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SULPHYDRYL-DEPENDENT REACTIONS OF D-PENICILLAMINE:

PHARMACOKINETICS AND EFFECTS ON MONOCYTE FUNCTION IN

RHEUMATOID ARTHRITIS

Submitted by

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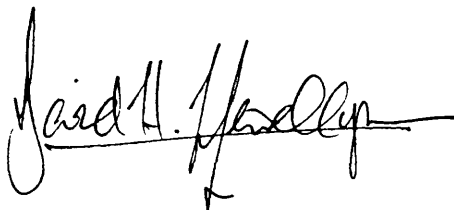
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ACKNOWLEDGEMENTS

DYFAL DONC A DYRR Y GARREG

- Welsh proverb.

(Translation; It's the steady tapping that breaks the stone)

This thesis is dedicated to my mother whose

unfailing love and encouragement throughout

the years have made it possible.

Also, to my brother and sister and the memory

of my late father, Arthur Llewellyn

(1921 - 1968).

SUMMARY

The project consisted of two major sections:-

(i) A high performance liquid chromatography assay to determine
total D-penicillamine in human plasma was developed and modified.
The final system comprised two columns together with a switching
valve, coupled with an electrochemical detector fitted with a
sulphydryl-specific amalgamated gold electrode. The resultant
chromatography was rapid, exhibited high sensitivity (<2.5 ng on
column) and very reproducible rendering it suitable for pharmaco-
kinetic studies. The pharmacokinetics of the drug were studied
in normal volunteers and rheumatoid arthritis patients. Absorption
of D-penicillamine was reduced when administered with food. The
drug was rapidly absorbed from the gut reaching its peak plasma
concentration at about two hours and exhibited two half-lives,
an initial rapid decline (5-6 hours) followed by a slower terminal
phase (55-60 hours). These results are discussed with regard to
possible correlations between steady-state drug levels and clinic-
al efficacy and toxicity exhibited by D-penicillamine.

(ii) Successful therapy with D-penicillamine leads to an improvement
in monocyte accessory function mediated, at least partially, by
an effect of the drug on cell surface sulphydryl groups. The
sulphydryl dependence of monocyte functions relevant to their
accessory function as possible targets for D-penicillamine was
investigated. Antigen presentation and Ca^{2+} ion mobilisation
were found to be unaffected by reversible blockade of cell mem-
brane sulphydryl groups by para-hydroxymercurisulphonic acid
(pHMPSA). Sulphydryl blockade by this agent inhibited reacylation
of free fatty acid but this effect was reversible by thorough

washing of the cells. Oxidative metabolism of monocytes was abolished by pHMPSA treatment. However, monocytes from rheumatoid patients treated with NSAIDs and with D-penicillamine generated higher amounts of H_2O_2 than cells from control subjects. The relevance of these findings in relation to rheumatoid arthritis and D-penicillamine therapy of the disease is discussed.

Chapter One

INTRODUCTION

This thesis has investigated different aspects of the pharmacology of a sulphydryl-containing anti-rheumatic drug, D-penicillamine. The work undertaken has comprised two major areas:-

(i) The development of a high performance liquid chromatography (HPLC) system to measure D-penicillamine in plasma, and

(ii) The delineation of Sulphydryl-dependent monocyte functions which may represent possible targets for D-penicillamine in rheumatoid patients.

This has necessitated the following layout for the introduction. First, there is a section (1A) on rheumatoid arthritis which discusses its pathology, immunopathogenesis etc. This section terminates with the chemotherapy of rheumatoid arthritis which is not intended to provide a comprehensive review of the topic but merely to give a brief overview of non-steroidal anti-inflammatory drugs and second-line anti-rheumatic agents. Section 1B is a detailed account of D-penicillamine, its pharmacology, use in rheumatoid arthritis and the development of assays to measure the drug. Finally, there is a section (1C) on the role of SH groups in the immune response and their observed disturbance in rheumatoid arthritis. This highlights a possible link between the aberrant immune response in rheumatoid patients and the immunomodulatory properties of sulphydryl-reactive anti-rheumatic drugs such as D-penicillamine.

1.A. RHEUMATOID ARTHRITIS

The term rheumatoid arthritis (RA) describes a chronic inflammatory disorder of unknown aetiology which characteristically affects the synovial joints (Short et al, 1957). The articular inflammation may go into remission but generally continues to cause progressive joint destruction and deformity leading to varying degrees of disability. Extra-articular features such as nodules, vasculitis and

splenomegaly which may accompany the joint symptoms emphasise the systemic nature of the disease. The most commonly affected joints are the proximal, interphalangeal and metacarpophalangeal joints, wrists, metatarsophalangeal joints and knees. The disease is about three times more common in females than in males. Onset of the disease can occur at almost any age but peak incidence occurs between ages 25-55.

In order to understand the pathological changes that occur in the joints in RA, it is necessary to describe the structure of normal synovial joints.

1.A.1. SYNOVIAL JOINT STRUCTURE

Synovial or diarthrodial joints possess a joint cavity which permits them to be freely movable. Such joints are encompassed by a capsule consisting of a dense, fibrous outer layer (fibrosum) which diminishes towards the articular cavity giving way to a thin lining of synovial membrane. This membrane encapsulates the whole of the articular cavity except at those places where the ends of the opposing bones are covered by articular cartilage. The synovial cavity is filled with a highly viscous fluid which lubricates the joint surfaces. A diagrammatic representation of a typical synovial joint is shown in figure 1.

The synovial membrane is considered to consist of two basic layers. The innermost layer lining the synovial cavity, known as the intima, is predominantly cellular with an abundant blood supply. The intima lies upon a less vascular sub-synovial layer consisting of a meshwork of connective tissue. This can be mainly areolar, fibrous or fatty in its nature depending upon its location in the joint. The outer part of this sub-synovium merges with the fibrosum of the capsule.
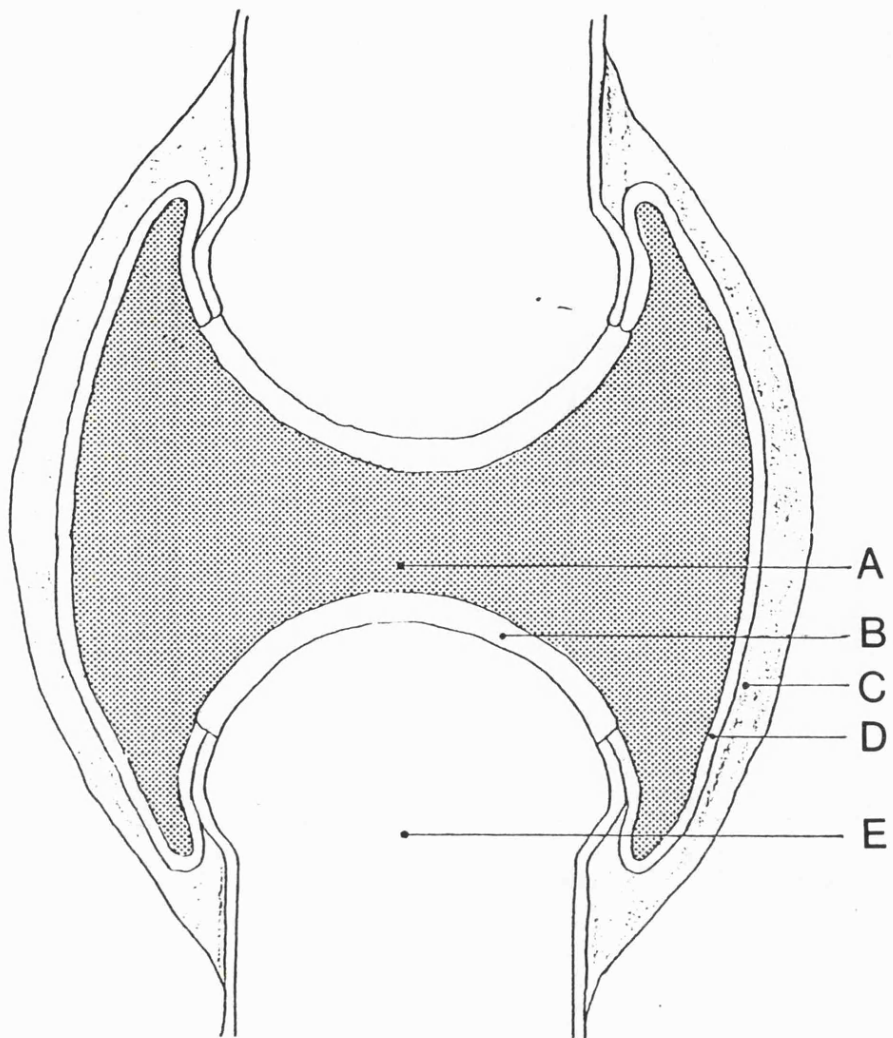
A = SYNOVIAL FLUID

B = ARTICULAR CARTILAGE

C = FIBROUS CAPSULE

D = SYNOVIAL MEMBRANE

E = BONE

FIG.1. Diagrammatical cross-section showing the structure of the typical synovial joint.

The basic functions of the synovial membrane are the
production of the synovial fluid and the removal of debris and
foreign material from the synovial cavity. Microscopic studies
of the intimal layer distinguish two major cell types (Ghadially
and Roy, 1969). The predominant cell type, termed type A, is
characterised by its large number of organelles including numerous
lysosomes and vacuoles and small amount of endoplasmic reticulum.
These cells resemble macrophages in their characteristics being
active in phagocytosis and secretion. In contrast, type B cells
have few organelles and possess abundant endoplasmic reticulum.
These cells are thought to produce the hyaluronate of the synovial
fluid. In addition there are some cells which share the features of
both major cell types. These intermediate cells, termed AB cells by
Ghadially (1983), suggest that type A and B cells are not distinct
races of cells but merely represent different functional phases of
the same basic cell.

Synovial fluid is essentially a dialysate of plasma to which
hyaluronate has been added. This hyaluronate, produced by cells of
the synovium, is a high molecular weight glycosaminoglycan respons-
ible for the viscosity of the fluid and hence essential to its lub-
ricating properties. The fluid is normally clear and does not clot
due to the absence of fibrinogen. It bathes the articular surfaces
serving as a source of nutriment for the cartilage.

The articular cartilage covering the ends of opposing bones
is the weight-bearing surface of the joint. In adults it is an
avascular, alymphatic and aneural tissue composed of a small number
of chondrocytes set in an abundant matrix. This matrix consists
basically of a framework of collagen fibrils (mainly type II collagen)
which entraps a ground substance rich in water and proteoglycans

manufactured by the chondrocytes. The functions of articular cartilage depend upon its resilience and load-bearing properties. These are due to the osmotic pressure of water taken up by the proteoglycans retained and restrained by the network of collagen fibrils. (Ghadially, 1983).

1.A.2. PATHOLOGY OF THE RHEUMATOID JOINT

Correct mechanical functioning of the joint depends upon maintenance of normal joint architecture. In RA, the normal structure of the joint becomes altered thus causing a disturbance in its normal function. This leads to the disability so characteristic of the disease, the degree of which is largely determined by the extent of joint damage.

It has been difficult to appraise the early lesions that occur in the rheumatoid joint since most patients do not present until the synovitis is quite well established. Also, synovectomies are not common, particularly in early RA. Amongst the earliest documented events are an increase in blood flow to the joint together with some oedema of the sub-synovium. Microvascular injury is evident as demonstrated by endothelial cell damage, gaps between the endothelial cells, thrombosis of small vessels and extravasation of erythrocytes. The synovial lining cells undergo a mild hyperplasia and, in some cases, there is a small degree of polymorphonuclear (PMN) cell infiltration into the superficial synovium and perivascular locations. The synovial fluid also contains small numbers of mononuclear cells.

As rheumatoid synovitis becomes established, the synovium undergoes gross histopathological changes. There is substantial hypertrophy and hyperplasia of the synovial lining cells. The intima, normally only 1-3 cells deep, can reach thicknesses of up to about 10 cells. Within the intimal layer there are multinucleated giant cells which are probably derived from type A cells (Ghadially and

Roy, 1969). The hyperplasia seems to involve all the different

synovial cell types but while Ghadially (1983) asserts that there

is a predominance of type AB and B cells, other workers maintain

that many of the hyperplastic cells appear to be the macrophage-

like type A cells (Meijer et al, 1977; Theofilopoulos et al, 1980).

The inflamed, oedematous synovium protrudes into the joint cavity

as slight villous projections extending towards the articular cartil-

age.

There is extensive mononuclear cell infiltration of the normally

acellular sub-synovial stroma. Sometimes these cells are collected

into follicles particularly around smaller blood vessels although true

germinal centres are rare. The dominant cell within such aggregates

in the B-lymphocyte (Meijer et al, 1977; Janossy et al, 1981).

The majority of mononuclear cells within the rheumatoid synovium,

however, are T-lymphocytes which account for up to 85% of the lympho-

cytes found there. (Meijer et al, 1977; Froland and Abrahamsen, 1979).

Along with the changes in cellular structure of the synovium,

there is a concomitant increase in the volume of the synovial fluid

which shows extensive cellular infiltration. The majority (75-90%)

of these are PMN cells with the remainder consisting mainly of lympho-

cytes together with some monocytes, macrophages and synovial lining

cells (Zvaifler, 1973). Plasma proteins such as fibrinogen which

are normally excluded from synovial fluid are found within rheumatoid

synovial fluid due to increased vascular permeability. Activation of

proteins such as fibrinogen and plasminogen, to form fibrin and

plasmin respectively, may be involved in articular inflammation

(Zvaifler, 1973).

The normal capacity of cartilage to rebound from a deforming

force is lost in the rheumatoid joint due to the loss of proteoglycans

from the cartilage matrix (seen as a loss of metachromatic staining).

Although most of the proteoglycan depletion occurs in regions where the inflamed synovium has encroached as pannus upon the cartilage surface, depletion is also seen at sites well removed from the advancing margins of the synovial membrane. Proteoglycan loss is probably a result of enzymatic digestion of the core protein of the proteoglycan subunit by proteases (Krane, 1979). Such proteases are found within the synovial fluid that bathes the cartilage.

Originally, the acid protease cathepsin D was proposed as the critical enzyme responsible for such damage (Fell and Dingle, 1963; Poole et al, 1976). Woessner, however, has demonstrated that this enzyme is only effective at acid pH^S, (Woessner, 1974). Thus cathepsin D is unlikely to play an important role in extracellular digestion of proteoglycans since such low pH^S are not found in rheumatoid synovial fluids (Falchuk, Goetzl et al, 1970). Extracellular destruction of proteoglycans is likely to be caused by neutral proteases such as those produced by human granulocytes characterised as an elastase and a chymotrypsin-like enzyme (Keiser et al, 1976). The activity of these enzymes is normally regulated by inhibitor proteins such as α_2-macroglobulin and α_1-antitrypsin. These inhibitors are present within rheumatoid synovial fluids thus making it unlikely that grossly uncontrolled degradation occurs (Krane, 1979). Proteoglycan loss may also be attributable to a depressed synthetic capacity of the cartilage in the inflamed joint. This inhibition may be due to soluble factors present in rheumatoid synovial fluid such as prostaglandins (Malemud and Solokoff, 1977).

Loss of proteoglycans from the cartilage is insufficient to cause irreversible structural damage since the skeletal framework is provided by collagen. Loss of collagen therefore is responsible

for structural damage. The triple helical configuration of the
collagen molecule imparts a resistance to proteolytic digestion
by enzymes such as trypsin, pepsin and chymotrypsin (Harris and
Krane, 1974). Formation of collagen fibrils further enhances this
resistance.

It has been demonstrated that explants of rheumatoid synovial
tissue in culture produce collagenases which are neutral metal pro-
teases able to digest collagen (Evenson, 1968). Proteolytic attack
by these enzymes cleaves the collagen chains into two fragments
which leaves the structure open to further digestion by other
proteases (Harris et al, 1972).

The greatest destruction of connective tissue in the rheuma-
toid joint occurs where the invading pannus extends over the surf-
ace of the articular cartilage. Pannus is a fibro-vascular gran-
ulation tissue comprising connective tissue, small blood vessels,
hyperplastic mesenchymal cells and various inflammatory cells,
which arises from the perichondral synovial membrane (Zvaifler,
1973). The cells in the pannus which are responsible for collag-
enase production are large, nucleated cells which have dendritic
processes but lack macrophage markers and are presumably syno-
viocytes. Collagenase and prostaglandin production by these syno-
viocytes is greatly enhanced by the cytokine, Interleukin-1,
(IL-1) which is produced by cells of the monocyte/macrophage lin-
eage in the pannus. (Mizel et al, 1981). The collagenase is re-
leased in an inactive form which can however bind to collagen
fibrils (Werb et al, 1977). It is unclear whether this latent form
of the enzyme is a true zymogen or the active enzyme complexed
with an inhibitor (Krane, 1979); however, activation is achieved
by the action of proteolytic enzymes produced by the cells of the

pannus. Another possible activator of the latent collagenase which may be of considerable importance in vivo is plasmin since rheumatoid synovial cells produce plasminogen activator which converts plasminogen found in rheumatoid joints into plasmin. (Werb et al, 1977). Plasmin may activate the inactive enzyme attached to collagen fibrils thus initiating the destruction of collagen containing tissues. Plasminogen activators can also be produced by endothelial cells of the small blood vessels in the pannus.

Once initiated, the collagenolytic process can be continued by enzymes produced by pannus cells such as cathepsin D which can degrade the collagen fragments produced by collagenase action. Collagen fibres are sometimes observed within phagolysosomes of cells within the pannus (Kobayashi and Ziff, 1975). Chondrocytes near the advancing edge of the pannus are able to replicate within their lacunae and can be activated to secrete enzymes capable of degrading the cartilage matrix (Harris, 1979). Again, IL-1 is able to induce such activity in the chondrocytes (Jasin and Dingle, 1981). Collagen loss from the cartilage is essentially irreversible since the chondrocytes have limited ability to regenerate the type II collagen (Krane, 1979).

