3.5 Low dose stimulation of phospholipase activity by chloroquine and mepacrine

On one peritoneal exudate phospholipase preparation both chloroquine and mepacrine at low doses (250 μM and 50 μM respectively) caused a stimulation of enzyme activity. These effects were striking (figure 3.27), reproducible only on this preparation and were lost after 2 months storage at 4°C. They were not observed on approximately twelve other preparations.

Stimulation of phospholipase activity by low doses of local anaesthetics has also been observed (Scherphof and Westenberg, 1975; Kunze et al, 1976). But these effects are only observed on micellar dispersions of phospholipids and not when the substrate was present in membranes. In this study, however even when extracted E.coli phospholipids were used as sonicated micellar dispersions, they failed to show low dose stimulatory effects by mepacrine and chloroquine if other enzyme preparations were used (table 3.12). These results also highlighted the differences in activity obtained when the same substrate is present as a micellar dispersion or bound in a biological membrane. Thus phospholipase activity towards E.coli membrane phospholipids was 8 - fold

higher than if the same phospholipids were present as sonicated dispersions.

3.6 Effects of other agents on the peritoneal exudate phospholipase A_2

Four other drugs associated with the treatment of inflammatory disorders were also screened at 1mM concentrations. The anti - arthritic drugs sodium aurothiomalate and D - penicillamine showed no effect against the peritoneal exudate phospholipase A_2 activity. However the anti - inflammatory drugs indomethacin and benzydamine were particularly effective showing almost total inhibition at 1mM concentrations. Further study evaluating IC_{50} values for the latter two drugs may be worthwhile to compare their effectiveness against their potencies towards the cyclooxygenase enzyme systems. Recently low concentrations of indomethacin have been reported to inhibit phospholipase A_2 of rabbit PMN leucocytes (Kaplan et al, 1978). 1 μM indomethacin reproducibly caused 14% inhibition, and 50 μM caused 70% inhibition. These findings are important as they indicate that inhibition of prostaglandin synthesis by indomethacin in leucocytes may be the result of an effect of the drug on prostaglandin synthetase and phospholipase A_2 activity.

3.7 Effects of chloroquine - like agents on the phospholipase A_2 activity of PMN leucocytes

Chloroquine - like agents showed completely contrasting effects when examined on the phospholipase A_2 activity of PMN neutrophils as compared with effects obtained on the peritoneal exudate PLA_2. Studies conducted on sonicated neutrophils, assaying PLA_2 activity at pH 6.5, showed stimulation of activity by chloroquine and mepacrine. This effect was dose - related and no biphasic effects, as observed with the peritoneal

exudate preparation, were seen. This finding appears to be unique as mepacrine and chloroquine have always been observed to inhibit phospholipase activities (Vargaftig and Dao Hai, 1972; Canedella et al, 1969; Blackwell et al, 1978). Mepacrine was again observed to be more active than chloroquine and in this case the difference was 10 fold.

Primaquine behaved differently. Although exhibiting a slight stimulation at low concentrations (at 0.5mM, 13%) a strong inhibition at doses greater than 1mM was observed.

The stimulatory action by chloroquine and mepacrine was further shown on other enzyme preparations obtained from neutrophils including (i) neutrophils suspended in water, (ii) 8,200g pellet granule preparations prepared in sucrose, and (iii) on phospholipase activity released from neutrophils exposed to zymosan - complement particles. Studies on the 8,200g pellet showed that the stimulation of activity by mepacrine was 2 to 3 fold greater at pH 6.5 than at pH 9.0 indicating that the diprotonated form of the drug was more important for this effect than the mono- charged species. This is the reverse situation to the inhibitory effects observed on the peritoneal fluid PLA_2 where the effect was greatest at alkaline pH's. Indeed at pH 9.0 chloroquine showed an inhibition of the zymosan - cpmplement released PLA_2 activity further supporting the inhibitory action of the monoprotonated species.

Studies using a partially purified PLA_2 preparation obtained by sulphuric acid extraction of neutrophils suspended in water revealed even more interesting results. Mepacrine and chloroquine no longer showed stimulation of PLA_2 activity at acid pH but only showed inhibition. This effect was not as strong as that obtained with the peri - toneal exudate preparation. Mepacrine as expected was more potent than

chloroquine, but primaquine showed to be the most potent and its inhibitory effect was of the same order and magnitude as that obtained with the neutrophil homogenate suspensions.

One explanation of the fact that both chloroquine and mepacrine stimulated PLA_2 activity at acid pH with all the other preparations except the sulphuric acid extracted enzyme, could be the involment of another factor present in neutrophils which was lost during the purifi - cation procedure.This of course assumes that the acid extracted PLA_2 is the <u>same</u> activity that is assayed in the homogenate. However the possibility that different populations of the phospholipases may exist should not be excluded. Thus in the presence of this endogenous factor chloroquine and mepacrine stimulated PLA_2 activity while in its absence only inhibition is observed. The unknown factor may be an anionic agent since it probably binds with the drugs which are cationic. The possibility that this could be a glycosaminoglycan such as heparin was investigated since heparin and to a greater extent the related agent, chondroitin - 4 - sulphate have been shown to be present in leucocytes in measurable quantities (Olsson and Gardell, 1976; Olsson <u>et al</u>,1968; Avila and Convit, 1976). The latter authors also found that the glycosaminoglycans were able to inhibit 30 different lysosomal enzymes including PLA_2. On a molar basis the inhibitory potency was heparin > chondroitin sulphate > hyaluronic acid, with only heparin showing activity against PLA_2. Thus glycosaminoglycans present a mode of control of leucocyte lysosomal enzyme activity.

Heparin is certainly able to interact with all three antimalarial drugs as clearly observed when solutions of the individual drugs are added to heparin and the resulting O.D. changes were measured (figure 3.31). On a molar basis mepacrine again was the most active drug.

Heparin itself was found to inhibit PLA_2 activity of neutrophils at acid pH in agreement with the findings of other workers (Avila and Convit, 1976; Franson et al, 1974). If however heparin was the endogenous factor then mixtures of mepacrine and heparin added together to reaction mixtures containing the acid purified PLA_2 enzyme, should show some antagonistic effects or even stimulation of activity. This statement again assumes that the acid purified enzyme preparation is the same as in the water homogenate. However this was not so and the anionic and cationic agents together showed additive inhibitory effects on the acid purified preparation.

It may be fruitful in future studies to examine the possibility that chondroitin sulphate may be involved. This requires much higher concentrations than heparin to effect PLA_2 activity and is also present in larger quantities. The interaction between chloroquine and heparin provide another factor for the lysosomotrophic effects of this drug as the glycosaminoglycans is thought to be present in leucocyte lysosomes.

Alternatively the stimulation of PLA_2 activity may be due to another factor or even another phenomenon. Franson et al, (1974) found the major portion of PLA_2 activity of rabbit neutrophils to be associated with the granules and membrane bound. Association of the enzyme with the membrane may be responsible for the stimulation of activity observed as chloroquine is a membrane - active agent. The purification procedure certainly provides the enzyme in a solubilised state which may account for the difference in response obtained. This however makes interpretation of the stimulatory effects of chloroquine and mepacrine

on the zymosan - complement released enzyme difficult unless one gets the release of membrane fragments aswell. The peritoneal exudate PLA_2 is also considered to be a soluble enzyme (Franson et al, 1978).

Heparin showed a completely contrasting effect on the peritoneal exudate enzyme. At acid pH heparin showed stimulation of PLA_2 activity at exactly the doses required to inhibit the neutrophil enzyme. This effect was pH dependant as at pH 7.5 the stimulatory activity shown by 10 units/ml heparin was less than half of the effect at pH 6.0 (table 3.19). Such differences in response of the two phospholipase activities can only be explained by the suggestion the peritoneal exudate PLA_2 is a different enzyme and not derived from neutrophils but from another source.

Recently rabbit serum too has been shown to be a potent source of PLA_2 activity (Kaplan - Harris et al, 1980). The described PLA_2 activity was maximally active at 2mM Ca^{2+} and higher concentrations led to a decrease of activity. This is very similar to the response obtained in this study, although no sigmoid behaviour was noted between 0 - 2mM Ca^{2+}. The stimulation of PLA_2 activity of the peritoneal exudate by heparin is shown primarily at pH 6.0 with a reduced effect at pH 7.5, thus the physiological significance of this is not known. Heparin administration to humans in large amounts (5000 units) leads to an increase in serum PLA activity although this has been associated with A_1 specificity (Vogel and Bierman, 1967).

3.8 Consequences of a stimulatory effect of PLA_2 of PMN leucocytes at acid pH

Stimulation of PLA_2 activity could be expected to lead to an increase in the release of polyenoic fatty acids and lysophospholipids.

Generation of both products leads to a potentiation of inflammation as arachidonic acid is the precursor for the prostaglandin related products, all of which are generated in stimulated neutrophils, and lysophospholipids are cytotoxic. Stimulation of PLA_2 activity by chloroquine at acid pH may be particularly important as (1) chloroquine accumulates in the lysosomes where the concentration achieved can be up to 700 times that in the medium (2) the pH of the lysosomes is acid therefore an acid PLA_2 activity is important and (3) the majority of the PLA_2 activity associated with rabbit neutrophils is associated with the granules with an acid and an alkaline pH optima.

Recently many studies have been published which relate products of PLA_2 hydrolysis and lysosomal enzyme release from inflammatory cells (Naccache et al,1979; Smolen and Weissmann, 1980; Smith, 1978). In particular, many studies involve the use of chemotactic peptides formyl - methionyl - leucyl - phenylalanine (FMLP) which also cause lysosomal enzyme release. Thus if human PMN's that have been treated with cyto - chalasin B are exposed to FMLP, then release of lysosomal acid hydrolases occurs. Release of acid hydrolases could be inhibited by 50μ M indomethacin, 12 μM 5, 8, 11, 14 eicosatetraynoic acid (ETYA, a structual analogue of arachidonic acid) or 18 μM para - bromophenacyl bromide (a PLA_2 inhibitor). Arachidonic acid itself has been shown to cause degrannulation of rabbit peritoneal PMN leucocytes although prior treatment with cytochalasin B is necessary (Naccache et al, 1979). The other product of PLA_2 action, lysophospholipids have also been implicated in the release process. They are themselves membrane active and thus can promote fusion of granules to phagosomes or plasma membranes. They also produce intense inflammation when applied locally (Thouvenot et al, 1974), intraperitoneally (Westphal et al, 1970) or subcutaneously (Philips et al, 1965). They have also been shown to stimulate guanylate cyclase (Shier et al, 1976) thereby

increasing cGMP levels which are known to elevate during release of lysosomal constituents.

Studies of the effects of chloroquine show that at relatively high concentrations it increases the release of lysosomal enzymes (Ringrose et al, 1975; Weissmann et al, 1975; Riches et al, 1981). This property may appear wrong for a drug that has been used to treat inflammatory disorders as most NSAID's and SAID's have generally been found to decrease release of lysosomal enzymes. Certainly if in PMN's acidic phospholipases are important in lysosomal enzyme release, then the effects of chloroquine observed in this study would explain the increase of release of lysosomal enzymes observed by previous workers. The actions observed by chloroquine occur at relatively high doses, and these levels can be achieved by virtue of its lysosomotrophic activity.

Stimulation of phospholipases and therefore of pros - taglandin biosynthesis may also have an anti - inflammatory action as very high levels of prostaglandins are known to have anti - inflammatory effects such as inhibition of rate of appearance of leucocytes and lysosomal enzymes at sites of acute inflammation (Zurier et al, 1973; Bonta and Parnham, 1978). In leucocytes these actions may arise through increases of cAMP levels. Thus the long lag - period required for the onset of action of chloroquine in inflammatory disorders may reflect the time required to build up sufficient concentrations of drugs to exert its effects. Future studies on the effects of chloroquine on prostaglandin production of neutrophil leucocytes may be worthwhile to evaluate this point.

PART D STUDIES OF AN ENDOGENOUS INHIBITOR OF PLA_2 IN RABBIT PERITONEAL PMN'S

4.1 Cellular localization and properties

When neutrophils are homogenised in sucrose and then centrifuged at 8,200g, then the majority (90%) of the PLA_2 activity was found to be associated with the pellet which consist in the main of nuclei and granules. The 8,200g supernatant contained little PLA_2 activity and this was lost upon storage at 4°C over five days. On the other hand the supernatant was also found to contain an inhibitory activity towards PLA_2 of the peritoneal exudate. The inhibition was dose - dependant to a maximum inhibition of 64% (figure 3.35). This inhibitory activity was independant of pH and similar dose - response curves were obtained between pH 5.5 and 9.0.

However inhibition was not independant of pH if inhibitory potency was assayed against PLA_2 activity of neutrophil granules (8,200g pellet). Indeed almost no inhibitory activity was present towards this preparation at pH 6.5. Activity at pH 7.5 has been reported (Franson *et al*, 1974) which was heat unstable ie. probably a protein. The different inhibition patterns obtained indicated another difference between the peritoneal exudate PLA_2 and the neutrophil granule PLA_2 activities. Indeed it is somewhat of an oddity that the neutrophil cytosol should contain an inhibitor which was active only at neutral and alkaline pH towards the PLA_2 from neutrophil granules and active at both acid and alkaline pH towards the peritoneal exudate enzyme.

Inhibition of PLA_2 activity by the 8,200g supernatant was not confined to the above mentioned preparations. Phospholipase A_2

of partially purified porcine pancreas and of crude Crotalus adamenteus venom were also inhibited to similar extents (table 3.23), and therefore appeared to show little selectivity of action. However the PLA_2 from Naja naja venom was not inhibited but a 58% stimulation was recorded. This stimulation was probably a non - specific protein effect as equivalent concentrations of BSA were able to stimulate the activities of all the preparations examined. This is probably due to binding and removal of products, ie. fatty acids and lysophospholipids, which are known to cause product inhibition (Franson et al, 1974).