In chronic, persistent RA there is characteristic erosion of the subchondral bone. Most of this damage is presumed to be caused by destruction by the pannus but some bone loss may also result from increased bone resorption by osteoclasts. The rheumatoid synovium may produce factors that possibly contribute to the enhanced resorptive activity such as prostaglandin E_2 (Robinson et al, 1975) and osteoclast activating factor (Krane, 1979).

The destructive changes to the cartilage and bone are the major factors in causing disruption of joint function. Weaken-

ing of the joint capsule and tendons can occur along with liga-
ment damage causing instability and subluxation. Fibrosis and
ankylosis of opposing articular surfaces can occur in severe joint
damage leading to decreased freedom of movement.

1.A.3. EXTRA-ARTICULAR MANIFESTATIONS OF RHEUMATOID ARTHRITIS

Although essentially a disease affecting synovial joints,
the systemic nature of RA is emphasised by the constitutional symp-
toms seen in the disease. These include general malaise, weight
loss and fever (Fye, 1978). Some degree of anaemia is found in al-
most all rheumatoid patients, a finding common to most chronic in-
flammatory conditions (Turnbull, 1971). In addition, extra-artic-
ular manifestations can occur involving a number of other tissues
of the body. The appearance of such features generally occurs in
seropositive patients i.e. patients whose serum contains rheuma-
toid factor (RF), immunoglobulin directed against the Fc portion of
IgG.

Probably the most common of these manifestations are subcut-
aneous nodules which appear at some time in about 20-25% of patients
who are almost invariably seropositive. The nodules are firm mass-
es, varying in shape and size, within subcutaneous or deeper con-
nective tissues. Histologically, the rheumatoid nodule is com-
posed of a central area of necrosis surrounded by a palisade of
epitheloid cells with variable degree of lymphocyte and plasma cell
infiltration and vasculitis. The nodules may appear, disappear and
reappear at any time. Although they rarely cause symptoms, they
can break down and become infected.

A wide range of vasculitic lesions often occur in rheumatoid
patients, generally in those patients with long-standing disease
with high RF titres and nodules. Most of the lesions are often

clinically benign such as those affecting the digital blood

vessels characterised pathologiclaly by intimal proliferation.

Rarely, some patients develop a vicious necrotising arteritis

causing necrosis of the skin, digital gangrene, neuropathic les-

ions and visceral infarction which may lead to death. Fortunately

this explosive arteritis occurs very infrequently and one or more

of these manifestations can appear insidiously without necessarily

being life-threatening. The pathogenesis of rheumatoid vasculitis

is unclear though it is thought that deposition of circulating imm-

une complexes may play a prominent role. However, their exact

relationship to vascular damage has yet to be fully understood.

Other extra-articular features include scleritis, muscle wasting,

lymphadenopathy and splenomegaly. Felty's syndrome is a symptom

complex in which RA is coupled with splenomegaly and neutropenia.

It has been suggested that hypersplenism is the cause of the neutro-

penia but in some patients the neutropenic abnormality does not

respond to splenectomy.

1.A.4. IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS

There is considerable evidence implicating both humoral and

cell-mediated immunological processes in the pathogenesis of RA.

The rheumatoid joint shows substantial infiltration by lymphoid and

phagocytic cells and interactions between these cells and their

products are thought to contribute to the tissue destruction so

characteristic of RA. Within the synovium, lymphocytes, plasma

cells and macrophages are found in both diffuse and nodular patt-

erns, an arrangement which has been likened to an antigenically

stimulated lymph node (Gardner et al, 1965).

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1.A.4.(i) HUMORAL IMMUNITY

In RA, serum immunoglobulin levels are raised whilst at the major site of inflammation, the rheumatoid synovium, there is generation of large amounts of immunoglobulin. About 25% of the immunoglobulin found in the synovial fluid is synthesised by the lymphocyte infiltrate of the synovial pannus (Sliwinski and Zvaifler, 1970). Excised rheumatoid synovium has also been shown to produce immunoglobulin in vitro (Smiley et al, 1968). These studies however did not define the antigenic specificity of the antibody produced. One study using synovial explants from RA patients previously immunised with tetanus showed the production of only very small amounts of specific anti-tetanus antibody. One reason for this might be that lymphoid cells of the rheumatoid synovium are immunologically committed to another antigen prior to tetanus challenge (Herman et al, 1971).

Egeland and colleagues have shown the proportions of immuno-globulin produced in the synovium to be IgG, 48%, IgM, 18%, and lgA, 34% (Egeland et al, 1982). Interestingly, IgG produced by the synovium shows a restricted electrophoretic mobility with consider-able enrichment of the IgG-3 subclass (Munthe and Natvig, 1972; Hoffman et al, 1982; Smiley et al, 1985). Some studies have also found an increased incidence of light chains (Lindstrom 1970, Munthe and Natvig, 1972). Such restricted responses might indicate a selective stimulation of the RA synovial lymphoid infiltrate (Smiley et al, 1985).

The major humoral immune reaction in RA is the production of rheumatoid factors (RF's) i.e. auto-antibodies directed against the Fc region of IgG molecules. RF's are found in the IgG, IgM and IgA classes (Johnson and Faulk, 1976). It is important, however,

to note that RF's are not specific for RA as they can be found in

the serum of patients with other rheumatic diseases such as SLE,

non-rheumatic diseases such as bacterial endocarditis as well as in

that of some normal individuals (Carson, 1982). In serum, IgM RF

may react with IgG to give complexes with a sedimentation coeffic-

ient of about 22S which are probably cleared by the reticulo-endo-

thelial system (Paget and Gibofsky, 1979). IgG RFs can obviously

self-associate to form intermediate-sized complexes, the presence

of which has been demonstrated in the rheumatoid synovium of both

seropositive and seronegative patients with active disease (Munthe

and Natvig, 1972). Such complexes may play an important role as

inflammatory stimuli in rheumatoid synovitis (Munthe and Natvig,

1972; Male and Roitt, 1981). Both IgM RF and IgG RF can fix com-

plement (Tanimoto et al, 1975; Brown et al, 1982) whilst IgG-IgG

RF complexes can stimulate local production of RFs and other immuno-

globulins in the synovium (Chattopadhyay et al, 1979; Egeland et al,

1982). Complexing of these immunoglobulins may also impair negative

feedback upon plasma cell antibody production causing a perpetuation

of the immune response and inflammation (Natvig and Munthe, 1975).

One possible explanation for RF production is the alteration of auto-

logous IgG leading to enhanced immunogenicity. It has been suggested

that free radicals generated by activated neutrophils can alter the

structure of IgG (Lunec   ·   1984).

The pathogenic importance of RF in RA remains controversial.

Synthesis of RF undoubtedly occurs in the rheumatoid synovium but

whilst early studies indicated that about 60% of synovial IgG pro-

ducing plasma cells produced RF (Smiley et al, 1968),more recent

evidence shows very few synovial plasma cells synthesising RF

(Egeland et al, 1982). Such observations might argue against any significant pathogenic role for RF in articular inflammation. Recently, an immunoregulatory role has been proposed for RFs as anti-idiotypic antibodies directed against anti-bacterial peptidoglycan antibodies (Johnson, Phua and Evans, 1985).

Whatever the specificity of antibodies produced by rheumatoid synovial lymphoid cells, one of the main postulates concerning the pathogenesis of inflammation in RA is the formation of immune complexes in the joints (Zvaifler 1973). Immune complexes (ICs) can cause activation of the complement cascade and complement fragments are demonstrable in rheumatoid synovial fluid. Complement levels, whilst apparently normal in the serum of rheumatoid patients, are reduced in the synovial fluids of most patients correlating inversely with IC levels (Luthra et al, 1975). Localisation of immunoglobulin and complement in articular cartilaginous tissues has been demonstrated in the majority of RA patients (Cooke et al, 1975). Complement fragments C3a and C5a are chemotactic for PMNs and so would help promote the influx of these cells into the rheumatoid synovium. These neutrophils, together with synovial pannus macrophages, phagocytose the complexes with concomitant release of hydrolytic enzymes capable of causing articular damage (Zvaifler, 1973). Activation of complement by proteolytic enzymes present in rheumatoid synovial fluid may be important in perpetuating the inflammatory response (Mellbye et al, 1983). Engulfment of ICs also causes the phagocytic cells to release oxygen-derived radicals (ODRs). The term oxygen-derived radicals here describes superoxide anion $O_2 \cdot^-$, hydroxyl radical $OH \cdot$, hydrogen peroxide H_2O_2 and singlet oxygen $^1O_2$ although chemically only $.O_2 \cdot^-$ and $OH \cdot$ are strictly radicals. ODRs have been implicated

. as possible mediators of tissue damage in chronic inflammation.
Goldstein et al, (1976), have shown that C5a induces ODR release
from neutrophils independent of phagocytosis. ODRs have been shown
to damage cultured endothelial cells (Weiss et al,1981; Niwa et al,
1982) and the phagocytic cells which had produced them (Sweder
von Asbeck et al, 1984). Niwa and colleagues (1983) have demon-
strated that ODRs released by synovial fluid neutrophils can de-
press the reactivity of synovial fluid lymphocytes in RA perhaps
explaining their disturbed immuno-reactivity. In vitro studies have
also shown that ODRs cam damage hyaluronic acid (McCord, 1974;
Monboisse, Braquet and Borel, 1984). ODR-induced depolymerisation
of hyaluronic acid may cause the reduction in synovial fluid vis-
cosity seen in inflamed joints (Greenwald and Moy, 1980). Much
attention has focussed on ODR-induced lipid peroxidation as a mech-
anism by which ODRs can cause tissue damage (Kellogg III and
Fridovich 1975; Kellogg III and Fridovich 1977; Dixit, Mukhtar and
Bickers 1982). Both the hydroxyl radical (OH·) and singlet oxygen
($^1O_2$) can mediate lipid peroxidation (Badwey and Karnovsky, 1980).
Polyunsaturated fatty acids are particularly susceptible to lipid
peroxidation which can lead to the formation of low molecular weight
aldehydes and alkenes etc. This may cause severe disruption of the
cell membrane structure and possibly, cell death. Peroxidation
of cell membrane lipids has been shown to be able to influence
arachidonate metabolism (Cook and Lands, 1976). Cleland (1984)
has suggested that in this way ODRs derived from phagocytic cells
may influence the metabolism of adjacent cells within an inflam-
matory focus.

The increased levels of lipid peroxidation products found
in rheumatoid synovial fluid suggests that this reaction might be

important in causing tissue damage in RA (Lunec et al,1981;Rowley and

Halliwell,1983). The formation of OH· radicals from O_2^- and H_2O_2 req-

uires the presence of transition metal ions, usually iron (Gutteridge,

Rowley and Halliwell,1982). In active RA there is increased iron dep-

osition in the synovium (Blake et al,1983) and iron complexes may cat-

alyse OH· formation in synovial fluid (Rowley et al,1984). Iron cataly-

sed OH· formation and lipid peroxidation are inhibited by the iron-che-

lator desferrioxamine. The ability of this chelating agent to suppress

inflammation in animal models (Gutteridge,Richmond and Halliwell,1979;

Blake et al,1983) further implicates iron-catalysed OH· radical format-

ion and, possibly, lipid peroxidation in mediating tissue damage. Rec-

ently it has been shown that administration of intravenous dextran-iron

to RA patients to treat anaemia causes a flare-up in synovitis together

with an increase in lipid peroxidation products in the synovial fluid

(Blake et al, 1985).

1.A.4.(ii) CELL MEDIATED IMMUNITY

An increasing number of observations suggest that cell mediated

immunity plays an important role in the pathogenesis of RA. Indeed, in

congenitally agammaglobulinaemic boys there is an increased incidence of

RA in which immune complexes are, presumably, of no importance(Taussig,

1979). Similarly, a chronic arthritis may be induced in bursectomised

chickens (Dumonde,1971). In the rheumatoid synovial membrane the predom-

inant infiltrating cell is the T-cell (Abrahamsen et al,1975) while

rheumatoid synovial fluid and supernatants of rheumatoid synovial tissue

show T-cell derived lymphokine activity (Stastny et al, 1975).

A number of studies have shown impaired T-cell function in

RA. Some patients show depressed cutaneous hypersensitivity to

recall antigens (Waxman et al, 1973) and several studies have dem-

onstrated significantly depressed in vitro lymphocyte activation

by T-cell dependent antigens, phytohaemagglutinin (PHA) and con-

canavalin A (Con A) with respect to normals (Griswold and

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McIntosh, 1973; Lance and Knight, 1974; Highton et al, 1981).

Although the synovium is the major site of inflammation in
RA it is not easy to obtain mononuclear cells from it and normal
cells cannot be obtained for control purposes. Consequently, studies
of the immune response in rheumatoid arthritis have had to use in
vitro culturing of peripheral blood mononuclear cells since such
cells can be obtained easily from both RA patients and control sub-
jects. The most common methods utilised for analysing immunoreg-
ulation in vitro are measurement of lymphocyte proliferation in res-
ponse to mitogens such as PHA and ConA, and, measurement of Pokeweed
mitogen(PWM) stimulated immunoglobulin production using an enzyme
linked immunosorbent assay (ELISA). Whilst some studies have shown
a normal proliferative response by RA cells (Reynolds and Abdou,
1973; Kinsella, 1974), others have demonstrated lower responses
than in normals (Griswold and McIntosh, 1973; Lance and Knight,
1974; Highton et al, 1981). Such discrepancies may be attributable
to patient heterogenicity and differences in the techniques employed
by the investigators.Some workers have suggested that decreased res-
ponses are peculiar to particular subsets of patients e.g. those
characterised by the presence of erosive joint disease (Silverman
et al, 1976) and those that display anergic delayed hypersensitivity
in vivo (Malone et al, 1984). Immunoglobulin production by rheuma-
toid cells has been shown to be lower than controls (Alarcon et al,
1982; Poikonen et al, 1982; Plater-Zyberk et al, 1983). McKeown
et al (1984a)have shown that RA patients on non-steroidal anti-
inflammatory drugs (NSAIDs) show defective immunoglobulin product-
ion in vitro, those on penicillamine therapy show normalised res-
ponses (this will be discussed later in section 1.C.3).

The hyporesponsiveness of rheumatoid mononuclear cells in vitro suggests defective immunoregulation in the disease. Such hyporesponsiveness may seem paradoxical in view of the apparent hyperactivity of the immune response in RA e.g. enhanced circulating levels of immunoglobulin. One possible explanation for this is that pre-activation of the cells in vivo renders them unable to be triggered fully in vitro.

Regulation of the immune response is achieved in part by the release of soluble mediators called cytokines produced by mononuclear cells. Recent evidence has indicated imbalances in the production of cytokines in RA which may be relevant to the immunopathogenesis of the disease. In response to antigens/mitogens and other stimuli, cells of the monocyte lineage produce a 12,000-15,000 MW factor called interleukin-1 (IL-1) (Mizel et al, 1981; Oppenheim and Gery, 1982). This induces T-cells to produce another cytokine, interleukin-2 (IL-2) (Smith et al, 1980) necessary for the proliferation of T-cells (Morgan et al, 1976). IL-2 synthesis leads to the production of gamma-interferon (γ-IFN) by T-cells (Torres, Farrar and Johnson, 1982) another cytokine which regulates the immune response. In addition to its effects on lymphocytes, IL-1 possesses many biological activities suggesting perhaps that it is more than a single molecule (Oppenheim and Gery, 1982). It is now widely accepted that IL-1 is probably identical to the fever inducing endogenous pyrogen and leucocyte endogenous mediator (LEM) (Sztein et al, 1981) a factor which induces hepatocytes to synthesise acute phase proteins (Kampschmidt, 1978).

IL-1 production has been demonstrated in the rheumatoid synovium (Mizel et al, 1981; Wood et al, 1983). IL-1 has several activities relevant to the pathological changes seen in the rheumatoid

joint; it causes collagenase and prostaglandin secretion by syno-viocytes (Mizel et al, 1981), the proliferation of fibroblasts (Schmidt, 1981) and induces chondrocytes to degrade the cartilage matrix (Jasin and Dingle, 1981). Disturbed cytokine production may be a significant factor in the pathogenesis of RA. Peripheral blood monocytes obtained from RA patients have recently been reported to produce significantly more IL-1 than cells from healthy controls (McKeown et al, 1984b;Tan et al, 1984; Nouri et al, 1985a).

Despite this increased IL-1 production, somewhat paradoxically, maybe peripheral blood mononuclear cells from RA patients show def-icient IL-2 production when compared to normal cells (McKeown et al, 1984b;Miyashaka et al, 1984; Nouri et al, 1985b,Combe et al, 1985). Stimulated T-lymphocytes express the Tac antigen which is the IL-2 receptor (Uchiyama et al, 1981). Decreased expression of this anti-gen has been reported on rheumatoid T-cells (Pirzad et al, 1985) correlating well with the finding that expression of Tac antigen is regulated by IL-2 itself (Welte et al, 1984).

The apparent hyporeactivity of T-cells in rheumatoid patients is supported by the finding of depressed IL-2 production. Indeed, this hyporeactivity has been attributed,at least in part, to def-ective IL-2 production since addition of exogenous, recombinant IL-2 has been reported to correct the depressed autologous mixed lymphocyte reaction (AMLR) in RA (McKenzie, Mills and Panayi, 1985). Combe et al (1985) have suggested that depressed IL-2 production may lead to defective T-suppressor cell activity in the rheumatoid joint since they have also demonstrated deficient IL-2 production by RA synovial lymphocytes. This same study also showed defective γ-IFN production in response to IL-2 by rheumatoid T-lymphocytes.