Why the enzyme from Naja naja venom was not inhibited by the neutrophil 8,200g supernatant is not certain. Since the substrate was the same in all cases, it would thus be due to differences in the enzymes. All the enzymes tested require Ca^{2+} for activity as 1mM EDTA was able to totally inhibit every enzyme preparation. This difference may be explained upon critical examination of the size and ionic nature of the proteins used.

Most venom phospholipases consist of different numbers of isozymes of slightly differing molecular weights and isoelectric points. There is generally an abundance of one or a few particular isozymes and the phospholipase of a particular venom maybe classified as a basic, neutral or acidic protein with a particular isoelectric point and molecular weight depending upon the abundent species. The more toxic venoms, as measured by LD_{50} values in mice, generally tend to have basic, cationic PLA's than the less toxic venoms which contain neutral enzyme proteins (Condrea et al,1980). Reports in the literature indicated that the PLA_2 activities used in this study ie. rabbit peritoneal exudate, rabbit PMN leucocyte, Crotalus adamenteus venom and pig pancreas are basic, cationic proteins (Franson et al, 1978; Weiss et al, 1975; Van Den Bosch, 1974;

De Haas et al, 1970; respectively). But the enzyme from Naja naja venom is primarily an acidic enzyme with the most abundant isozyme having an isoelectric point of 4.95 (Salach et al,1971). It is also a larger protein of MW = 20,000 while the rest are all below 15000MW. This may also indicate that inhibitory activity present in the neutrophil cytosol is an acidic protein. Furth r verification of this suggestion requires study with other known acidic phospholipases.

Recently work by Hirata et al,(1980) has also demonstrated the existence of a phospholipase A_2 inhibitory protein in the polymorphs whose synthesis could be induced by glucocorticoids. PMN leucocytes treated with glucocorticoids were shown to have a reduced response to the chemo - tactic peptide f Met - Leu - Phe (FMLP) which was related with an inhibition of phospholipase activity. The induced phospholipase inhibitory protein was isolated from water lysed neutrophils which had previously been incubated with flucinoline acetonide for 16 hours. The precipitate arising from water lysed neutrophils was solubilised and subsequent gel filt ration on sephadex G200 columns revealed a molecular weight of 40,000. This protein exhibited inhibitory activity towards PMN and pancreatic PLA_2. However there is one notable difference: the phospholipase inhibitory protein as described by Hirata, was lost when the polymorphs were treated with pronase. As pronase acts on the outer surface of the cells, the PLA_2 inhibitory protein resides on the plasma membrane. Franson et al, (1974) suggested that the PLA_2 inhibitory protein is present in the soluble portion of the cell as it was also present in 100,000g supernatant preparations of leucocyte homogenates.

4.2 Endogenous inhibitor of PLA_2 activity and the mode of action of steroidal anti - inflammatory drugs

Much interest has recently been shown in the inhibition of prostaglandin biosynthesis by steroidal anti - inflammatory drugs (SAID), which occurs only in intact cells. The fact that glucocorticoids do not inhibit microsomal cyclooxygenase (the site of action of NSAIDS) was shown by Flower et al,(1972). In 1975 two hypothes s were proposed. Lewis and Piper suggested that SAID's prevented the release of prostaglandins from the cell by virtue of their membrane stabilizing effects and Gryglewski et al, suggested that glucocorticoids prevented the release of polyenoic fatty acids from phospholipids, and hence to the microsomal enzyme system for prostaglandin biosynthesis. Therefore glucocorticoids were suggested to interfere with phospholipase A_2 activities.

Since then much evidence has been put forward substan - tiating the latter theory. Danon and Assouline (1978) showed that inhibition of prostaglandin biosynthesis in rat renal papillae by SAIDS involved RNA and protein synthesis. Thus the glucocorticoids may work by the same mechanism exhibited by the steroid hormones on their target cells ie. combination of the steroid with a cytosolic receptor protein, translocation of this drug - receptor complex to the nucleus, stimulation of transcrip - tion and consequent biosynthesis of specific proteins. In 1979 Flower and Blackwell, using the guinea - pig perfused lung preparation showed that the glucocorticoids induced the synthesis of a polypeptide or protein that inhibited the release of arachidonic acid from phospholipids. Indeed inhibitors of protein synthesis such as cycloheximide were able to block the effects of the glucocorticoids. Potent inhibition of phospholipase activity also explained why the glucocorticoids are more potent as anti - inflammatory agents than NSAIDS, as they prevent availability of substrate

for both the cyclooxygenase and lipoxygenase enzyme systems whereas the NSAID's only block cyclooxygenase. These effects have been further demonstrated on rat peritoneal macrophages where incubation with the drugs not only induced the biosynthesis of an inhibitor of PLA_2, but also released it into the medium (Carnuccio et al,1980), which was then assayed against prostaglandin production by leucocytes undergoing phagocytosis of killed bacteria. Evidence that this inhibitor was preformed and stored within macrophages and released upon incubation with hydrocortisone was provided by Blackwell et al, (1980a) whose group showed that the inhibitory factor, isolated from both guinea - pig lungs and rat peritoneal macrophages was a polypeptide (designated "macrocortin") of approximately 15,000MW.

An inhibitor of phospholipase activity in the cytosol of rabbit PMN leucocytes was first observed by Franson et al,(1974) and this observation has been extended by this study showing that it was active against other basic cationic phospholipases but not against the acidic,anionic phospholipase from Naja naja venom. Recent work by Dray et al,(1980) suggested that the rat leucocytes may be a cell type insensitive to the actions of steroidal anti - inflammatory drugs. In their study rat peritoneal PMN leucocytes were incubated with dexamethasone for 2 hrs at 37°C. Then the cells were seperated from the media and further incubated with killed Bortella pertussis in the presence of dexamethasone and the prostaglandin levels at the end of this incubation were measured by radioimmunoassay. Cells treated with dexamethasone up to 10^{-4}M showed no reduction in the amount of prostaglandins produced.

These observations were furthur substantiatedby work in our own laboratory (Traynor, Solanky and Authi, unpublished observations). Rat peritoneal PMN leucocytes were incubated with and without hydrocortisone for 2 hrs at 37°C. The phospholipase A_2 inhibitory activity present in the

8,200g supernatant of leucocyte homogenates towards rat peritoneal exudate PLA_2 was assayed using (1 - ^{14}C) oleic acid labelled E.coli. Hydrocortisone up to $10^{-5}M$ showed no increase in the PLA_2 inhibitory activity of the 8,200g supernatant preparations.

However work by Hirata et al,(1980) showed that rabbit PMN's do contain an inhibitor of phospholipase activity whose synthesis could be induced by glucocorticoids but at least 5 - 10 hours of preincubation with the drugs was necessary. The phospholipase inhibitory protein in this case had a molecular weight of 40,000. Thus information obtained so far reveals the PMN leucocytes to differ from macrophages in that longer incubations with the steroids were necessary to show an effect and the resultant inhibitory protein has a higher molecular weight.

Recently the importance of this PMN phospholipase inhibitory protein has been further highlighted by the finding that some patients suffering from chronic inflammatory conditions such as systemic lupus erythematosis or rheumatoid arthritis contain in their plasma an autoantibody towards the phospholipase inhibitory protein (Hirata et al, 1981). Thus in such patients this autoantibody allows increased formation of arachidonic acid and subsequently the formation of inflammatory metabolites.

The anti - inflammatory steroids have also been shown to enhance the ability of tissues to breakdown prostaglandins (Moore and Hoult, 1980).

CONCLUSIONS

Phospholipase A_2 enzymes are belived to play an important role in inflammation by their role in providing polyenoic fatty acids which are the precursors for prostaglandin - like compounds. The present research shows that one cell type known to accumulate at damaged tissues, namely the polymorphonuclear leucocyte, does release a phospholipase A_2 enzyme under various conditions of stimulation. Indeed the degree of phagocytosis by the cells correlates with the degree of release of phospholipase A_2.

The released phospholipase A_2 activity has been characterised and compared with a phospholipase A_2 activity found in the peritoneal inflammatory exudate. Close examination of the properties and responses to specific pharmacological agents reveals striking differences between the two enzymes. Table 4.2 summarises the described properties of the two enzymes.

In agreement with the findings of other workers using other tissues, chloroquine and mepacrine were found to inhibit phos - pholipase A_2 of the peritoneal exudate. Inhibition of PLA_2 activity may explain some of the anti - rheumatic effects of chloroquine as this prevents the generation of prostaglandin - like mediators. It may also explain the major side effect, lipidosis leading to retinopathy, which is a build up of polar lipids within lysosomes.

However the effect of chloroquine on phospholipases is not simply inhibition as described in the literature, but biphasic

TABLE 4.2

COMPARISON OF PROPERTIES OF THE RABBIT NEUTROPHIL RELEASED PHOSPHOLIPASE A_2 AND THE RABBIT PERITONEAL EXUDATE PLA_2. ENZYME CHARACTERISTICS AND EFFECTS OF PHARMACOLOGICAL AGENTS.

ENZYME CHARACTERISTICS	PMN PLA_2	PERITONEAL EXUDATE PLA_2
PH OPTIMA	6.0, 9.0	6.0, 8.0
Ca^{2+} REQUIREMENT	OPTIMUM = 5mM Ca^{2+} NORMAL HYPERBOLIC CHARACTERISTICS	OPTIMUM = 1.2mM SIGNOID KINETICS AND GREATER THAN 2mM INHIBIT S
Km	$\simeq 25 \times 10^{-6}$M	$\simeq 670 \times 10^{-6}$M
STABILITY AT 4°C	ACID ACTIVITY MORE UNSTABLE THAN ALKALINE	POSSIBILITY THAT ENZYME EXISTS AS A ZYMOGEN
MOLECULAR WEIGHTS (FRANSEN ET AL,1974)	15,000	14,800
EFFECTS OF PHARMACO - LOGICAL AGENTS		
CHLOROQUINE AND MEPACRINE	STIMULATION AT ACID PH (INHIBITION ON ACID - PURIFIED PREPARATION)	INHIBITION, BIPHASIC EFFECTS
PRIMAQUINE	STIMULATION AT LOW CONC. STRONG INHIBITION AT HIGH CONC.	INHIBITION
HEPARIN	INHIBITION AT ACID PH.	STIMULATION AT ACID PH
NEUTROPHIL 8,200g SUPERNATANT	INHIBITION AT NEUTRAL PH(FRANSON ET AL,1974)	INHIBITION INDEPEND - ANT OF PH

dose - response curves were observed. These effects were further repeated when physical effects on membrane stability such as hypotonic haemolysis of erythrocytes were examined. Indeed chloroquine and mepacrine were observed to stimulate PLA_2 activity of PMN leucocytes. This finding may be very important as it explains the observed increase of lysosomal enzymes by chloroquine from PMN leucocytes and macrophages. Further studies extending this to prostaglandin synthesis may be worthwhile to elucidate this point.

Recently much interest has been shown in endogenous inhibitors of phospholipase activity. Within the lysosomes the glucosaminoglycan heparin may provide regulation of enzyme activity at acid pH. However at neutral pH the major control of phospholipase A_2 activity is by a phospholipase inhibitory protein whose biosynthesis can be enhanced by glucocorticoids. This protein appears to have little selectivity of action towards phospholipases from different sources except perhaps those that are acidic proteins. The phospholipase inhibitory protein or the lack of its activity may be of great importance in inflammatory conditions as recently an autoantibody to this protein has been found in some patients with rheumatoid arthritis and systemic lupus erythrematosis. Thus the presence of this autoantibody may lead to inadequate regulation of phospholipase A_2 activities in these diseased states.

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PHOSPHOLIPASE A_2 ACTIVITY OF LYSOSOMAL ORIGIN SECRETED BY POLYMORPHONUCLEAR LEUCOCYTES DURING PHAGOCYTOSIS OR ON TREATMENT WITH CALCIUM

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1. Peritoneal neutrophil leucocytes, derived from the rabbit, release phospholipase A (EC 3.1.1.4) activity during phagocytosis of complement-coated zymosan particles, or during treatment with Ca^{2+}. This enzyme is able to release [1-^{14}C]oleate from the membrane phospholipids of *Escherichia coli*. 2. The release of phospholipase A paralleled that of the known lysosomal marker enzymes β-glucuronidase and lysozyme. The phospholipase A would thus appear to be derived from the lysosomal granules of the cells. 3. The released enzyme is of A_2 specificity, has an absolute requirement for divalent cations, and is active over a broad pH range (pH 6–9).

Introduction

The importance of prostaglandins and related metabolites of arachidonic acid in inflammatory reactions is well documented, as indeed is the role of phospholipase A_2 in freeing arachidonic acid from phospholipids [1,2]. The appearance of prostaglandins in some inflammatory exudates is paralleled by infiltration of the inflamed area with polymorphonuclear leucocytes. A close correlation between the release of lysosomal enzymes from these cells and the appearance of prostaglandins led to the suggestion that lysosomal phospholipases A_2, derived from these cells, cause the release of precursor fatty acids for prostaglandin production [3].

A variety of physiological and pharmacological stimuli are able to cause the selective release of lysosomal contents from neutrophils. We have investigated the ability of two such stimuli to cause the release of phospholipase A_2 activity from rabbit peritoneal polymorphonuclear leucocytes. The stimuli we have used are Ca^{2+} [4] and the phagocytosis of zymosan particles coated with complement [5]. This current paper presents the results of these findings and a discussion of the properties of the released enzyme.

A preliminary account of parts of this work has been presented [6].

Materials and Methods

All reagents and solvents used were of analytical grade.