Decreased γ-IFN production by rheumatoid T-lymphocytes has also been reported by Hasler et al (1983) who attributed it to the inhibitory effects of monocyte-generated prostaglandins. However, Combe and his colleagues (1985) have shown that removal of the adherent cells does not alter the γ-IFN production suggesting that excessive prostaglandin production is not responsible for the decreased γ-IFN production. These workers suggest that T-cell hyporeactivity with respect to IFN and IL-2 production may cause B-cell hyperactivity with the resultant immunological injury seen in the rheumatoid joint.

The importance of immune mechanisms in RA is emphasised by the observed clinical improvements by immunosuppressive regimens of therapy. Thoracic duct drainage (Paulus et al, 1977), total lymphoid irradiation (Kotzin et al, 1981) and leukapheresis (Lockwood, 1979) often cause a remission in disease activity whilst use of immunosuppressive drugs, such as corticosteroids and cytotoxic drugs e.g. azathioprine, often has the same result. However, the generalised immunosuppression in RA patients as a consequence of this therapy leaves them susceptible to infections, so caution is indicated.

1.A.5. AETIOLOGY OF RHEUMATOID ARTHRITIS

Despite many years of intensive research, the aetiology of RA remains obscure. The generally accepted view is that RA arises out of a deranged regulation of the immune response to an antigenic stimulus in a genetically susceptible host. This can be viewed in two basic ways; firstly, an inability to eradicate the offending antigen such that it persists leading to chronic immunity, and, secondly, a failure to regulate the immune response after a normally transient stimulation by antigen (Paget and Gibofsky, 1979). Although the identity of the antigen is still unknown, a microbial infection remains an attractive proposition. However, as yet, no firm

microbiological evidence has been produced to sustain such a view. Circumstantial evidence favouring this idea however is available. Chronic arthritis can accompany a number of natural and experiment- ally-induced infections in animals e.g. pannus formation along with other features of rheumatoid synovitis are seen in some streptococ- cal infections of rabbits (Paget and Gibofsky, 1979). Bacteria can cause some forms of arthritis e.g. infection by S.aureus (Phillips, 1982). Studies to isolate bacteria from Rheumatoid synovial tissue has met with little success (Phillips, 1982).

Lyme disease, a systemic, inflammatory disorder also causes some changes in the synovium similar to those seen in RA (Steere et al, 1977). This disease is now thought to be transmitted by the tick Ixodes damini (Steere and Malawista, 1979) which carries the presumed infective agent, a spirochaete (Burgdorfer et al, 1982).

Perhaps more likely candidates are viruses and particular interest has been expressed in a role for infection by Epstein- Barr Virus (EBV). This is based on the high incidence of antibodies in RA patients directed against nuclear antigens (RANAs) found in EBV-transformed lymphoblastoid cell lines (Alspaugh and Tan, 1976; Alspaugh et al, 1978). This antibody was found in 67% of RA pat- ients compared with only 8% of normals in these studies. A later study by Ng and colleagues (1980) found anti-RANA activity in more than 90% of both seropositive and seronegative patients as opposed to 16% of normals but the specificity of the assay utilised has been challenged (Catalano et al, 1980).

Lymphocytes from RA patients are stimulated by EBV, a poly- clonal B-cell activator, to produce more IgM-RF than control lympho- cytes (Alspaugh et al, 1978). The same study also demonstrated that RA lymphocytes transformed more spontaneously (without EBV) and

showed more rapid transformation by EBV than normal cells. Since

T-cells can suppress EBV transformation of animal cells these

results might indicate defective T-cell function at least in

vitro in RA (Bardwick et al, 1980). The idea that RA is initiated by

some sort of polyclonal B-cell activator such as EBV causing dis-

turbed IgG production, including auto-antibodies such as RF, is

quite an attractive one. However, the rather restricted nature of

the immunoglobulin produced in RA (Smiley et al, 1985) would argue

against such a suggestion.

1.A.5.(i) IMMUNOGENETICS

The idea of some form of genetic predisposition to RA has

been mooted for a long time but it is only since the discovery of

an association between antigens of the major histocompatibility

complex (HLA antigens) and certain diseases that this suggestion

has had any firm basis. The important contribution that genetics

can make to rheumatology was first demonstrated by Brewerton et al

(1973) who showed a strong association between HLA-B27 and ankylos-

ing spondylitis. Recently it has been shown that RA is associated

with an increased incidence of HLA-DR4 and DW4 in whites (Stastny,

1978). Interestingly, the increased incidence is not seen in sero-

negative RA (Stastny, 1978). The exact nature of the link between

these antigens and the pathogenesis of RA is uncertain; however,

since DR products are expressed on macrophages, activated T

lymphocytes and B-lymphocytes (Winchester and Kunkel, 1979) and

are involved in immunoregulation then DR-4 positive cells in RA

patients might lead to an aberrant response to particular antigen(s).

Recent evidence has suggested that DR-4 is associated with disease

severity as opposed to pre-disposition (Jones et al, 1983) agree-

ing with the observation that patients with severe RA and extra-

articular manifestations are almost invariably HLA-DR4 positive

(Dinant et al, 1980).

1.A.6. CHEMOTHERAPY IN RHEUMATOID ARTHRITIS

While the exact cause remains unknown,treatment of RA with

drugs continues to be empirical. Such therapy usually follows a well-

established regimen. Initially, non-steroidal anti-inflammatory

drugs (NSAIDs) are used to achieve pain relief and some reduction in

the inflammation thereby allowing better joint function and improv-

ing the patient's general well being. In cases of less active dis-

ease NSAID therapy alone is often adequate. If the disease remains

aggressive so-called "second-line anti-rheumatic" agents such as

gold and penicillamine may be used in an attempt to suppress dis-

ease activity. In some cases these drugs appear to halt the prog-

ression of the disease so preventing further disability. However

treatment with these drugs is hampered by the manifestation of toxic

side-effects which has limited their widespread use. In extremely

severe cases the use of cytotoxic/antimetabolic drugs and/or cortico-

steroid therapy may be considered in an effort to suppress immune

activity in RA, thus, hopefully curbing the disease process. The

nature of these agents means there are obvious problems with toxicity

and generalised immunosuppression which severely restricts their

application.

Non-steroidal anti-inflammatory drugs

NSAIDs do not appear to have any profound effects upon the under-

lying disease process in RA and so do not reverse the arthropathy.

However, by virtue of their analgesic and anti-inflammatory effects

they are of benefit clinically since this permits better joint function

in reducing pain, stiffness and swelling. Aspirin and the salicylates

have been used for a long time whilst newer classes of NSAIDs such as

derivatives of the organic acids, proprionic acid (ibuprofen) and acetic acid (indomethacin), have been developed in recent years.

Their anti-inflammatory action is proposed to be due to the inhibition of the synthesis of prostaglandins (PGs) (Vane, 1971). The PGs are a family of biologically active compounds derived from unsaturated fatty acids (generally arachidonic acid) by the action of the enzyme cyclo-oxygenase. This enzyme converts arachidonic acid into prostaglandin (PG) G_2 which can be reduced to form PGH_2. PGG_2 and PGH_2 are short-lived endoperoxides which undergo rapid enzymatic converstions to the prostaglandins (PGD_2, PGE_2 and PGI_2) and thromboxanes (TXA_2, TXB_2). The PGs are involved in producing the cardinal signs of inflammation - they are vasodilatory and pyretic and synergise with other inflammatory mediators e.g. C5a and bradykinin to produce oedema and pain (Ninneman, 1984).

NSAIDs directly interact with cyclo-oxygenase to inhibit PG synthesis which accounts for their anti-inflammatory actions. All NSAIDs show this inhibitory action in vitro although their potencies vary greatly (Crook et al, 1976). The variation in their potency does not correlate, however, with their rather equivalent clinical efficacy suggesting perhaps that their action may be more than simple inhibition of PG synthesis. Such a view is also sustained by the inability of aspirin to produce noticeable anti-inflammatory effects in rheumatoid synovial tissue at a dose (600 mg./day) which totally abolishes cyclo-oxygenase activity.

In addition to their inflammatory actions, a number of studies have shown that PGs may have immunomodulatory properties. Thus PGE_2 can suppress a number of T-lymphocyte functions at least in vitro including mitogen responsiveness (Goodwin et al, 1977) and IL-2 production (Rappaport and Dodge, 1982). Recent evidence has

also shown that human B cell activation may be regulated by PGE_2
(Staite and Panayi, 1982; Jellinek et al, 1985). In all these
studies PGE_2 has been demonstrated to have such effects at physio-
logically relevant concentrations. So, by inhibiting PGE_2 synthesis,
NSAIDs may also exert an immunomodulatory effect which may be rele-
vant to their mode of action in the treatment of RA. However NSAIDs
do not have any profound effect upon the rheumatoid process making
it unlikely that PGE_2 modulation of the immune response plays a major
role in the pathogenesis of this disease.

Second-line anti-rheumatic drugs

A number of drugs have been shown to be effective in the treat-
ment of RA by clinical experience despite lacking any direct anti-
inflammatory properties (Huskisson, 1976). These drugs including
gold, penicillamine and anti-malarials viz. hydroxychloroquine differ
from the NSAIDs in that they appear to affect the progress of RA.
Clinical effect with these agents only becomes apparent after a delay
of several weeks, and, often, months which has led to their descript-
ion of "slow-acting". Additionally, inflammation may continue to be
quiescent for some time after discontinuation of therapy. Although
sometimes called "remission-inducing" drugs, total clinical remission
with them is an extremely rare event and perhaps it is better to
describe their action in RA as suppressive. Their therapeutic use
has been somewhat hampered by their ability to induce toxic side-
effects which can be life-threatening in some cases e.g. thrombo-
cytopenia and neutropenia. The major second line drugs used are
gold salts and penicillamine and in this introduction only these
agents are discussed.

Gold Compounds

Gold salts have been used in the treatment of RA since the

1920s, their introduction being based on their anti-tuberculosis effect in the belief of a link between tuberculosis and RA. Despite initially favourable results it was not until 1960 that the first controlled clinical trial established the efficacy of chrysotherapy (Research sub-committee of the Empire Rheumatism Council, 1960).

The mechanism(s) by which gold compounds work in RA is obscure. Explanations based on biochemical effects observed in some model systems have included inhibition of the activity of lysosomal and other enzymes (Persellin and Ziff, 1966), interference with complement activation (Burge et al, 1978) as well as various non-specific anti-inflammatory effects (Vernon-Roberts,1979). The disease modifying nature of the compounds however suggests a more profound immunomodulatory action. Lipsky and Ziff (1977) showed that gold sodium thiomalate (GST) inhibits the antigen/mitogen-induced proliferation of normal human blood mononuclear cells in vitro by interfering with monocyte function which is essential for such proliferation (Rosenberg and Lipsky, 1979a,b). They demonstrated that the gold atom itself inhibited monocyte function as opposed to thiomalate since GST and gold chloride were effective as inhibitors whilst thiomalate per se was not. These results suggest that gold compounds might work in RA by an immunosuppressive effect upon monocytes affecting their function in both initiation and expression of the chronic, immunologically mediated inflammatory reaction (Lipsky, 1982). However, it should be noted that many second-line anti-rheumatic agents contain thiol groups or are converted to thiol groups in vivo (Hunneyball, 1980) suggesting a role for the thiol moiety of GST. Indeed,it has been pointed out that all the gold salts used in controlled long-term trials have been thiols (Munthe et al, 1978). Therefore it may be that GST has two active moieties involved in

the mechanism(s) by which it works, the gold atom and the thiol

group. The role of thiol groups in RA and second-line anti-rheuma-

tic agents is more extensively discussed in section 1.C.

Gold has a number of side-effects which limits its use in

rheumatoid arthritis. The most common side-effect with chrysotherapy

is a rash occurring early after the commencement of treatment in

about 30% of patients. Proteinuria can occur later in therapy and

may necessitate withdrawal of the drug. Potentially more serious

toxic reactions sometimes occur such as agranulocytosis, thrombo-

cytopenia and aplastic anaemia. Fortunately, however, such side-

effects are rare.

1.B.D-PENICILLAMINE

Penicillamine was first identified by Abraham and colleagues

in 1943 as a penicillin-hydrolysis product during studies to eluci-

date the structure of penicillin. Penicillamine is a thiol-contain-

ing amino-acid similar in structure to the naturally occurring amino-

acid, cysteine (see fig. 2   ). Walshe's speculations that the

compound might be therapeutically useful in Wilson's Disease (hepato-

lenticular degeneration) (Walshe, 1956),and cystinuria, an inherited

disorder of amino-acid transport (Crawhall et al, 1963) were sub-

sequently proved correct. As with Wilson's Disease, the chelating

properties of the drug have caused it to be used successfully in the

treatment of some forms of heavy-metal poisoning including lead poison-

ing (Goldberg et al, 1963) and mercury poisoning (MacGregor and Clark-

son, 1974). Recently it has been used in a number of other condit-

ions such as chronic active hepatitis (Stern et al, 1977) and primary

biliary cirrhosis (Dickson et al, 1977) although its efficacy in

these conditions is, as yet, questionable. A list of therapeutic

applications of penicillamine is given in Table 1.
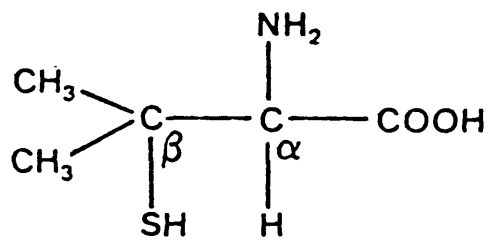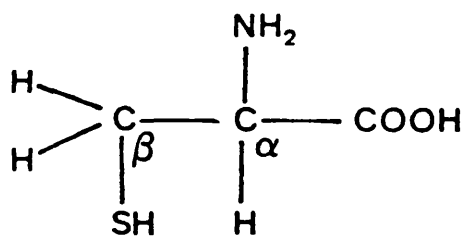
FIG.2. Comparison of the structures of D-penicillamine
        and L-cysteine.

$\alpha =$ α carbon atom

$\beta =$ β carbon atom

USUALLY EFFECTIVE	USUALLY INEFFECTIVE
Wilson's disease	Systemic sclerosis
Cystinuria	Cystinosis
Rheumatoid Arthritis	Ankylosing Spondylitis
Lead Poisoning	Psoriatic Arthritis
Mercury Poisoning	Waldstrom's Macroglobulinaemia
Morphoea	
INEFFECTIVE	**DISPUTED OR UNDER TEST**
Huntington's chorea	Primary biliary cirrhosis
Duchenne muscular	Active chronic hepatitis
dystrophy	Schizophrenia
Cadmium poisoning	Oculocutaneous syndrome
	Arsenic poisoning
	Antimony poisoning
	Darier's disease
	Idiopathic pulmonary fibrosis

TABLE 1   THERAPEUTIC APPLICATIONS OF PENICILLAMINE

(established, discarded or under test)

Adapted from Lyle (1979)

In 1960, Ritzman, Coleman and Levin demonstrated the ability of penicillamine to dissociate macroglobulins in vitro due to disulphide bond cleavage. Two years later, Jaffe assumed that penicillamine might act similarly upon macroglobulin rheumatoid factor (RF) in patients with RA. Injection of the drug into the knee joint caused a reduction in the synovial fluid RF titre but no relief of symptoms (Jaffe, 1962). However, systemic administration of the drug did bring about a lowering of the serum RF after a few months treatment with prior clinical improvement being evident. Cessation of the treatment led to an increase in RF levels to pre-treatment levels but this did not occur until after many weeks (Jaffe, 1965). In subsequent years a number of groups carried out studies which confirmed Jaffe's observations (Miehlke and Kohlhardt, 1967; Golding et al, 1968). Thus, penicillamine was demonstrated to produce a clinical improvement in RA although such effects were manifest only after a latent period of several weeks. However, the degree of responsiveness to the drug varied greatly amongst the patients with some responding dramatically and others showing little or no improvement with the drug. Another striking aspect was the incidence of adverse reactions associated with the drug in RA patients ranging from mild skin rashes to more severe effects such as leucopenia. In 1973, results from a multicentre trial confirmed the efficacy of the drug in RA but again provided evidence of its ability to induce adverse reactions (Andrews et al, 1973).

Commencement of penicillamine therapy in a rheumatoid patient follows generally the same indications for the employment of chrysotherapy. Thus, patients whose disease is sufficiently active and who have failed to respond to treatment with non-steroidal anti-inflammatory drugs are candidates for treatment with penicillamine.

The drug frequently not only leads to an amelioration of symptoms
but also brings about an improvement in serological correlates of
disease activity such as titres of rheumatoid factor and erythrocyte
sedimentation rate (ESR). The drug, together with other second-line
agents such as gold, also appears to be able to prevent further eros-
ions of peri-articular bones (Gibson et al, 1976).

1.B.1. POSSIBLE MECHANISMS OF ACTION OF PENICILLAMINE IN RA

The chemical reactions of D-penicillamine (see later, p 35)
clearly explain its action in conditions such as Wilson's Disease and
cystinuria. However, in RA the mechanism(s) by which D-penicillamine
acts is far from clear. It is pertinent to ask whether its mode of
action can be explained by its chemical reactions.

In active RA, levels of serum copper and caeruloplasmin are
elevated but fall to normal upon suppression of disease activity by
ACTH, aurothiomalate or penicillamine (Scudder et al, 1978). Jayson
et al (1976) have shown that serum copper and caeruloplasmin are
similarly raised in ankylosing spondylitis. Since penicillamine
has no effect in this disease then it is unlikely to act in RA mere-
ly by promoting copper loss although it has been shown to increase
copper excretion (Lyle et al, 1977). However, detachment of copper
from protein-bound sites by the drug causes copper to form complexes
of varying stability and mobility and such copper redistribution
might be of importance in the inflammatory process.