Neutrophil suspension

Female rabbits of the New Zealand White strain, weighing 2–3 kg, were lightly anaesthetised with diethyl ether and injected intraperitoneally with 100 ml sterile thioglycollate medium (U.S. Pharmacopea), supplied by Oxoid Ltd. (Basingstoke, U.K.). After 19–22 h each rabbit was anaesthetised by intravenous injection of 80 mg · kg^{-1} sodium pentobarbital, then given 150 ml sterile isotonic saline, containing 4 I.U./ml heparin, into the peritoneal cavity. The abdomen was lightly massaged and the peritoneal fluid allowed to drain from the cavity via a 16 gauge perforated plastic catheter. The cells in the fluid were collected by centrifugation at room temperature for

Abbreviation: Hepes, *N*-2,-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.

4 min, at 200 × *g*. The cell pellet was resuspended in 0.83% NH_4Cl in water (pH 7.2) to lyse contaminating erythrocytes. After 5 min, the cells were collected and washed by resuspension and centrifugation in a buffered medium containing: 150 mM NaCl, 3 mM KCl, 10 mM glucose, 5 mM Hepes, adjusted to pH 7.4 with NaOH. Microscopic examination of the final suspension showed that neutrophils accounted for more than 95% of the total leucocyte population.

Release experiments

Aliquots of the leucocyte suspension, containing approximately $1 \cdot 10^7$ cells in 1 ml Hepes buffered medium were incubated in the presence of varying amounts of complement-coated zymosan particles (0–5 mg) prepared as described by Hensen [5], or calcium (0–5 mM) added in 0.2 ml buffered medium. Incubations were performed with shaking at 37°C for 60 min, unless stated otherwise. After incubation the tubes were cooled in melting ice and the cells removed by centrifugation at 3 000 × *g* for 5 min. An aliquot of the supernatant was taken for subsequent assay of released enzymes. Protein in the samples was determined by the method of Lowry et al. [7] using bovine serum albumin as standard. In experiments using complement-coated zymosan particles the uptake of particles by the neutrophils was determined by phase-contrast microscopy, counting at least 100 cells.

Enzyme assays

The marker enzymes β-glucuronidase, lysozyme and lactate dehydrogenase were assayed by methods described by others [4]. The results of all assays were expressed as a percentage of the activity released in the presence of Triton X-100 at a final concentration of 0.2%. It was assumed that Triton released all leucocyte enzyme activity into the suspending medium.

Phospholipase A_2 activity was determined by the ability to remove [1-^{14}C]oleic acid (The Radiochemical Centre, Amersham, U.K.) from the membrane phospholipids of *E. coli* prepared as described by Patriarca et al. [8]. More than 95% of the incorporated 1-^{14}C label was in the 2-position of the membrane phospholipids, as shown by treatment with the phospholipase A_2 of crude *Crotalus adamanteus* venom.

Enzyme assays were performed using an incubation medium consisting of, unless otherwise stated, $7.5 \cdot 10^8$ autoclaved labelled bacteria, containing approximately 15 nmol of phospholipid (determined as described in Ref. 9). 40 mM Tris-malic acid buffer, pH 6.0, 5 mM $CaCl_2$ and an appropriate aliquot of the supernatant, in a total volume of 0.5 ml. Blanks without enzyme were included in all assays. Incubations were carried out for 60 min at 37°C in a shaking water bath, then stopped and hydrolytic activity determined as described previously [10]. Radioactivity in substrates and products was counted in a medium containing 3 ml water and 7 ml Unisolve 1 (Koch-Light, Colnbrook, U.K.). Enzyme activities were corrected for non-enzymic hydrolysis and expressed as initial velocities or, in release experiments, as a percentage of the total phospholipase A_2 activity released after sonication (6 μm, 3 min). This was preferred to Triton X-100 treatment since detergents are known to interfere with the activity of phospholipase A. Control experiments with the marker enzymes showed that there was no difference between the total amounts of enzyme released by the two treatments. Phospholipase A_2 activity due to the complement-coated zymosan particles preparation was 1% or less of total cellular enzyme.

Enzyme activity was also determined against *E. coli* labelled with [1-^{14}C]palmitic acid (The Radiochemical Centre, Amersham, U.K.) of which 77% was incorporated into the 1-position of membrane phospholipids as determined by treatment with the phospholipase A_2 of *C. adamanteus* venom.

Results

Release of phospholipase A during phagocytosis

Neutrophil leucocytes incubated with serum-treated zymosan, and therefore particles coated with complement, at 37°C over 60 min demonstrated the ingestion of particles and the release of phospholipase A activity. The release was concentration-dependent, being linear up to 0.5 mg coated zymosan particles and continuing up to a maximum release at 5 mg zymosan. By this method a maximum of 30% of total cell phospholipase A (activity released by sonication) was obtained. The release of phospholipase A activity was paralleled by the uptake of

TABLE I

COMPARISON OF PHOSPHOLIPASE A RELEASE WITH PHAGOCYTOSIS OF COMPLEMENT-COATED ZYMOSAN PARTICLES

Neutrophil leucocytes ($1 \cdot 10^7$) were incubated in the presence of varying amounts of complement-coated zymosan particles for 1 h at 37°C. The degree of phagocytosis was determined by light microscopy, counting at least 100 cells. Phospholipase A activity released into the incubation medium was assayed as described in the text. Values represent mean ± S.D.

Complement-coated zymosan particles (mg)	% Cells with particles	No. particles/cell	Phospholipase A release (%)
0	0	0	8.3 ± 2.0
0.25	53	1.4 ± 0.5	9.9 ± 2.8
0.5	62	2.0 ± 1.2	16.0 ± 4.0
1.0	83	2.4 ± 1.7	20.3 ± 4.8
2.5	85	2.8 ± 1.2	25.0 ± 2.0
5.0	96	3.2 ± 1.5	30.0 ± 4.0

zymosan granules by the cells, determined either as the % of cells undergoing phagocytosis, or as the number of particles ingested by each cell (Table I). The lysosomal marker enzymes β-glucuronidase and lysozyme (not shown) were released in a similar concentration-dependent manner to the phospholipase A, however, no significant discharge of the cytoplasmic enzyme lactate dehydrogenase was observed (Fig. 1). These findings suggest a lysosomal location for the phospholipase.

The secretion of phospholipase A in the presence of 5 mg complement-coated zymosan particles increased linearly up to 10 min, with maximal secretion being obtained after 1 h. A similar response was seen in the degree of phagocytosis with time, demonstrating that release of the phospholipase occurred during phagocytosis (Table II). The same pattern of release was seen for the lysosomal marker enzymes.

Release of phospholipase A by Ca^{2+}

Addition of Ca^{2+} to the neutrophil leucocytes at

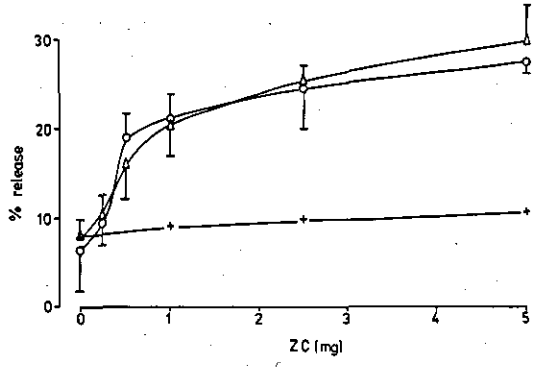

Fig. 1. Release of neutrophil enzymes in the presence of complement-coated zymosan particles (ZC). Neutrophils were incubated in Hepes buffered medium in the presence of varying amounts of complement-coated zymosan particles. After 60 min at 37°C aliquots of the suspending medium were taken for enzyme assays as described in Materials and Methods ○, β-glucuronidase; △, phospholipase A; +, lactate dehydrogenase. Points are means of three observations. Verticalbars represent 1 S.E.

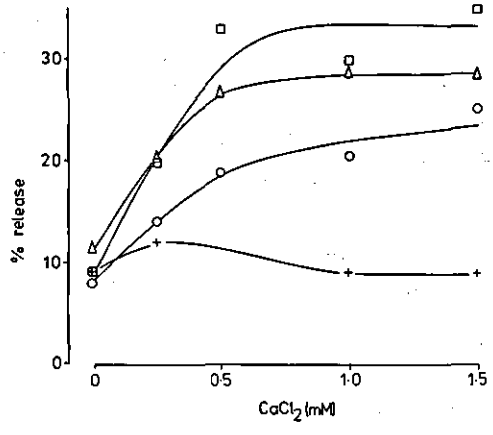

Fig. 2. Release of neutrophil enzymes in the presence of Ca^{2+}. Neutrophils ($1 \cdot 10^7$ cells) were incubated in Hepes buffered medium in the presence of varying amounts of Ca^{2+}. After 60 min at 37°C aliquots of the supernatant were examined for enzyme release as described in text. ○, β-Glucuronidase; △, phospholipase A; □, lysozyme; +, lactate dehydrogenase. Points are means of duplicate determinations.

TABLE II

TIME-DEPENDENT RELEASE OF PHOSPHOLIPASE A, β-GLUCURONIDASE, AND LYSOZYME FROM RABBIT NEUTROPHILS IN THE PRESENCE OF COMPLEMENT-COATED ZYMOSAN PARTICLES

Neutrophil leucotyes ($1 \cdot 10^7$) were incubated with complement-coated zymosan for varying times at 37°C. The degree of phagocytosis was determined by light microscopy, counting 100 cells. Released enzyme activities were assayed as described in the text. Values represent mean ± S.D. Single figures are means of duplicate determinations. n.t., not tested.

Incubation time (min)	% Cells with particles	Enzyme release (% total cell enzyme)		
		Phospholipase A_2	β-Glucuronidase	Lysozyme
0	0	1	5	2.5
3	33	10.3 ± 2.5	7.5	11.8
5	39	14.8	8.3 ± 2.4	16.8
10	58	20.7 ± 4.7	15.3 ± 1.6	20
20	n.t.	n.t.	22.0 ± 3.3	24.5
40	84	26.0 ± 4.0	24	35.5
60	n.t.	26.0 [a]	29 [a]	n.t.

[a] Cells incubated in the absence of complement-coated zymosan particles for 60 min release 8.8 ± 0.26% of total phospholipase A_2, and 10.0 ± 4.6% of total β-glucuronidase.

37°C over 1 h caused the release of phospholipase A from the cells (Fig. 2). The release of this enzyme followed the pattern of release of β-glucuronidase and lysozyme, whilst lactate dehydrogenase was not released. Maximal release of all lysosomal enzymes occurred at approximately 1.5 mM $CaCl_2$. Using 2.5 mM $CaCl_2$ a time-dependent release of the enzymes is observed (Fig. 3).

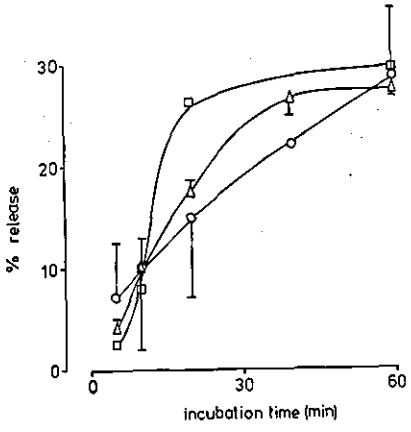

Fig. 3. Effect of incubation time on the Ca^{2+}-induced release of lysosomal enzymes from neutrophils. Neutrophils ($1 \cdot 10^7$ cells) were incubated in Hepes buffered medium containing 2.5 mM Ca^{2+} at 37°C. Aliquots of the suspending medium were taken for enzyme assay (see text) at times shown. ○, β-Glucuronidase; △, phospholipase A; □, lysozyme. Points are means of two or three observations. Vertical bars represent 1 S.E.

Properties of the released phospholipase A

The properties of the phospholipase A obtained from the release experiment were examined without further purification. The enzyme caused the release of [1-^{14}C]oleic acid from the 2-position of the membrane phospholipids of *E. coli*. All of the radio-

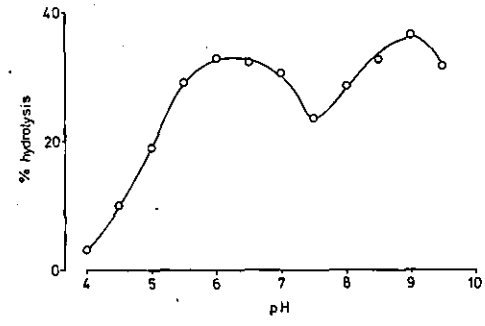

Fig. 4. Effect of pH on the hydrolysis of [1-^{14}C]oleate-labelled phospholipids of *E. coli* by a lysosomal phospholipase A released by neutrophils in the presence of complement-coated zymosan particles. The complete reaction mixture (0.5 ml) contained 5 mM $CaCl_2$, 5 nmol phospholipid ($2.5 \cdot 10^8$ *E. coli*) 60 μg protein and 40 mM appropriate buffer (pH 4–6, sodium acetate; pH 5–7.5, Tris-maleate; pH 7.5–9, Tris-HCl). Incubations were performed for 1 h at 37°C.

TABLE III

EFFECTS OF NEUTROPHIL LEUCOCYTE PHOSPHOLIPASE A TOWARDS DIFFERENTLY LABELLED PHOSPHOLIPIDS OF *E. COLI*

The complete incubation mixture contained 10 nmol phospholipid ($5 \cdot 10^8$ *E. coli*), 40 mM Tris-maleate (pH 9.0), 5 mM $CaCl_2$ and 96 μg released enzyme protein (for [1-^{14}C]oleate) or 20 nmol phospholipid ($1 \cdot 10^9$ *E. coli*), 40 mM Tris-HCl (pH 6.0), 5 mM $CaCl_2$ and 0.18 mg leucocyte homogenate protein (for [1-^{14}C]palmitate) in a total volume of 0.5 ml. Incubations were performed for 60 min at 37°C.