The relationship between serum levels of other trace metals
and D-penicillamine therapy in RA have been studied e.g. Zinc
(Aasethet al, 1976; Jepsen et al, 1984). Whilst alterations in
zinc levels are seen, the studies indicate that penicillamine does
not act in this way in exerting its clinical effects.

Another possible mode of action of D-penicillamine is the scavenging of oxygen derived radicals (ODRs) produced in the synovium by phagocytic cells. ODRs have been increasingly implicated in the inflammatory process and can cause effects relevant to RA e.g. depolymerisation of hyaluronic acid (McCord, 1974), as outlined earlier in section 1.A.4. The hydroxyl (OH·) radical, generally considered to be the most reactive of these species, is formed from superoxide anion O_2^-· and H_2O_2 in the presence of transition metal ions, generally iron. (Gutteridge, Rowley and Halliwell, 1982). The chelate that D-penicillamine forms with copper has been reported to have potent superoxide dismutase activity, thus removing the O_2^-· necessary for OH· formation, and can prevent depolymerisation of hyaluronic acid (Greenwald and Moy, 1980) which might account for some of the beneficial effects seen with D-penicillamine. Chelation of the iron that catalyses OH· formation may also protect the joint from the destructive effects of these radicals. However, it should be remembered that chelation of iron by D-penicillamine is relatively poor in comparison with that seen with copper or lead ions (Doornbos and Faber, 1964) so such a mechanism is questionable. ODRs can also inactivate serum α_1 -proteinase inhibitor resulting in the loss of its inhibitory effect upon lysosomal proteinases (Carp and Janoff, 1980). Skosey and Chow (1982) have shown that D-penicillamine can protect the enzyme's inhibitory capacity against the effects of neutrophil derived free radicals and those derived by a cell-free system i.e. xanthine oxidase acting upon acetaldehyde. This again might be relevant to a chelating and scavenging action for D-penicillamine in RA.

Penicillamine can affect collagen cross-linking in two major ways (Nimni, 1977). Since it contains both a thiol and an amino

group it can form thiazolidines with lysyl and hydroxlysyl der-
ived aldehydes (Nimni, 1977; Siegel, 1977). The significance of
such an effect is speculative and among the ideas proposed is a
stabilising effect on collagen making it less susceptible to de-
gradation (Crawhall et al, 1979). The other way in which D-penicil-
lamine can affect collagen cross-linking is by chelating the copper
necessary for the activity of the enzyme lysyl oxidase which is
responsible for the cross-linking (Nimni, 1977). However, the very
high concentrations of D-penicillamine needed to do this are un-
likely to be attained in vivo (Siegel, 1977; Ruckner and Murray,
1978). Gerber (1978) has proposed that D-penicillamine may mimic
the action of collagenase inhibitors due to disulphide exchange
reactions thereby regulating collagenase activity in the rheumatoid
joint. This same author has shown that in vitro the drug inhibits
the sulphydryl dependent heat denaturation of IgG (Gerber, 1978)
and so might prevent the formation of autoantigenic IgG aggregates in
vivo . Similarly, free radical associated IgG aggregation may be
inhibited by the scavenging of these radicals (Wickens and Dormandy,
1984).

While these biochemical effects might explain some of the
benefits achieved by D-penicillamine in RA, the drug appears to have
a more profound effect on the immune response in the disease sugg-
esting an immunomodulatory mechanism of the drug. In vitro mito-
genic-induced lymphocyte proliferation can be enhanced at low doses
of penicillamine and inhibited at higher doses (Room et al, 1979).
D-penicillamine has also been shown to synergise with copper ions
in vitro to suppress lymphocyte function by inhibiting T-cell
function (Lipsky and Ziff, 1980). This has been attributed to the
generation of H_2O_2 by D-penicillamine-copper complexes which suppress

T-cell activity (Lipsky, 1984). However, no convincing demonstration

of inhibition of T-cell activity in vivo by D-penicillamine has yet

been shown. Evidence has been produced indicating a possible immuno-

regulatory role for D-penicillamine in RA by affecting SH-dependent

reactions of monocytes (McKeown et al, 1984a). The role of SH

groups in the immune response and their significance in RA are dis-

cussed in section 1. C.

1.B.2. SIDE EFFECTS OF PENICILLAMINE

The use of penicillamine as a therapeutic agent in RA has been

complicated by its ability to induce adverse side-effects (Andrews

et al, 1973). These effects are unpleasant but most of them are not

serious and are generally reversible upon cessation of treatment. Per-

haps the most common problems encountered with penicillamine therapy

are nausea, rashes and loss of taste. There are two main types of

rashes seen. One of them occurs early after the start of treatment

but generally stops upon cessation of the drug and does not recur

upon recommencement. The other rash occurs much later and is more of

a problem although, fortunately, it occurs less commonly. As with

rashes, loss of taste occurs in about 25% of patients. It generally

returns whether the therapy is discontinued or not so cessation of

treatment is unnecessary. Proteinuria is a more important complic-

ation of penicillamine therapy because it may lead to withdrawal of

the drug. It occurs in about 15% of patients and is of variable

severity. In some of these cases nephrotic syndrome can occur. It

is essential to monitor renal function during treatment with penicil-

lamine. Haematological abnormalities can also occur in patients

receiving penicillamine of which thrombocytopenia is the most

common. When this occurs, treatment should be discontinued to fac-

ilitate recovery but can generally be cautiously restarted. Neutro-

penia is much more serious but fortunately less common. There are

some blood dyscrasias which continue despite cessation of treatment

and in some of these cases deaths have occurred. Immunological ab-

normalities have been reported with penicillamine e.g. drug-induced

systemic lupus erythematosus (SLE) but fortunately these are very

rare.

The occurrence of side effects in RA patients treated with

penicillamine and the variation in efficacy seen with the drug may

be related to plasma levels of the drug (or its metabolites). How-

ever, technical difficulties in developing assays to measure the

drug in biological fluids have led to a lack of information regard-

ing this situation. Before discussing these problems and approaches

to assaying D-penicillamine, it is necessary first to outline the

chemistry of the drug.

1.B.3. STRUCTURE AND CHEMISTRY OF PENICILLAMINE

Penicillamine is a tri-functional amino acid containing car-

boxyl, amino and sulphydryl groups (Fig.2). It differs from cysteine

in that the two hydrogen atoms attached to the β-carbon atom in

cysteineare substituted by methyl groups so that penicillamine may

be designated β-β dimethyl cysteine. The asymmetrical α-carbon atom

means that penicillamine can exist in D- and L-stereoisomeric forms

as a DL form if synthesised from a racemic precursor.

Early studies indicated metabolic and toxicological differences

between the different forms of the drug (Kuchinskas et al, 1957).

Due to its lesser toxicity it is the D-form which is solely used

for pharmacological purposes. The optical laevorotation of D-pen-

icillamine in aqueous solutions means it is properly designated D(-)

penicillamine, although in this thesis (-) is omitted.

The main chemical reactions in which D-penicillamine can part-
icipate are characteristic of its three functional groups. The main
reactions are listed in Table 2.

The stability of complexes with divalent ions falls in the
following order (Kuchinskas and Rosen, 1962; Doornbos and Faber,
1964); Hg, Pb, Ni, Cn, Zn, Cd, Co, Fe, Mn. The exact nature of the
linkages is unclear and appears to vary from metal to metal (Doornbos
and Faber, 1964). The chelating properties of the drug have led to
its use in Wilsons Disease and heavy metal poisoning.

In aqueous solutions, it is slowly oxidised to penicillamine
disulphide this reaction being facilitated by the presence of air,
light, alkaline pH, raised temperatures and traces of heavy metals
(Kucharchyk and Shahinan, 1981). The ability of penicillamine to
undergo disulphide formation and take part in thiol-disulphide ex-
change reactions means it can react with both cysteine and cystine
to form the mixed disulphide, cysteine-penicillamine disulphide.
This is more soluble than cystine and its formation reduces "cystine
stone" formation in renal tubules, this being the rationale behind
penicillamine therapy in cystinuria (Crawhall et al, 1963).

1.B.4. ASSAYS FOR PENICILLAMINE IN BIOLOGICAL FLUIDS

The inherent reactivity of D-penicillamine has proved to be a
considerable problem in the development of reliable, accurate assay
procedures. Only in recent years have procedures been adopted which
have eliminated problems in penicillamine analysis. These have been
mainly the use of EDTA to chelate any metal ions present, thereby
preventing penicillamine oxidation and chelation, and manipulation
of the pH to keep the drug in its desired form (Bergstrom et al,1980).
Most of the more recent assays developed have been high performance
liquid chromatographic (HPLC) methods coupled with some form of

(A) COMPLEX FORMATION.

    (i) P-SH + heavy metals (Cu, Zn)   ⇌   (P-SH) - metal

    (ii) P-SH + pyridoxal   ⇌   thiazolidine derivative

(B) DISULPHIDE FORMATION

    (i) P-SH + thiol-SH (e.g.P-SH,cysteine) ⇌ P-S-S-thiol

    (ii) P-SH + protein-SH   ⇌   P-S-S-protein

(C) THIOL-DISULPHIDE EXCHANGE

    (i) P-SH + disulphide (thiol-S-S-thiol) ⇌ P-S-S-P + thiol-SH

    (ii) P-SH + protein-S-S-thiol   ⇌   P-S-S-protein + thiol SH

(D) METHYLATION

    (i) P-SH + CH_3 (methyl donor)   ⇌   S-methyl penicillamine

    P-SH = D-penicillamine

TABLE 2

BIOCHEMICAL REACTIONS OF D-PENICILLAMINE

electrochemical detection (Rabenstein and Saetre, 1977; Saetre and Rabenstein, 1978). Abounassif and Jeffries (1983) developed sample preparation methods together with an HPLC system utilising glassy carbon based electrochemical detection which allowed measurement of total drug in plasma. However, there are still many problems associated with this assay. In this project, the aim was to modify and further develop this basic technique to determine penicillamine concentrations in plasma and utilise the method to study the drug's pharmacokinetics in normal volunteers and RA patients. This should allow possible correlations between the pharmacokinetics and metabolism of the drug and its efficacy and toxicity to be studied.

1.C. THE ROLE OF SH GROUPS IN RHEUMATOID ARTHRITIS

1.C.1. THIOL CONTAINING COMPOUNDS AS ANTI-RHEUMATIC DRUGS

Many of the second-line anti-rheumatic drugs have free sulphydryl groups or are converted to SH-containing compounds in vivo (Hunneyball 1980). Both penicillamine and aurothiomalate, perhaps the most widely used second-line agents, are thiols and it has been pointed out that all the gold salts used in controlled, long-term clinical studies have been thiols (Munthe et al, 1978). Levamisole, another second-line drug with anti-rheumatic actions, has been shown to have similar biological and clinical effects to D-penicillamine and gold (Symoens and Schuermans, 1979). In vivo, the drug is metabolised to a number of products including DL-2-oxo-3-(2-mercaptoethyl-5-phenyl-imidazolidine) (OMPI) which has a free SH group (DeBrabander et al, 1978). It has been proposed that the effectiveness of these second line drugs may be due to their SH groups (Jaffe, 1980). Such a hypothesis seems eminently reasonable in view of the disturbance in SH levels seen in RA.

1.C.2. SULPHYDRYL LEVELS IN RA

Depressed serum SH groups are a feature of active RA (Lorber et al,

1964) and serial measurements show an inverse correlation with disease

activity (Haataja, 1975). In addition, erythrocytes from RA patients

have low SH levels which correlate with serum SH levels (Lorber and Chang,

1968). Serum SH levels rise to normal in those patients showing good

clinical response to D-penicillamine and aurothiomalate treatment (Dixon

et al, 1980).

In the serum,most of the SH groups are on albumin molecules. Oxidation

of these groups with small thiols such as cysteine to form mixed disulph-

ides is apparently the reason for the depressed SH levels observed in RA.

(Thomas and Evans, 1975). Although albumin levels are reduced in RA and

may be partially responsible for the depressed SH levels, lack of correl-

ation between albumin and thiol concentrations indicates it is not a major

contribution (Banford et al, 1982). A number of reasons for the disulph-

ide formation are possible. Caeruloplasmin, an acute phase reactant,

oxidises low molecular weight thiols to disulphides (Albergoni and Cassini,

1978) and may play a role in albumin-cysteine disulphide formation. Rel-

ease of reactive oxygen intermediates from inflammatory phagocytic cells

has been suggested as another mechanism to catalyse such a reaction (Hall

et al, 1984).

1.C.3. ROLE OF SH GROUPS IN THE IMMUNE RESPONSE

Disturbed thiol expression in RA may be involved in the defective imm-

unoregulation observed in the disease which may play a role in its patho-

genesis. Several aspects of the immune response appear to involve sulph-

ydryl groups. In animal studies, simple thiols such as 2-mercaptoethanol

(2-ME) have been shown to potentiate the immune response in vitro includ-

ing lymphocyte proliferation (Broome and Jeng, 1973; Bevan et al, 1974)

and antibody responses (Click et al, 1972). Click et al showed that 2-ME

affected an early event in the immune response since it was unnecessary

to add the reagent to the cultures daily. In humans, lectin-induced

lymphocyte proliferation _in vitro_ can be inhibited by the addition of SH-blocking agents such as diamide and para-hydroxymercuri-phenylsulphonic acid (pHMPSA) (Chaplin and Wedner,1978). Again, these authors concluded that SH groups were involved in an early event since addition of the SH-blocking agents was required at the start of culture to have inhibitory effects. Several lines of evidence suggest that the important SH groups are present on the cell surface and are not intracellular. Diamide enters cells only very slowly (Chaplin and Wedner 1978) and pHMPSA does not apparently permeate cell membranes (Tsan and Berlin 1971). Similarly, glutathione, an impermeant thiol, enhances lymphocyte proliferation in a fashion akin to 2-ME whereas cysteamine phosphate, a compound which is only converted to a SH-containing compound intracellularly, has no such effect (Noelle and Lawrence,1979).

These observations suggest that in RA the oxidation of functional SH-groups may be involved in the observed aberrant immune response. Such a view is enhanced by the immunomodulatory nature of SH-containing second-line anti-rheumatic drugs such as aurothiomalate and penicillamine. Previous studies in this laboratory have sought to analyse the possible role of SH groups in immunoregulation in RA which might be relevant to the pathogenesis of the disease (McKeown _et al_, 1984a). These showed that peripheral blood mononuclear cells (PBMCs) obtained from RA patients treated with NSAIDs (RA-NSAID) produced significantly less IgG in response to PWM _in vitro_ than normal cells. Addition of 2-ME (5 x 10^{-5}M) to the RA-NSAID PBMCs stimulated IgG synthesis to normal values, a procedure which had no significant enhancing effect upon IgG production by PBMCs from healthy controls or from D-penicillamine treated patients (RA-D-pen). When monocytes were pre-incubated with the SH-blocking agent pHMPSA and added back to lymphocytes in reconstitution experiments,IgG production was abolished showing that monocytes require free cell surface SH groups

for their accessory function in the immune response. Further experiments in which monocytes were titrated back to lymphocytes demonstrated that monocytes from RA-NSAID patients exhibited impaired accessory cell function with respect to normal monocytes. This could be corrected by the addition of 2-ME. RA-D-penicillamine monocytes showed normal accessory function which, like those from normal controls, was unchanged by 2ME treatment. These studies strongly suggest that defective monocyte accessory cell function in RA results from reversible oxidation of the cell surface SH groups. Support for this comes from other studies which have shown a significant enhancement of proliferation of PBMCs from RA patients to PHA in the presence of 2-ME (Corrigall and Panayi, 1979). Depressed SH levels on PBMCs probably result from the same oxidative mechanisms that diminish serum SH groups and erythrocyte membrane thiols in RA. In vitro, these can be re-exposed following chemical reduction by 2-ME leading to enhancement of the accessory cell function. PBMCs from D-penicillamine treated patients show normal IgG synthesis and monocyte accessory cell function and are unresponsive to enhancement by 2-ME in vitro. It may be that D-penicillamine exerts its beneficial effects in RA by modulating SH-dependent mononuclear cell functions especially those of monocytes active in the immune response.

The aim of this part of the project was to delineate monocyte functions dependent on the expression of free SH groups on the cell surface relevant to their accessory cell function since they represent possible targets for penicillamine therapy in RA.

Chapter Two

MATERIALS AND METHODS

2.A.1. MATERIALS

Solvents

All solvents were of HPLC grade (Fisons Ltd.,
Loughborough, U.K.). The water used for aqueous mobile
phases was deionised and doubly-distilled by means of a Fi-stream
pre-deio niser and water still (Fisons Ltd.)

Reagents and Solutes

Reagents were supplied as follows:

Reagent	Supplier
D-penicillamine dithiothreitol, citric acid (anhydrous)	Sigma Chemicals Ltd. Poole, U.K.
Disodium hydrogen ortho- phosphate, sodium dodecyl sulphate (primar grade)	B.D.H. Ltd., Poole, U.K.
para-methoxyphenylaectic acid	Koch Light Labs, Colnbrook, U.K.
Spherisorb-NH$_2$ (5 μm)	Jones Chromatography, Llanbradach, Wales, U.K.