	Radioactivity (%) in lipid fractions of *E. coli* labelled with:			
	[1-^{14}C]Oleate		[1-^{14}C]Palmitate	
	Control	+Phospholipase	Control	+Phospholipase
Phospholipid	97	49	73	17.7
Fatty acid	2.5	50	16.5	27.3
Lysophospholipid	0.5	1	9.5	55.0

activity released was, after thin-layer chromatography [10], recovered in the fatty acid fraction, and none in the lyso product (Table III), suggesting the enzyme to be of A_2 specificity. Using *E. coli*, the phospholipids of which were labelled in the 1-position with [1-^{14}C]palmitate, and a preparation of sonicated neutrophils this specificity was further confirmed. Although in the substrate only 77% of the label was specifically incorporated into the 1-position, and some fatty acid remained unincorporated it can be seen (Table III) that after enzymic hydrolysis the radioactivity in the lyso fraction markedly increased, indicating the removal of the non-labelled fatty acid in position 2. Of the activity lost by the phospholipid 80% was recovered in the lyso fraction, which compares well with the specificity of the original preparation.

The hydrolytic activity of the released lysosomal contents towards [1-^{14}C]oleate-labelled *E. coli* lipids showed two pH optima, a broad peak at pH 6 and an alkaline optimum at pH 9.0 (Fig. 4). The phos-

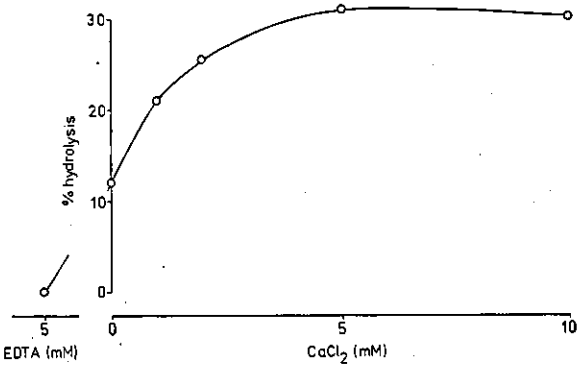

Fig. 5. Effects of Ca^{2+} concentrations on the hydrolysis of [1-^{14}C]oleate-labelled phospholipids of *E. coli* by lysosomal phospholipase A released from neutrophils during phagocytosis of complement-coated zymosan particles. The complete reaction mixture (0.5 ml) contained 5 nmol phospholid ($2.5 \cdot 10^8$ *E. coli*), 40 mM Tris-HCl (pH 6.0), 64 μg protein and varying amounts of $CaCl_2$ or EDTA. Incubations were carried out for 60 min at 37°C.

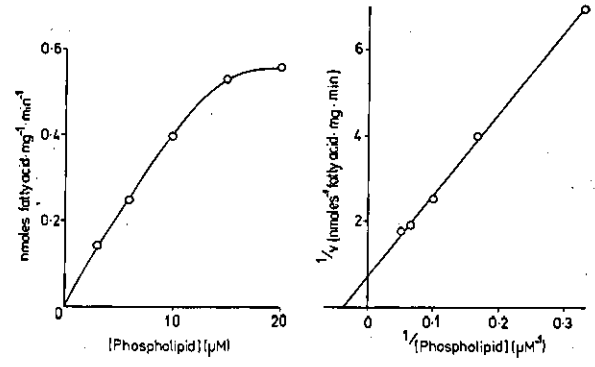

Fig. 6. Hydrolysis of [1-^{14}C]oleate-labelled *E. coli* phospholipids by lysosomal phospholipase A of neutrophils, released during phagocytosis of complement-coated zymosan particles, at various substrate concentrations. The complete reaction mixture (0.5 ml) contained 40 mM Tris-maleate (pH 9.0), 96 μg enzyme protein, 5 mM $CaCl_2$ and 3.0–20 μM phospholipid ($(0.75-5) \cdot 10^8$ *E. coli*). Incubations were carried out at 37°C for 60 min.

pholipase A activity had a requirement for calcium. At both pH optima maximal activity occurred at a calcium concentration of 5 mM and above. Fig. 5 gives the curve obtained at pH 6.0. In the absence of added Ca^{2+}, activity was less than 50% of the maximal value. This was possibly due to the presence of endogenous divalent cations since the addition of 5 mM EDTA abolished activity totally.

Enzymic activity of the phospholipase A_2 was linear up to 40 min and up to 64 μg protein. The effect of substrate was examined at concentrations varying from 3 to 20 μM *E. coli* phospholipid per assay. The reaction remained linear up to 12 μM phospholipid, equivalent to $3 \cdot 10^8$ labelled, autoclaved *E. coli* (Fig. 6). The Lineweaver-Burk plot of the data [11] (Fig. 6) gives an apparent K_m of $26 \cdot 10^{-6}$ M.

The released phospholipase A activity was relatively stable to storage at 4°C if assayed at pH 9.0, losing only 20% of its original activity over 20 days. However during this same time period 78% of the original activity was lost if assayed at pH 6.

Discussion

The present results demonstrate the release of phospholipase A_2 activity from rabbit neutrophil polymorphonuclear cells during particle uptake. The release of the enzyme follows the phagocytosis of zymosan in a concentration-dependent manner. The source of the enzyme would appear to be the lysosomes since its release follows closely the release of two lysosomal marker enzymes β-glucuronidase and lysozyme. The lack of appearance of the cytoplasmic lactate dehydrogenase in the incubation medium demonstrates that the cells remain intact. The release of phospholipase A_2, along with the other lysosomal enzymes, is time-dependent, and follows the time-dependency of phagocytosis, indicating that release occurs during phagocytosis. This is to be expected if the phospholipase is of lysosomal origin, since the mechanism of release of lysosomal contents during phagocytosis is by a process termed 'regurgitation during feeding', the lysosomal contents being released through incompletely closed phagosomes [12].

The Ca^{2+}-induced release of lysosomal marker enzymes, but not cytoplasmic enzymes, from rabbit neutrophil leucocytes previously suspended in Ca^{2+}-free medium agrees with previous findings [5]. Since the release of phospholipase A_2 follows that of the lysosomal markers, in relation to both Ca^{2+} concentration and time, we conclude that the phospholipase A_2 is of lysosomal origin and is released by exocytosis [15] from polymorphonuclear cells. However, rabbit polymorphonuclear leucocytes possess at least two distinct enzyme-containing granules. The azurophil granules contain most of the β-glucuronidase, whilst the specific granules contain most of the lysozyme [13,14]. From our findings it is not possible to say with which granule phospholipase A_2 is associated. We are looking into the possibility of repeating our experiments on human peripheral neutrophils since such cells, when treated with Ca^{2+}, are provoked to release lysozyme, but not β-glucuronidase [15].