2.A.2. EQUIPMENT

HPLC Instruments

Liquid chromatographs were assembled from commercially available components. Constant flow pumps were used (Consta Metric III pumps, L.D.C. Ltd., Stone, Staffs, U.K.) fitted with additional custom-made pulse dampeners to maximise a pulse-free solvent flow. Samples were introduced by means of a high-pressure injection valve fitted with a constant volume loop (50 μls or 100 μls) (Rheodyne 7125, Rheodyne Ltd., Cotati, California, U.S.A.). All column fittings, tubing and plumbing were supplied by H.E.T.P. Ltd., Macclesfield, U.K.

The final column configuration is shown in the results section. Switching of the solvent flow in the penicillamine determination was achieved by means of a high-pressure switching valve (Rheodyne 7000, Rheodyne Ltd.).

Temperature of the HPLC system was maintained at 25°C by a heated water bath (Type 400.010, Gallenkamp, Loughborough, Leics, U.K.).

Mobile phases were degassed under vacuum, greater than 500 mmHg for 20 minutes. The degassed condition was maintained for up to 24 hours by use of a Dupont Flotation degassing system (Jones Chromatography, Llanbradach, Wales, U.K.).

Detection Systems

The column eluent was monitored by passing it through the flow-cell of an electrochemical detector (BAS LC-4A, Bio-analytical systems, West-Lafayette, U.S.A.) fitted with a thin-layer cell. In the final system for penicillamine determination the cell contained a gold-mercury electrode (BAS, TL-6A). The initial HPLC system utilised a glassy carbon electrode (TL-4A). The reference electrode in all

cases was an Ag/AgCl electrode (RE -1).

Recorder

The detector output was recorded on a potentiometric chart recorder (JJ CR650S recorder, J.J. Ltd., Southampton, U.K.). The recorder voltage was changed in accordance with the voltage of the detector employed.

2.A.3. METHODS

HPLC grade solvents and doubly distilled, de-ionised water were used to make up the mobile phases to minimise contamination. Mobile phases were freshly prepared to avoid concentration changes and filtered using $0.45\,\mu m$ filters (Type HA, Micropore filters, Millipore Ltd., Harrow, Middlesex, U.K.) Mobile phase pH was checked using a Howe 6031 pH meter, V.A.Howe Ltd., London, SW18, U.K.).

Mobile phases were passivated by degassing under a vacuum exceeding 500 mmHg for 20 minutes. Timing of the degassing was precise to minimise any organic solvent loss. The degassed condition was maintained by a Du-Pont flotation degassing system which is effective for at least 24 hours.

Procedures for the determination of penicillamine

Calibrations

A stock solution of D-penicillamine in mobile phase containing $1gL^{-1}$ EDTA was used to prepare dilutions in the range $1 \times 10^{-6}M$ (0.1492 μgml^{-1}) to $1 \times 10^{-3}M$ (149.2 μgml^{-1}). An identical calibration was performed for D-penicillamine spiked into plasma, the required amount of D-penicillamine in mobile phase being added in 0.01 ml aliquots to 0.99 mls of plasma. The spiked plasma samples were then treated according to the method for total drug in clinical plasma samples as described below.

Total D-Penicillamine in plasma

Clinical plasma samples and spiked plasma samples (both 1ml) were pipetted into glass flow tubes to which 0.36 mls of dithiothreitol (10 mgml^{-1}) was added, followed by 1.64 mls of disodium hydrogen phosphate buffer (0.05M) to give a final volume of 3 mls. After thorough mixing of the contents the tubes were incub-

ated at $60^{\circ}C$ for 3 hours in a water bath. Upon completion of the

incubation period, the contents were again thoroughly whirlimixed

and samples spun at 4500 r.p.m. for 25 minutes in a Hettich Uni-

versal Centrifuge (Arthur Horwell Ltd., London, U.K.). The super-

natants were removed and pipetted into Eppendorf microfuge tubes

(Sterilin Ltd., Feltham, Middlesex, U.K.) which were then spun in

an Eppendorf microfuge for 1 minute at maximum speed to further re-

move any debris. The supernatants were then filtered through Amicon

micro-filters (0.2 μm pore size) (Amicon, Woking, Surrey, U.K.).

The filtrates (400 μls) was then added to 100 μls of mobile phase

containing $1gL^{-1}$ EDTA in small sample tubes (TSP $\frac{1}{2}$, Fison's Ltd.,

Loughborough, U.K.). 50 μls of the samples were injected onto a

15 cm. pre-column containing Spherisorb-NH_2 (5 μm) by means of the

injection valve. At $8\frac{1}{2}$ minutes precisely, the running samples were

transferred to the main analytical 5 cm Spherisorb-NH_2 column by

means of the switching valve. After elution of the penicillamine

peak the flow was switched back using this valve so that the next

sample could be introduced onto the pre-column. In this way, "over-

lapping" of samples was achieved thereby reducing the effective analy-

sis time. All samples were injected in duplicate.

Concentrations of unknown clinical samples were determined

by use of an external standard method. Spiked plasma samples contain-

ing a known drug concentration were treated in the same way as clin-

ical samples and their filtrates were injected before and after dup-

licate clinical samples. The mean peak height of these known stand-

ards was taken to be the peak height relating to that particular con-

centration. The concentration of the unknown clinical sample was

then calculated by the following equation:-

$$\frac{\text{peak height (unknown)}}{\text{peak height (known standard}} \times \text{concentration of known standard}$$

This system was employed due to the lack of an internal standard
and also to compensate for any changes in detector sensitivity
throughout the analysis.

2.A.4. HUMAN STUDIES

Volunteers

Five healthy volunteers, 3 males and 2 females (age range 22
to 42 years) were each given an oral dose of 500 mg. D-penicillamine
on 2 separate occasions. The first of these was following an overnight
fast with no food or milk allowing for two hours after dosing. On
the second occasion the drug was taken in the middle of a light break-
fast. On each occasion blood samples were obtained by venepuncture
at 1, 2, 3, 4, 6, 8 and 24 hours post-dosing (approximately) and placed
in EDTA tubes. The plasma obtained by centrifugation was then assayed
for total drug as described previously. Area under the curve (AUC)
values were calculated using the trapezoidal rule.

Statistical analysis was performed using the Wilcoxon's rank
sum test.

Patients

Patients with active RA were admitted to the Royal National Hos-
pital for Rheumatic Diseases, Bath, to commence penicillamine therapy.
Clinical assessment of the disease state was performed by consultant
rheumatologists at the hospital as was the assessment of their suitabil-
ity to undertake such therapy. Treatment was dictated by the physicians
and not influenced in any way by the study for which full clinical app-
roval was obtained. The patients gave their consent for the study to
be undertaken. Their previous case histories were taken as were notes
of any drugs being administered or which had been previously administ-
ered.

After an overnight fast, patients were given 125 mg. penicilla-

mine by mouth next morning without food. Blood samples were with-

drawn by venepuncture at regular intervals (or as necessitated by

hospital routine) and the plasma obtained for determination of drug

concentrations.

Wherever possible, patients returned to out patient clinics

after an initial stay in hospital and samples were obtained at such

clinics in order to follow up the concentration of the drug to steady

state levels.

Total drug was assayed for as previously outlined and AUCs

similarly calculated.

2.B.1. <u>MATERIALS</u>

<u>Materials</u>	<u>Supplier</u>
All reagents and disposable tissue culture plastics, except where otherwise stated:	GIBCO Bio-cult Limited, Paisley, Scotland.
All chemicals, except where otherwise stated (including radiochemicals):	Sigma Chemical Company, Poole, England.
Ficoll-paque, Percoll, Sephadex G-10:	Pharmacia (GB) Limited, Milton Keynes.
Leukotriene B_4	Miles Laboratories Limited, Slough, Berkshire.
Scintillation grade toluene:	British Drug Houses Limited, Poole.
Scintillation fluid (Liqui-scint):	National Diagnostics Limited N.J. USA.
Scintillation vials:	Richardsons of Leicester, Leicester.
Lab-Tek tissue culture: chamber/slides (Type 4808):	Miles Laboratories Limited, Slough, Berkshire.

2.B.2. EQUIPMENT

Equipment	Supplier
MSE Chilspin 2 centrifuge, MSE Mistral centrifuge:	MSE Scientific Instruments, Sussex.
Skatron cell harvester:	Flow Laboratories Limited Irvine, Scotland.
Laborlux 12 microscope:	E. Leitz (Instruments) Limited, Luton.
Dynatech Automatic micro ELISA reader	Dynatech Instruments. California, USA.
Packard Tri-carb scintill- ation counter 3255:	United Technologies, Packard, Berkshire.
Perkin-Elmer MPF 44A Fluorimeter:	" " "

2.B.3.   ROUTINE BUFFERS AND MEDIA

Phosphate buffered saline (PBS) x 10

	gLitre^{-1}
Sodium chloride	80.000
Potassium chloride	2.000
Disodium hydrogen phosphate	11.500
Potassium dihydrogen phosphate	2.00

The above constituents were dissolved in 1 litre distilled water
and the pH adjusted to 7.3 by use of 3M NaOH.   The buffer was
then filter sterilised (Millipore filter, 0.22 μm pore size)
and stored at 4°C.

In some cases, the buffer was diluted 10-fold before use to
give 1 x buffer and the pH again corrected to 7.3.

Calcium and Magnesium Free Salt Solution (CMFSS)

	gLitre^{-1}
Sodium Chloride	8.000
Potassium Chloride	0.400
Glucose	1.600
Potassium dihydrogen phosphate (anhydrous)	0.060
Disodium hydrogen phosphate (anhydrous)	0.048

The above constituents were dissolved in 1L of distilled water and the pH adjusted to 7.3. The solution was then filter sterilised (Millipore filter, 0.22 μm pore size) and stored at 4°C until use.

Cell Culture medium RPMI 1640

	volume (mls)
RPMI 1640 (10x strength)	100
7.5% sodium bicarbonate solution	27
Penicillin and Streptomycin solution (5000 Unitsml^{-1})	20
200 mM glutamine	20

The above were mixed and the total volume made up to 1L using sterile distilled water. The pH was corrected to 7.3 using sterile 3M NaOH.

Where appropriate, 10% foetal calf serum (FCS) was added replacing 100 mls of the distilled water.

Hank's Balanced Salt Solution (HBSS) + 15% FCS

	volume (mls)
H.B.S.S. (10 x strength)	100
7.5% sodium bicarbonate solution	27
Penicillin and streptomycin solution (5000 Unitsml^{-1})	20
Foetal calf serum	150

The above were mixed and the total volume was made up to 1L using sterile distilled water. The pH was adjusted to 7.3 using sterile 3MNaOH, the solution was then filtered and stored at 4°C.

Ca^{2+} ion assay simplified medium

The following were added to 1 x PBS (1 litre)

Calcium chloride	1 mM
Glucose	5 mM

PRS buffer

The following were added to 100 mls. of distilled water

Sodium chloride	1.0226 g
Potassium dihydrogen phosphate	0.1701 g
Glucose	0.1238 g

32 mls of this buffer was added to 4 mls Horse-Radish peroxidase (Type II) (0.0123 g/100 mls. distilled water) and 4 mls phenol red (2gL^{-1} distilled water). The pH was adjusted to 7.3 by 3MNaOH. Solutions were made freshly on each occasion.

ELISA buffers

Coating buffer

	gL^{-1}
Sodium carbonate	1.59 g
Sodium hydrogen carbonate	2.93 g
Sodium azide	0.2 g

The above constituents were dissolved in sterile distilled water and the pH adjusted to 9.6. The buffer was stored at room temperature for not more than two weeks.

PBS/Tween

Tween 20 (Polyoxyethylene sorbitan monolaurate)	0.5 ml
Sodium azide	0.2 g

The above constituents were added to 1L PBS and the buffer stored at room temperature.

Diethanolamine

Diethanolamine	97 mls
Distilled water	800 mls
Sodium azide	0.2 g
Magnesium Chloride hexahydrate	0.1 g

This solution was then adjusted to pH 9.8 with 1MHCl. The total volume was made up to 1L with distilled water and the buffer stored in an amber bottle for not more than 2 weeks at room temperature.

<u>PBS/BSA</u>

    0.5 g of BSA (>99% globulin free) were added
to 100 mls of 1 x PBS buffer.

<u>Scintillation Fluid</u>

    To 2.5 litres of toluene was added:-

    2.5 - Diphenyloxazole (PPD)   12.50 g

    1.4-di-2-(4 methyl 5-phenoxazolyl) benzene

    (POPOP)   0.75 g

2.B.4. CELL PREPARATION

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinised blood by density centrifugation on Ficoll Paque (Boyum 1968). Blood was diluted 1:1 with CMFSS and 7 mls. layered onto 3 mls. of Ficoll-Paque in sterile, polystyrene tubes which were then centrifuged at 400 g. for 30 mins. at 18°C. The PBMCs were removed from the interface, washed three times with CMFSS and resuspended in 1 ml. RPMI 1640. The cells were counted in a haemocytometer and the concentration adjusted to 1 x 10^6 cells per ml.

In some experiments, these cells were separated into monocyte and lymphocyte-enriched populations by centrifugation through 52%·($\frac{v}{v}$) Percoll. PBMCs in 1 ml. RPMI 1640 were carefully layered onto 2 mls. of Percoll in sterile, polycarbonate tubes which were centrifuged at 750 g for 30 mins. at 18°C. Monocytes were obtained from the supported band and lymphocytes from the pellet. Lymphocytes were then further depleted of contaminating monocytes by passage through a 10 ml. Sephadex G-10 column equilibrated with HBSS + 15% FCS. Cells were allowed to remain on the column for 5 minutes before elution with HBSS + 15% FCS. Purity of the cell populations was assessed by non-specific esterase staining which showed the monocyte population to be > 80% pure and monocyte contamination of the lymphocytes to be < 1%. All tissue culture experiments were performed using aseptic technique.

2.B.5. CELL CULTURE AND ASSAYS

Lymphocyte Proliferation

PBMCs (1 x 10^6 ml^{-1}) were cultured in 0.2 ml aliquots of RPMI 1640 containing 10%· FCS in round bottomed microtitre plates. Cultures were set up in the presence or absence of PHA (1 $\mu g ml^{-1}$) and incubated for 72 hours at 37°C in a humid atmosphere of 5% CO_2 in air. Cells were pulsed with 0.5 μCi ^{3}[H]– thymidine (sp. 5Ci $mmol^{-1}$) 4 hours

before termination of the culture incubation. The cells were harvested onto glass fibre filter discs and dried. These were placed into vials containing 2 mls scintillation fluid and the uptake of radio-isotope determined by liquid scintillation spectrometry.

IgG production

PBMCs, lymphocytes and monocytes were cultured alone or in various combinations. PBMC (1×10^6 cells ml^{-1}) were cultured in 1 ml. aliquots of RPMI 1640 + 10% FCS in large-well flat-bottomed culture plates. Incubations were set up in the absence or presence of PWM (final dilution $\frac{1}{200}$). Cultures were incubated for 7 days (except where stated) at $37^{\circ}C$ in a humid atmosphere of 5% CO_2 in air. The supernatants were harvested and assayed for IgG content by enzyme-linked immunosorbent assay (ELISA).

In reconstitution experiments, 50 μls monocytes (1×10^6 cells ml^{-1}) were added to 950 μls of lymphocytes (1×10^6 cells ml^{-1}) and controls performed in the absence of monocytes.

Enzyme-linked immunosorbent assay (ELISA) for IgG

100 μls of an optimal dilution of a goat anti-human polyvalent immunoglobulin were dispensed in each well of a flat-bottomed micro-ELISA plate. The plates were covered and incubated for 1hr at $37^{\circ}C$ under humid conditions. The plates were used immediately or stored at $4^{\circ}C$ until needed. They were washed three times with coating buffer, dried thoroughly and 100 μls of PBS/BSA added to each well. After incubation for 30 minutes at $37^{\circ}C$ in humid conditions 100 μls of each doubling dilution of the test supernatants were added to the appropriate wells. Standard IgG containing solutions (100 μls) were also added and doubly diluted to construct a standard curve. Following incubation for 1 hr at $37^{\circ}C$ in humid conditions, the wells were washed thoroughly with PBS/Tween and dried. 100 μls of an alkaline phosphatase

conjugated anti-human IgG $\frac{1}{100}$ (100 dilution) were then added to each well

and the plates incubated for 1 hr at $37^{\circ}C$ in humid conditions before

extensive washing with PBS Tween and thorough drying. 100 μls of

alkaline phosphatase substrate (p-nitrophenyl phosphate) in diethan-

olamine buffer (1 mg ml^{-1}) were then added to each well and the colour

allowed to develop at room temperature. Once the maximum known IgG

concentration well had reached an absorbance reading of 1.0 at 405 nM,

the plates were read at this wavelength in a Dynatech micro-ELISA

reader. IgG concentrations of unknown supernatants were determined

by reference to the standard IgG calibration curve simultaneously con-

structed.

Blockade of cell membrane SH groups

Cell surface SH groups were blocked by pre-incubating cells for

1 hr at $37^{\circ}C$ with an irreversible non-penetrating SH-blocking agent,

p-hydroxymercuriphenyl sulphonic acid (pHMPSA) (Tsan and Berlin, 1971).

The cells were then washed three times with CMFSS and resuspended in

RPMI 1640.

Monocyte-lymphocyte clustering

Monocytes and lymphocytes were obtained as previously outlined.

Both populations were incubated in absence (unblocked) or presence of

5 x 10^{-5}M pHMPSA (blocked) for 1 hr at $37^{\circ}C$. Monocytes (unblocked

and blocked) were then diluted in RPMI 1640 + 10% FCS to 400,000 cells

per ml. and 0.4 mls of these cell solutions added to appropriate com-

partments in Lab-Tek tissue culture chamber/slides (type 4808) which

were incubated for 1 hr at $37^{\circ}C$ in 5% CO_2 in air under humidified

conditions. At the end of the incubation, non-adherent cells were

flushed away by washing three times with RPMI 1640 + 10% FCS. 0.4 ml.

aliquots of lymphocytes (4 x 10^{5} cells ml^{-1}) were added to appropriate

chambers in cross-over reconstitution experiments (i.e. blocked mono-

cytes with blocked lymphocytes, blocked monocytes with unblocked

lymphocytes etc.). Cultures were then incubated in the absence and

presence of $\frac{1}{200}$th dilution of PWM and in some cases, purified protein

derivative of tuberculin (PPD) (100 μgml^{-1} final concentrations) for

16 hr at 37°C in 5% CO_2 in air under humidified conditions. At the

end of the incubation period, the cells were fixed by the addition of

1% glutaraldehyde in 0.1M Cacodylate buffer (pH 6.8) for 30 mins at

37°C. The slides obtained were then stained using a Difquick differ-

ential stain and the number of monocyte-lymphocyte clusters counted

under light microscopy.

Measurement of intracellular Ca^{2+} concentrations

PBMCs were prepared as previously outlined and the concentration

adjusted to 2 x 10^7 cells ml^{-1}. The fluorescent quinoline Ca^{2+} ind-

icator, Quin-2 (Tsien et al, 1982 a,b) was added in its ester form

(Quin-2/AM) at a final concentration of 20 μM and incubated with the

cells for 20 minutes at 37°C. At the end of this period, the cells

were diluted 10-fold with RPMI 1640 and the incubation at 37°C con-

tinued for another 40 minutes to complete Quin-2 loading. The cells

were then incubated with 5 x 10^{-5}M pHMPSA or CMFSS (in controls) for

1 hr at 37°C and resuspended at a concentration of 1 x 10^7 cells per

ml. in RPMI 1640. After being spun in a microfuge (low-speed), the

cell pellets were resuspended in 2 mls simplified medium and the sus-

pensions rapidly transferred to quartz micro-cuvettes. The background

fluorescence due to basal intracellular Ca^{2+} ions was monitored in a

fluorimeter (Perkin-Elmer MPF 44A) (excitation 339 nM, emission 492nM)

to achieve a stable baseline before 1 μg.ml.$^{-1}$ PHA was added and the

subsequent increase in fluorescence monitored.

Measurement of [3H]-oleic acid uptake into mononuclear cells

PBMCs were incubated with 9,10 (n) [3H] oleic acid (sp.act

4.8 Curies/mmol) at a concentration of 4 μCi ml^{-1} for 1 hour at

37°C. Following this the cells were washed three times with RPMI

+ 0.5% fatty acid free BSA, counted and the concentration adjusted

to 5 x 10^6 cells per ml. 1 ml. aliquots of cell suspensions were

added to 2 mls scintillation fluid (Liqui-scint, National Diagnostics,

N.J. USA). The ^{3}(H)oleic acid incorporated into the cells was

then determined by measuring the radiolabel incorporated using a

scintillation counter.

In some experiments, no washing of the cells was performed after

the incubation step and the cells were counted, adjusted to 5 x 10^6

cells per ml. and aliquoted into Liqui-scint.

Hydrogen Peroxide Assay

This assay is based on that developed by Pick and Keisari

(1981). It is based on the hydrogen peroxide-dep-

endent horse-radish peroxidase-mediated oxidation of phenol-red which

results in the formation of a compound which has an increased absorb-

ance at 610 nM at alkaline pH compared to native phenol-red

(Figure 3).

Standardisation of Assay

The assay was standardisedby using hydrogen peroxide solutions

in . CMFSS . of known concentrations. Appropriate hydrogen per-

oxide dilutions in 100 μl. aliquots were added to 100 μls of PRS-

buffer in the wells of a flat-bottomed micro-titre plate to give hydro-

gen peroxide concentrations in the range 1 μM to 100 μM. The plates

were incubated at 37°C for 5 minutes and 10 μls of 3MNaOH added to

each well to bring the pH to 12.5. The absorbance at 610 nM was

read in a micro-ELISA reader against blanks containing 100 μls PRS-

buffer, 10 μls 3MNaOH and 100μls H_2O_2 (added after the 3MNaOH). The

standard curve constructed from this assay was found to be linear in

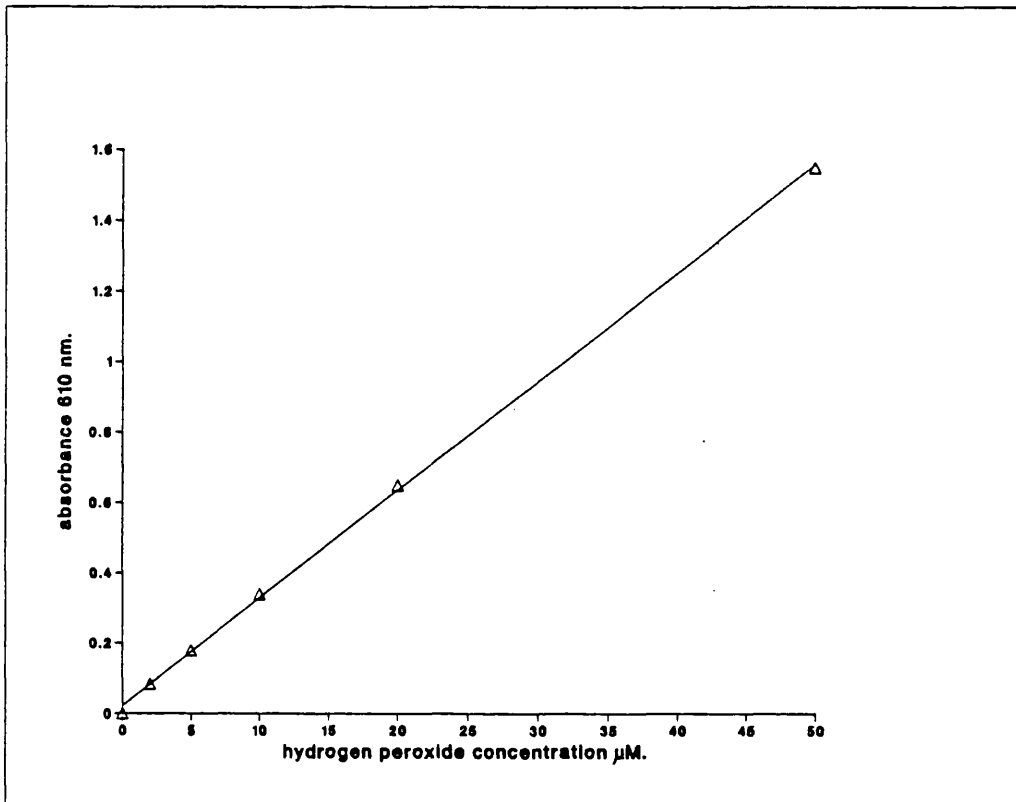FIG. 3. Curve to standardise the H_2O_2 micro-assay.

the range 1 μM and 50 μM (Figure 3   ).

Hydrogen Peroxide production by monocytes

100 μls aliquots of monocytes (2 x 10^6 cells ml^{-1}) were added

to the wells of a flat-bottomed microtitre plate containing 100 μls

of PRS-buffer with HRPO together with 5 x 10^{-5}M FMLP where approp-

riate. Wells without stimulus (FMLP), wells plus catalase (1500 Uml^{-1})

and wells with stimulus plus catalase served as controls. Each det-

ermination was performed in triplicate. The plates were incubated

at 37°C for 30 minutes and the reaction stopped and pH adjusted to

12.5 by the addition of 10 μls 3MNaOH. The absorbance was then read

immediately in a micro-ELISA reader. H_2O_2 production by the cells

was calculated by reference to the standard curve constructed using

known H_2O_2 concentrations.

Superoxide production by monocytes

100 μls aliquots of monocytes (2 x 10^6 cells ml^{-1}) were added

to the wells of a flat-bottomed microtitre plate containing 100 μls

of a phenol-red free balanced salt solution with 160 μM ferri-cytochrome

c together with 50 μg heat-aggregated IgG (HAGG) where appropriate.

Wells without stimulus (HAGG) and stimulus plus superoxide dismutase

(SOD) (300 U ml^{-1}) served as controls. Each determination was per-

formed in triplicate. The plates were incubated for 1 hr at 37°C

and the absorbances read in a micro-ELISA reader at 550 nM against

a blank containing no cells. Results were expressed in terms of n moles

cytochrome C reduced using the formula

A = E c l where A = absorbance

c = concentration (mM)

l = length of light path (cm)

E = Extinction coefficient = 21 mM^{-1} cm^{-1}

The length of light path (1) was determined by measuring the

radius (r) of the wells and then applying the formula:

$$\text{Volume} = r_2 h$$

where h is the height of liquid in the well and therefore the length

of the light path.

Chapter Three

RESULTS A

3. RESULTS

3.A. DEVELOPMENT OF A MODIFIED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM FOR THE DETERMINATION OF PENICILLAMINE.

The inherent reactivity of D-penicillamine has caused considerable problems in the development of reliable, accurate assay procedures. A number of pharmacokinetic studies measuring free D-penicillamine in biological fluids, mostly by HPLC coupled with mercury based electrochemical detection, have been performed but no studies have been performed measuring total D-penicillamine except for that carried out by Muijsers et al (1979) using an amino-acid analyser. This method is limited, however, by the long analysis times involved. Table 3 shows a list of published pharmacokinetic studies. In 1983, Abounassif and Jefferies developed sample preparation procedures coupled with a HPLC system to detect total D-penicillamine in human plasma. However, a number of problems associated with the system made routine analysis difficult. The initial aim of this study was to develop and modify this system to eliminate these problems rendering it suitable for routine analysis.

By initially treating plasma samples with the reducing agent Dithiothreitol (Cleland's Reagent) (Cleland, 1963), Abounassif and Jefferies reduced the oxidised, disulphide forms of penicillamine to the reduced SH-form of the drug and measured total drug in this form by their HPLC system. The chromatographic conditions employed by these workers are shown in Table 4 . One of the major difficulties of the system was the high detector voltage employed to detect D-penicillamine (1.2V). At high voltages (1.0V) there is a high background current and consequently high background noise which caused problems in detecting the D-penicillamine peak at low concentrations of the drug where high detector sensitivity was needed. A voltamagram was constructed whereby a fixed concentration of drug in mobile phase was injected onto the column and the voltage i.e. the potential difference (p.d.) between the elect-

		Study
Saetre and Rabenstein	1978	Total PSH in non-protein fraction, free PSH
Muijsers et al	1979	Total PSH
Bergstrom et al	1981	Free PSH
Wiesner et al	1981	Free PSH
Butler et al	1982	Free PSH
Kukovetz et al	1983	Free PSH
Schuna et al	1983	Free PSH
Brooks et al	1984	Free PSH

Key   PSH = penicillamine

TABLE 3. Previous studies of D-penicillamine pharmacokinetics.

PARAMETER	ASSAY CONDITIONS
Mobile phase	5% methanol $(\frac{v}{v})$, $0.1gL^{-1}$ EDTA, and 5×10^{-3} sodium dodecyl sulphate in 0.05M citrate phosphate buffer
Stationary phase	Spherisorb NH_2 $5\mu m$ 25 cm
Temperature	$25^{o}C$
Flow-rate	2 ml min^{-1}
Injection volume	100 μl.
Applied potential	+ 1.2V vs. Ag/AgCl
Chromatography time	20 minutes
Type of electrode	TL4A Glassy carbon

TABLE 4.　Initial Chromatographic conditions for analysis of total and free penicillamine in plasma

rodes of the electrochemical detector altered. This was to ascertain the voltage needed to give a maximum response to D-penicillamine to find if it was possible to reduce the detector voltage employed but still retain the ability to detect D-penicillamine maximally. The voltamagram is shown in fig.4. There was only a minimal response to D-penicillamine at low voltages below +0.9V i.e. the potential difference was insufficient to cause significant ionisation of the D-penicillamine molecule (possibly carboxy-and/or amino-groups). Between 0.95V and 1.15V there was a sharp increase in the response which continued to increase beyond 1.2V although there was some decrease in the rate of response at this point. These results demonstrate the need for a high p.d. to detect using the glassy carbon electrode.

Similar experiments were performed to find the effect of increasing the potential upon the background current and peak to peak noise. These results are shown in Figs. 5 and 6. The background current and noise remain low at potentials below 1.0V. After this, vast increases were observed in both parameters in response to relatively small increases in the p.d.

The results of all these experiments showed that a high detector voltage was necessary to detect D-penicillamine maximally but at such voltages high background currents and noise were encountered. The only possible solution to the problem was to use another electrode which would detect D-penicillamine at lower voltages. (This was eventually done - see later).