Treatment with either Ca^{2+} or with zymosan-complement caused the release of approximately 30% of the total cellular phospholipase A_2 activity, as assayed at pH 6.0. The lysosomal marker enzymes were released to a similar degree. These findings are in agreement with degrees of release obtained by other workers [4,5].

The lysosomal phospholipase A_2 activity released by neutrophil leucocytes is evident over a broad range of pH from 5.5 to 9.5, although with a consistent drop in activity at pH 7.5. Even so, considerable activity at neutral pH is retained. The enzyme activity has an absolute requirement for divalent cations, and shows linear hydrolytic activity up to $3 \cdot 10^8$ microorganisms, representing 12 μM phospholipid.

The soluble phospholipase A_2 described here has marked similarities to a membrane-bound phospholipase A_2 associated with azurophil and specific lysosomal granules [16]. This latter enzyme has two pH optima at pH 6 and 7.5, although it has considerably less activity at pH 7, requires Ca^{2+}, has a similar optimum for substrate, but differs in that activity at both acid and alkaline pH is stable, although the apparent instability of the released enzyme may be due to the presence of released proteases. An unstable acid phospholipase activity has been described in leucocyte homogenates [17].

The above differences may suggest that the soluble enzyme described here might not be derived from the membrane-bound phospholipase A_2 of azurophil and

specific lysosomal granules. A possibility is that it may be contained in a less dense, ill-defined heterogenous group of granules [13,18] which, indeed, do retain a considerable portion of the phospholipase A_2 activity of the original leucocyte homogenate [16].

The phospholipase A_2 activity released by polymorphonuclear leucocytes during phagocytosis or treatment with Ca^{2+} bears a close resemblance to a soluble phospholipase A_2 found in sterile peritoneal exudates of the rabbit after intraperitoneal injection of glycogen [19]. This enzyme is also Ca^{2+}-dependent, phospholipase A_2-specific and has a broad optimum at pH 6–8, with slightly less activity at pH 6.5. The released lysosomal enzyme may well be the source of this phospholipase since at the time this enzyme is harvested 95% of the infiltrating cells are neutrophils [6,18].

The lysosomal phospholipase A_2 selectively released by neutrophils may be of importance in the pathogenesis of inflammatory diseases.

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Release of a lysosomal phospholipase A from rabbit neutrophil leucocytes

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The appearance of prostaglandins in some inflammatory exudates is paralleled by infiltration of the inflamed area with polymorphonuclear leucocytes (PMN). A close correlation between the release of lysosomal enzymes from these cells, and the appearance of prostaglandins led to the suggestion that lysosomal phospholipases A cause the release of precursor fatty acids for prostaglandin production (Anderson, Brocklehurst & Willis, 1971). The importance of phospholipase A_2 in prostaglandin biosynthesis has been confirmed (Blackwell, Flower, Nijkamp & Vane, 1978). This communication describes the release and properties of a phospholipase A liberated from PMN leucocytes during phagocytosis of zymosan-complement (ZC) (Henson, 1971), and by calcium (Northover, 1977).

PMN's were obtained from female White New Zealand rabbits (2.5 kg) 18 h after intraperitoneal injection of sterile thioglycollate medium, U.S.P. (100 ml) as described by Northover (1977). Neutrophils (1×10^7) were incubated at 37° in medium containing (mM) NaCl 150, KCl 3, glucose 10, Hepes 5, pH 7.4, with varying amounts of ZC or calcium. Aliquots of the medium were incubated with [1-^{14}C]-oleate labelled *E. coli* for 60 mins to assay phospholipase A activity (Authi & Traynor, 1979). β-Glucuronidase, lysosyme and lactate dehydrogenase activities were also determined (Northover, 1977). Results of enzyme assays are expressed as percentages of the activities released after sonication (6 μm, 3 min) of the cells (for phospholipase activity), or treatment with Triton X-100 (0.2%).

ZC and calcium caused a time and concentration dependant increase in the release of phospholipase A activity. Maximal release of approximately 30% of the total activity was obtained at 2.5 mM calcium, or 2 mg/ml ZC. The release of phospholipase A followed that of β-glucuronidase and lysosyme, confirming a lysosomal location for the enzyme. No such release of the cytoplasmic marker lactate dehydrogenase was observed.

The released phospholipase A has two pH optima at pH 6 (broad) and pH 9 and a requirement for calcium. The enzyme appears to be of A_2 specificity since 95% of the label incorporated into phospholipid was in the 2-position and no radioactivity was recovered in the lyso product. A phospholipase A with similar properties has been found in rabbit peritoneal exudates after injection of glycogen (Fransen, *et al.*, 1978).

The lysosomal phospholipase A described may be of importance in the observed production of prostaglandins by PMN leucocytes during phagocytosis (Higgs, McCall & Youlten, 1975) and in the pathogenesis of inflammatory diseases generally.

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Effects of antimalarial drugs on phospholipase A_2

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Antimalarial drugs of the chloroquine-type inhibit lipolytic processes in fat tissue *in vitro* (Markus & Ball, 1969) and phospholipase activity in various tissues (Blackwell, Flower, Nijkamp & Vane, 1978) including the malarial parasite (Cenedella, Jarrell & Saxe, 1969). Several of these drugs have been used in the treatment of rheumatoid conditions. We report the effects of three antimalarial drugs, chloroquine, mepacrine and primaquine, on the activity of a crude phospholipase A_2 enzyme obtained from an inflammatory peritoneal exudate (Fransen, Dobrow, Weiss, Elsbach & Weglicki, 1978).

Phospholipase A_2 activity was assayed against E. coli labelled with [1-^{14}C]-oleate. Greater than 95% of the incorporated label was in the 2-position of membrane phospholipids. Radiolabelled E. coli were autoclaved for 15 min at 2.7 kg/cm^2 to inactivate endogenous bacterial phospholipases and render the membrane more susceptible to enzymic attack. Assays were performed at pH 6.0 in tris buffer 4×10^{-2} M containing calcium 5×10^{-3} M at 37°C for 5 min. Lipid products were extracted, separated by TLC and areas of plates containing radioactive lipids were scraped off and radioactivity determined by scintillation counting.

All three drugs inhibited the enzymic hydrolysis of E. coli phospholipids. IC_{50} for mepacrine was 33×10^{-5} M, primaquine was twice and chloroquine five times less active. In addition the drugs chloroquine and mepacrine showed a stimulation of hydrolysis at approximately 20-fold lower doses, whilst at doses above those causing maximum inhibition a reversal of the inhibitory effects was seen.

The antimalarial drugs examined are amphiphilic cationic drugs. Other drugs of this general type inhibit phospholipase enzymes, possibly by effects on the substrate (Kunze, Nahas, Traynor & Wure, 1976). In relation to this we have studied the effects of the drugs on the stability of guinea-pig red blood cells to hypotonic haemolysis. All three drugs again showed paradoxical dose-dependent inhibitory and stimulatory effects.

The findings described may be of relevance to the use of the drugs in the treatment of malaria and rheumatoid diseases and in their effects in causing drug-induced lipidoses.

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