Fig.7 shows a chromatogram from the initial system. As can be seen there was a considerable number of endogenous "plasma" peaks which eluted prior to the penicillamine and Internal standard peaks. At low D-penicillamine concentrations it was necessary to increase the sensitivity of the electrode to detect the drug. However, this also had the
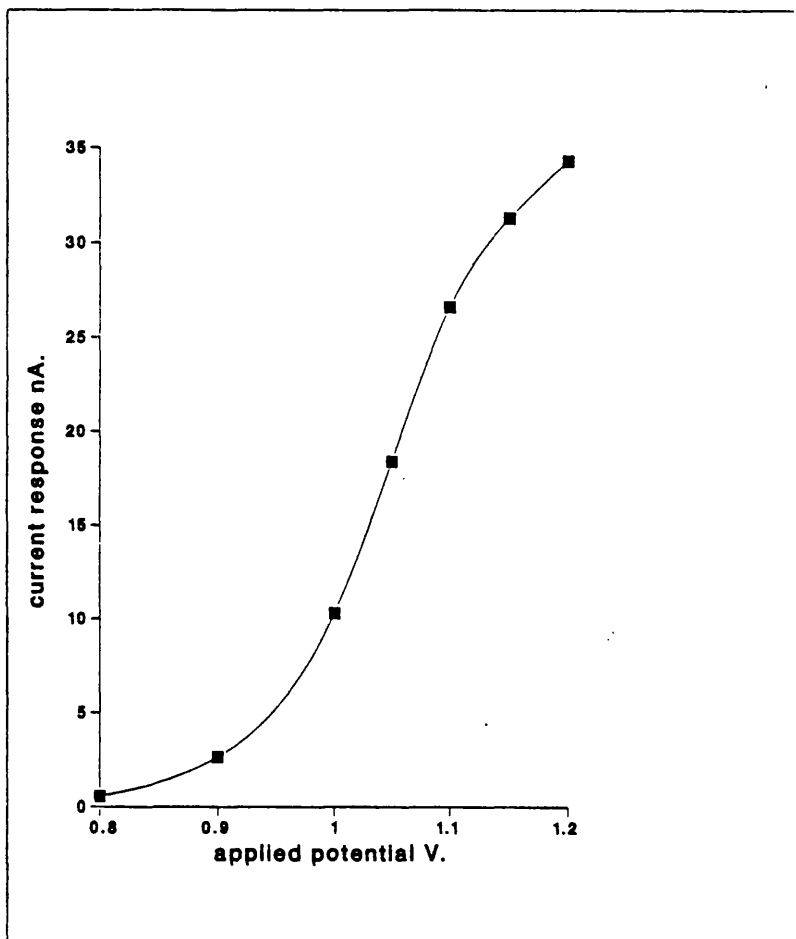
FIG.4. Voltamagram showing current response to D-penicillamine
vs. applied potential.
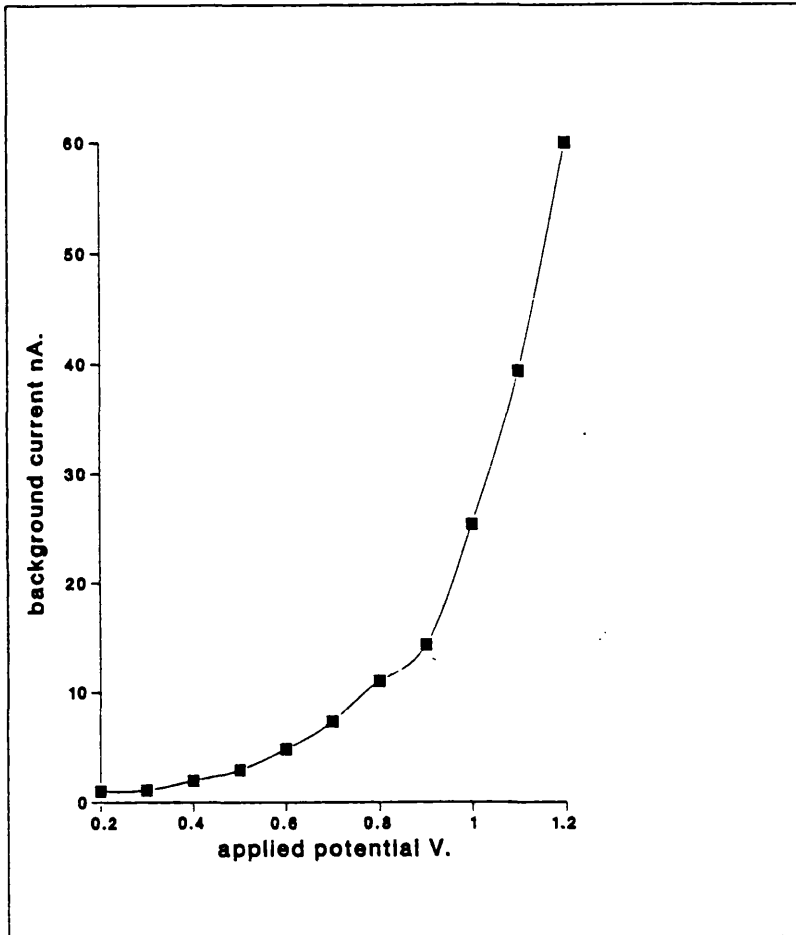
FIG. 5.   Effect of increasing applied potential upon
          the background current of the detector.
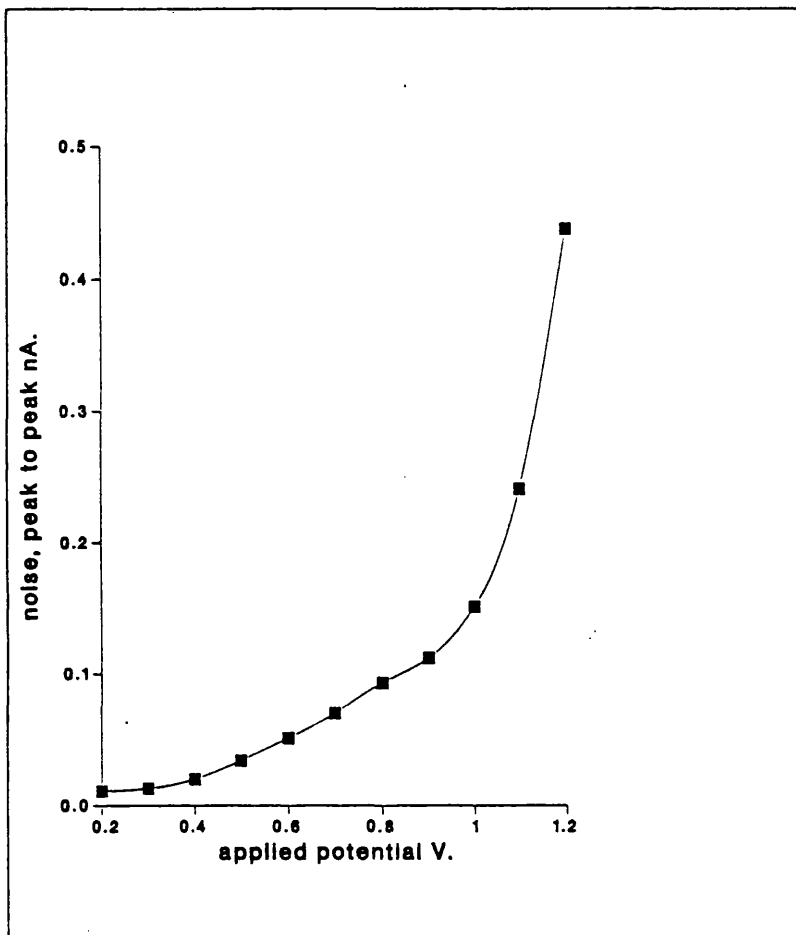
FIG.6. Effect of increasing applied potential upon noise, peak to peak, of the detector.
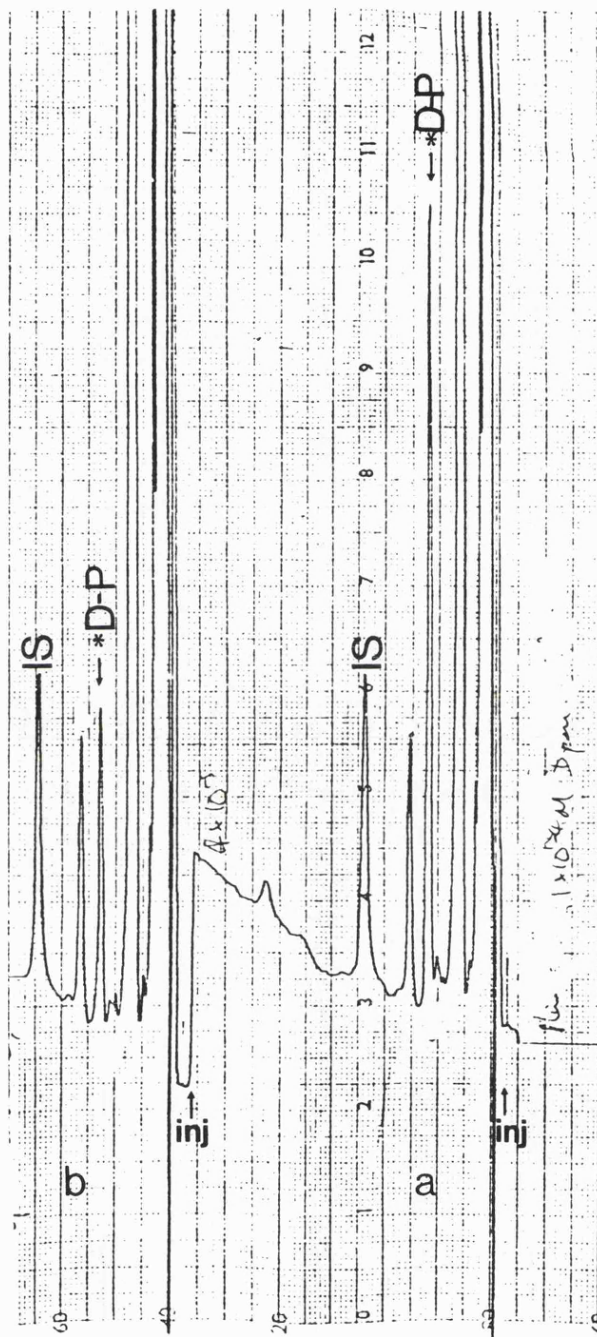
FIG.7.   Original chromatograms from the one-column system.
The D-penicillamine peak (*D-P) elutes after the "plasma"
peaks at about 13 minutes.   The internal standard peak
(IS) elutes at about 20 minutes.   The D-penicillamine peaks
correspond to (a) 1×10^{-4}M (14.92 μgml^{-1}) and (b)
4×10^{-5}M (5.97 μgml^{-1}).   Inj $\uparrow$ represents point of
injection.

effect of increasing proportionately the size of the "plasma" peaks.

Sometimes this led to these peaks partially masking the penicillamine

peak making the determinations of low concentrations very difficult.

These plasma peaks also caused another problem. In order to maintain

the sensitivity of the electrode it had to be cleaned and polished at

frequent intervals, again, interfering with use of the system on a rout-

ine basis. It was thought these plasma compounds that were being det-

ected might be contributing to the electrode's loss of sensitivity by

"coating" the electrode surface. In order to eliminate or, at least,

minimise this problem and generally clean up the chromatography, a

two-column system was devised.

As the majority of interfering peaks eluted prior to D-penicill-

amine, a two-column system linked by a switching valve was devised to

divert these unwanted compounds away from the detector but allow D-

penicillamine to pass to the detector. The sample was initially in-

jected onto a pre-column whose outlet flushed to waste. Just prior to

the elution of D-penicillamine from this column the flow of the eluate

was diverted by a switching valve to another column (analytical column)

whose outlet directly connected to the detector. In this way, unwanted

compounds which eluted early were flushed to waste whilst D-penicilla-

mine was passed to the detector for analysis. To reduce the analysis

time and to avoid problems with band spreading giving poor peak reso-

lution, smaller columns were tried in the system and investigations

showed that a 15 cm. pre-column (flow rate 1.45 ml/min) and an analyt-

ical column (flow rate 1.35 mls/min) gave good peak resolutions and

separation with an elution time for D-penicillamine only 30 seconds

later than the initial one-column system. The final column arrange-

ment is shown in Fig. 8 .

Such a configuration also meant that immediately after elution

PUMP 1   PUMP 1

A

PUMP 2 →   → W

PC   B

AC

detector.

(i)   (ii)

KEY

PC = pre-column
AC = analytical column
W   = waste
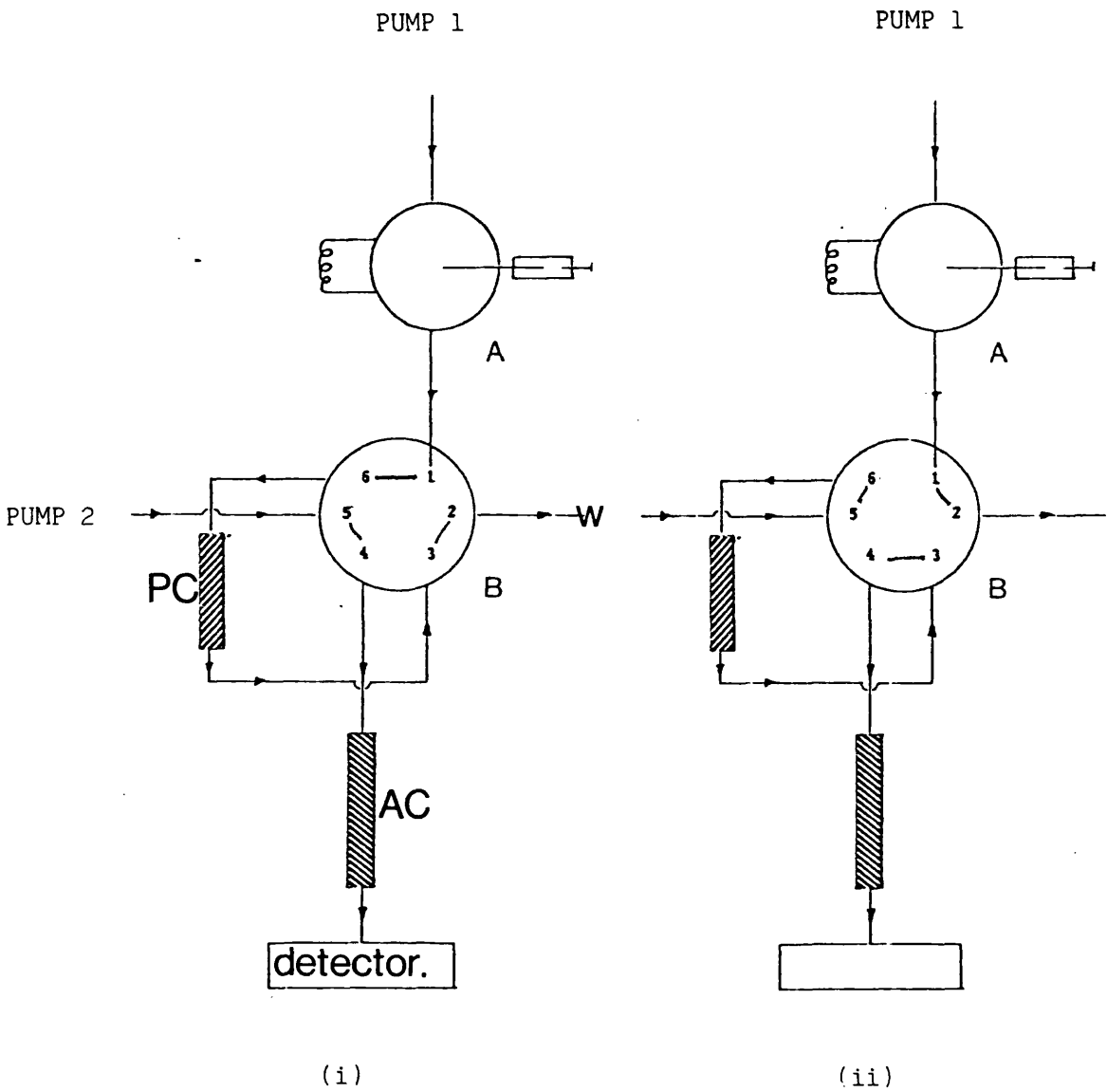
A= Rheodyne injection valve 7125

B= Rheodyne switching valve 7000

FIG.8.   Column arrangement and port positions for switching
         system.
         (i) Loading pre-column and flushing to waste
         (ii) Transfer from pre-column to analytical column

of D-penicillamine from the analytical column the flow could be reverted

back to the initial pattern and the next sample loaded onto the pre-col-

umn. So, samples were overlapped and the effective analysis time reduced.

This two-column method initially used the glassy-carbon electrode

of the original method. This meant that whilst the problems of cleaner

chromatography and maintained sensitivity were solved, the problems with the

high applied potential persisted. This was overcome when a mercury-

gold amalgamated electrode became available and utilised in the system.

This electrode is specific for thiols, chelons and halideions and can

be used at very low voltages (+ 0.2V) so eliminating background current

and noise problems. Its detection of thiols relies on the reaction of

the mercury with the sulphydryl group. The electrode was compatible

for the analysis of total penicillamine in plasma since the total (Oxi-

dised) drug is reduced to the free, reduced form by di-thiothreitol

(DTT) prior to analysis and measured in this form. However, this red-

uction step posed a potential problem since di-thiothreitol is used in

excess in this reaction to ensure completion of the reduction. Di-

thiothreitol possesses two free SH groups and so is capable of detection

by the amalgamated gold electrode and since it is in excess could have

given a large signal. The elution of DTT was found to occur at 3 min-

utes from the pre-column but the signal large enough to continue until

very close to the switching time of 8 minutes. For this reason, the

methanol content of the mobile phase was reduced from 5% to 4% $(\frac{v}{v})$

which lengthened the elution time of D-penicillamine from 8'30" to

9'15" without affecting DTT elution. This allowed the switching time

to be delayed to 8'30" thus avoiding any possibility of DTT passing to

the analytical column. So, the DTT was flushed to waste from the pre-

column.

The calibration for penicillamine spiked into plasma was linear

over the range from 1 x 10^{-6}M (0.149 μgml^{-1}) to 1 x 10^{-4}M (14.92 μgml^{-1}) having the following values : slope (3.034) and intercept (0.045). When compared with a similar calibration in mobile phase, slope (4.015) and intercept (0.019), this represents a 75.6% recovery of penicillamine. This yield was found to be constant and stable. Both the calibrations were linear with r values > 0.995 and are shown in Fig. 9 . The system was found to be able to measure < 2.5 ng on-column whilst the coefficient of variation for spiked plasma samples (0.149 μg/ml and 5.968 μg/ml) was less than 2% throughout the day.

An applied potential of 0.15V was employed as a voltamagram (data not shown) showed this voltage to give a maximal response to D-penicillamine although only small differences were observed between 0.05V and 0.15V. The final conditions employed in the system are shown in Table 5 . Chromatograms from the system are shown in Figs. 10 .. and 11 . The actual clinical sample chromatogram (Fig. 11 .) when compared with one from the original system (Fig. 7 .) shows the improvement achieved with the new system.
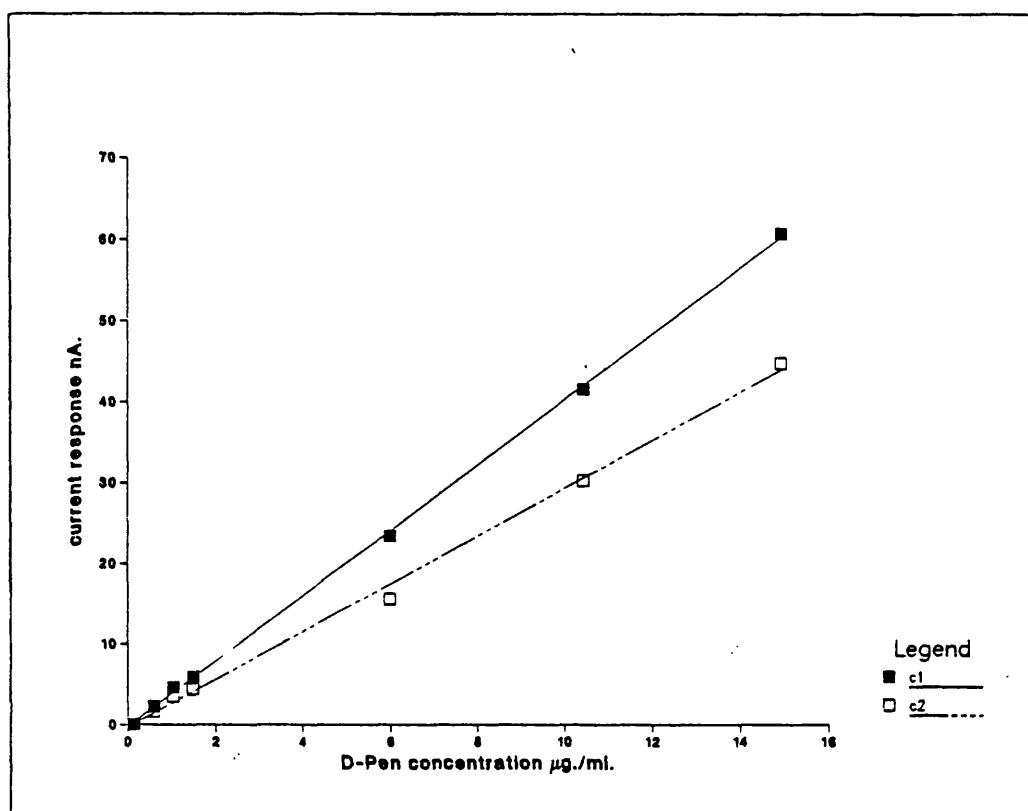
FIG.9. Calibrations for D-penicillamine in mobile phase (c1)
and plasma (c2). The r values in each case exceeded
0.995.

PARAMETER	ASSAY CONDITIONS REQUIRED
Mobile phase;	$4\%(\frac{v}{v})$ methanol. 0.1 gL^{-1} EDTA and $5 \times 10^{-3}M$ sodium dodecyl sulphate in $0.05M$ citrate-phosphate buffer, pH3.2
Stationary phases	
Column (i); analytical column	Spherisorb-NH$_2$, 5μm, 5c m
Column (ii);pre-column	Spherisorb-NH$_2$, 5μm, 15c m
Temperature	35°C
Flow rates;	
analytical column;	1.35 mlmm^{-1}
pre-column;	1.45 mlmin^{-1}
Injection volume	100 μls.
Applied Potential	$+0.15$V vs. Ag/Ag Cl
Penicillamine elution time	13 minutes
Effective chromatography time*	15 minutes
Type of electrode	TL6A, HgAu electrode

* see methods

TABLE 5.   Chromatographic conditions for the analysis of total penicillamine in mobile phase and plasma
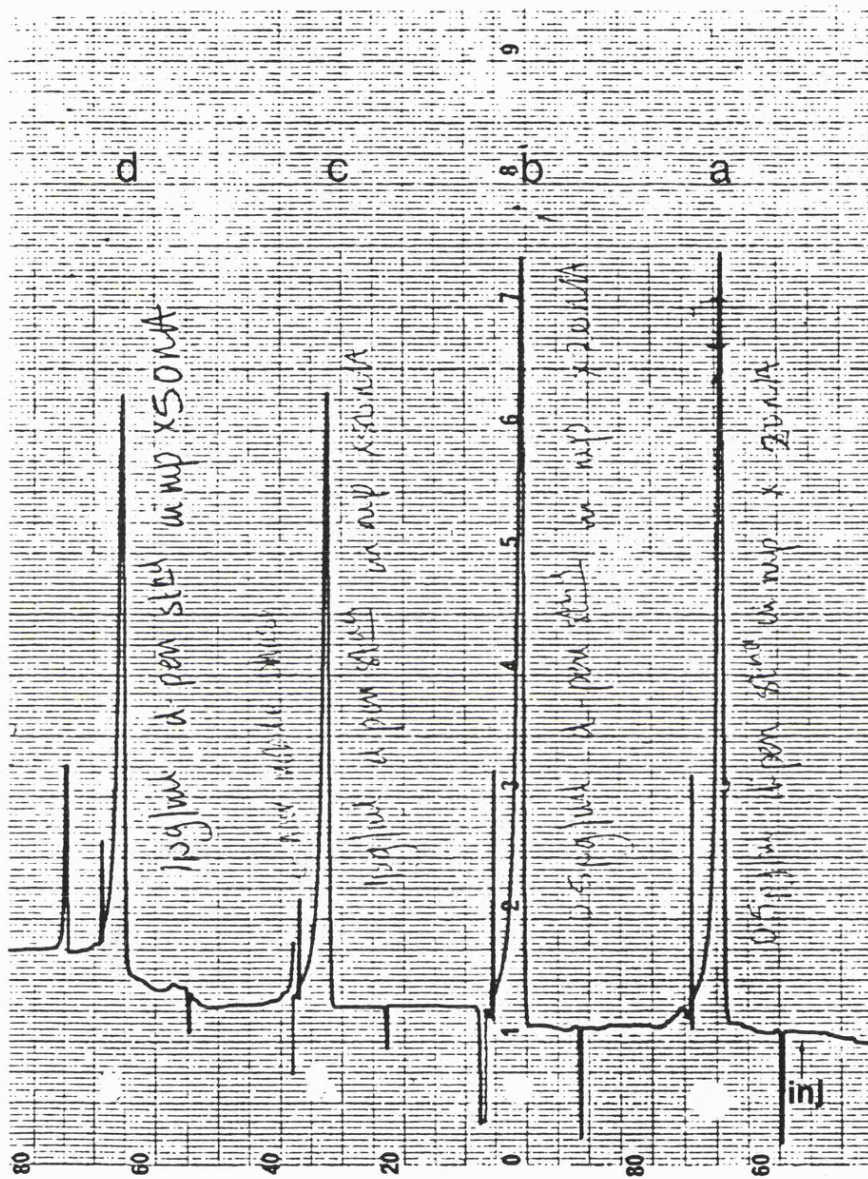
FIG.10. Chromatograms for D-penicillamine in mobile phase:
S1 indicates time for switching to analytical column;
S2 indicates switching time to revert to original flow
pattern. Chromatograms (a) and (b) show peaks for D-
penicillamine corresponding to 0.5 μgml^{-1} (20 nA sensi-
tivity and (c) and (d) show peaks for D-penicillamine
corresponding to 1 μgml^{-1} (50 nA sensitivity). Inj$\uparrow$
shows point of injection.
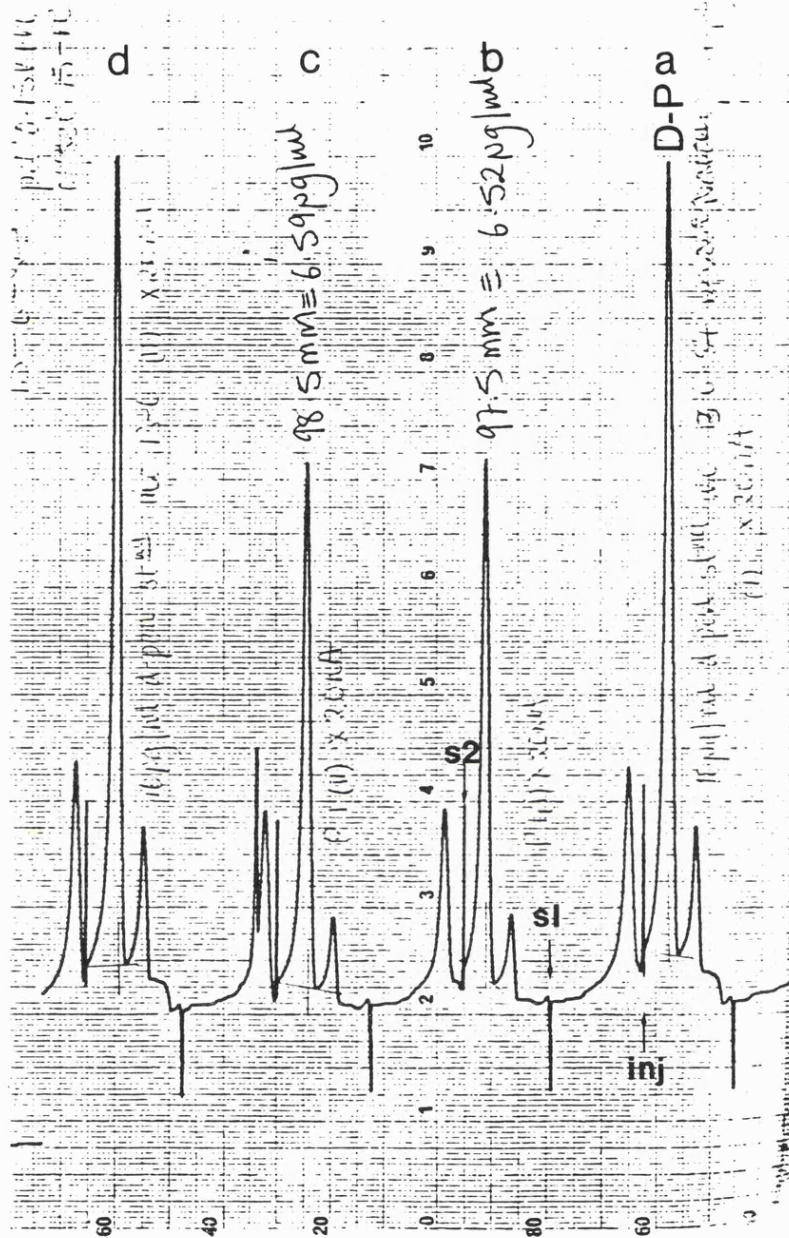
FIG.11. Authentic chromatogram for clinical sample :
S1 indicates time for switching to analytical
column; S2 indicates switching time to revert to
original flow pattern. Chromatograms (a) and (d)
are spiked standard samples for calibration purposes
corresponding to 10 μg1ml D-penicillamine. Chromato-
grams (b) and (c) are duplicate samples for an actual
clinical sample corresponding to 6.55 μg/ml. Inj $\uparrow$
shows points of injection.

3.B. EFFECT OF FOOD UPON THE ABSORPTION OF PENICILLAMINE IN NORMAL VOLUNTEERS

The bioavailability of many drugs including some of the penicillins is reduced when they are administered with food (Welling, 1980). Since D-penicillamine binds extensively to proteins and chelates metal ions, an investigation was performed to see if its bioavailability was similarly affected.

The protocol of the study is detailed in the methods section and the pooled data are shown in Table 6. (Whole data are displayed in Appendix 1). Figures 12 and 13 show pharmacokinetic profiles ($\pm$ food) for two of the subjects.

D-penicillamine was rapidly absorbed from the gastro-intenstinal tract with peak plasma concentrations observed at about 2 hours post-dosing. In the fasting state, the peak concentration was 8.04 ± 0.34 μgml^{-1} (mean $\pm$ 1.s.d.) which was significantly greater than that in the non-fasting state which was 5.54 ± 0.43 μgml^{-1} (Wilcoxon's rank Sum test, significant at 1% level). The decline in the plasma drug concentration was biphasic in each individual with an initial half-life of 5.19 ± 0.58 hours (mean $\pm$ 1.s.d.) followed by a much slower decline (γ-phase half-life) of 59.43 ± 9.06 hours. The half-life values in both fasting and non-fasting states did not differ significantly (see Table 6). It is important to note that the values for the final half-life can be criticised since they were calculated over a time period actually less than the $t_{\frac{1}{2}}$ value. The area-under-the-cruve (AUC) value (0 - 24 hours), as calculated using the trapezoidal rule, in the fasting state was significantly higher than that in the non-fasting condition (Wilcoxon's rank Sum test, 1% level). These results indicate that absorption of penicillamine is significantly reduced when administered following a light meal.

	fasting state	non-fasting state
Peak plasma concentration ($\mu g ml^{-1}$)	8.04 $\pm$ 0.34	5.36 $\pm$ 0.43 *
Area-under-the-curve (0 - 24 hours; μg $h^{-1}ml^{-1}$	83.97 $\pm$ 1.60	73.19 $\pm$ 2.99 *
Plasma elimination half-life (β phase; hours)	5.01 $\pm$ 1.15	5.37 $\pm$ 0.42 mean 5.19 $\pm$ 0.58 hours
Plasma elimination half-life (γ phase; hours)	56.09 $\pm$12.53	62.76 $\pm$14.39 mean 59.43 $\pm$ 9.06 hours

TABLE 6 .   Effect of food upon the absorption of D-penicillamine
(figures represent mean $\pm$ l.s.d. n = 5).

* p $<$ 0.01 (Wilcoxons Rank Sum test)
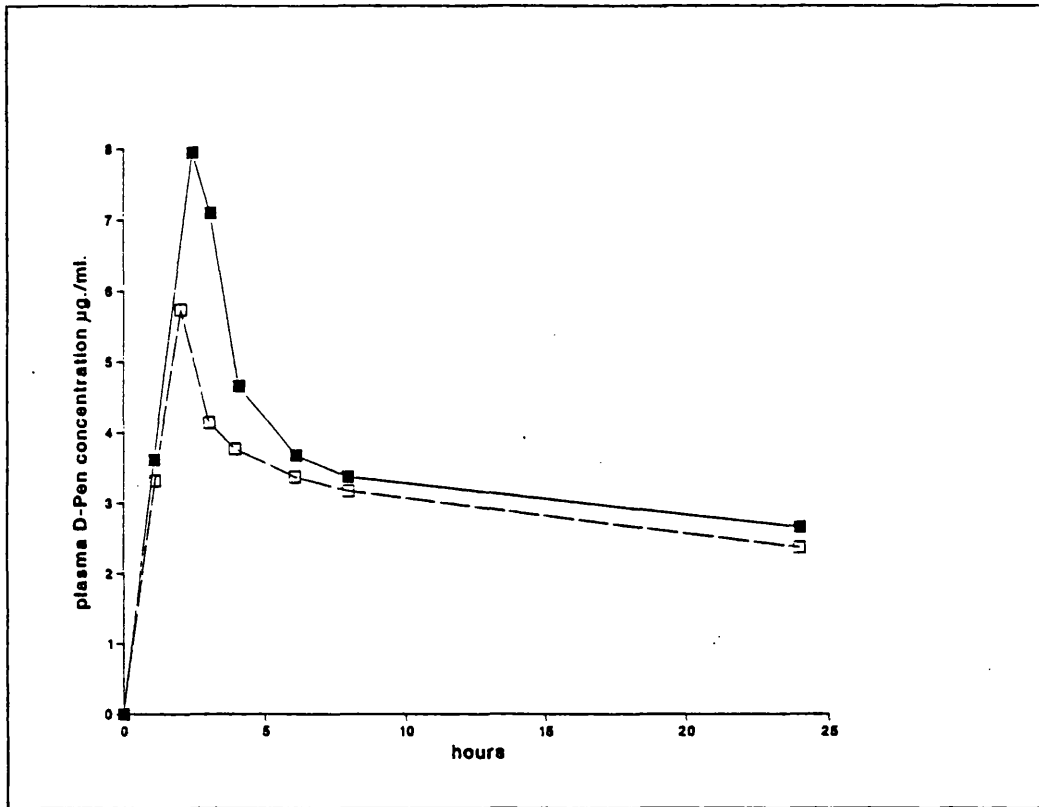
FIG.12. Pharmacokinetic profile of normal subject after
        receiving 500 mg. D-penicillamine by mouth with
        food (□) and without food (■).
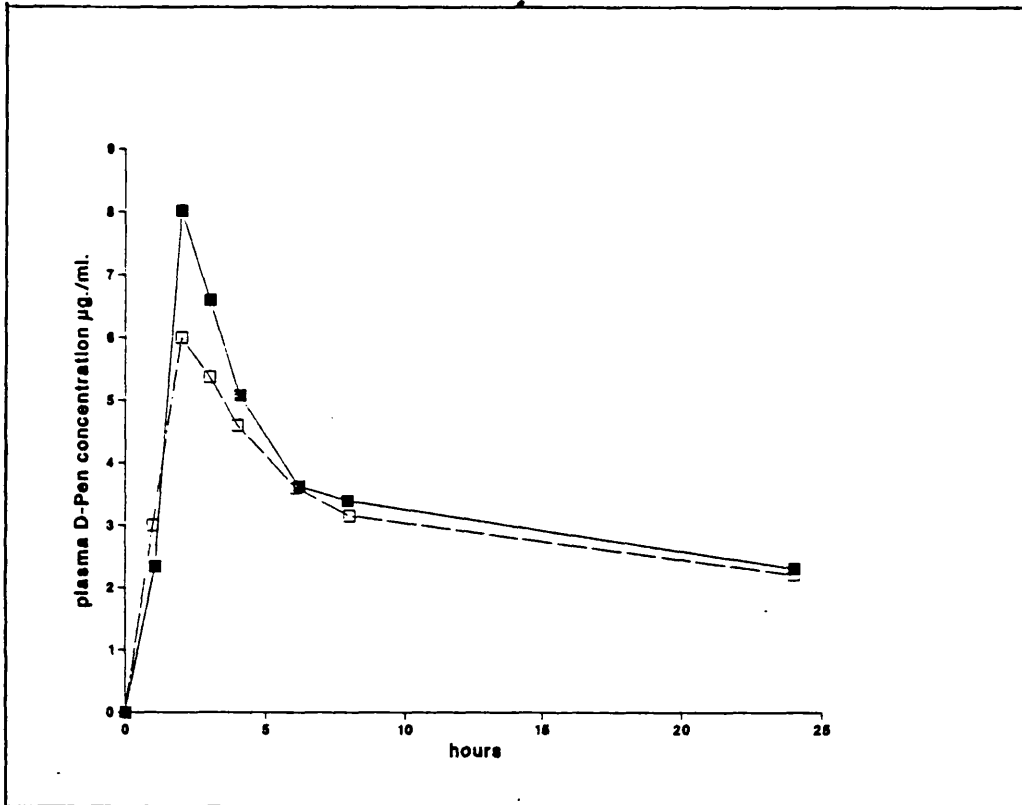
FIG.13. Pharmacokinetic profile of normal subject after receiving 500 mg. D-penicillamine by mouth with food (□) and without food (■).

3.C. PHARMACOKINETICS OF PENICILLAMINE IN RHEUMATOID ARTHRITIS

PATIENTS

The total penicillamine levels in the plasma of 16 RA patients were determined in the first 24 hours after being challenged with 125 mg. drug (by mouth) for the first time following an overnight fast. Pooled data are shown in Table 7 (Whole data are displayed in Appendix 2).

Peak plasma concentrations were observed at about 2 hours after dosing. The mean plasma concentration was 2.32 ± 0.79 μgml^{-1} (mean $\pm$ 1.s.d.) - this was calculated for 14 patients where sufficient samples were obtained. The decline in plasma concentration was biphasic with an initial phase of 6.82 ± 0.74 h (mean $\pm$ 1.s.d.) followed by a longer half-life of 56.22 ± 3.79 h. The γ-phase half-life can be criticised for the reason expounded previously in the study with normal volunteers. The half-life values are not significantly different from those obtained in that previous study. The area-under-the-curve for the 24 hour period was 45.70 ± 6.56 $\mu gh^{-1}ml^{-1}$ (mean $\pm$ 1.s.d.).

The dose administered in RA patients (125 mg.) was a quarter of that given to normal volunteers. The mean peak plasma concentrations and AUC (0 - 24h) values (for the fasting condition in volunteers) did not show proportionality but elimination kinetics in each case were similar. Pharmacokinetic profiles for four of the RA patients are shown in figures 14 and 15.

Peak plasma concentration ($\mu g ml^{-1}$)	2.32 ± 0.79	(n = 14)
Area-under-the-curve (0 - 24 hours; $\mu g h^{-1} ml^{-1}$)	45.70 ± 6.56	
Plasma elimination half-life (β- phase; hours)	6.82 ± 0.74	
Plasma elimination half-life (γ phase; hours)	56.22 ± 3.79	

TABLE 7.Pharmacokinetics of a single dose of D-penicillamine (125 mg. by mouth) in rheumatoid arthritis patients.

(values = mean $\pm$ l.s.d.)

(n = 16 except where stated)
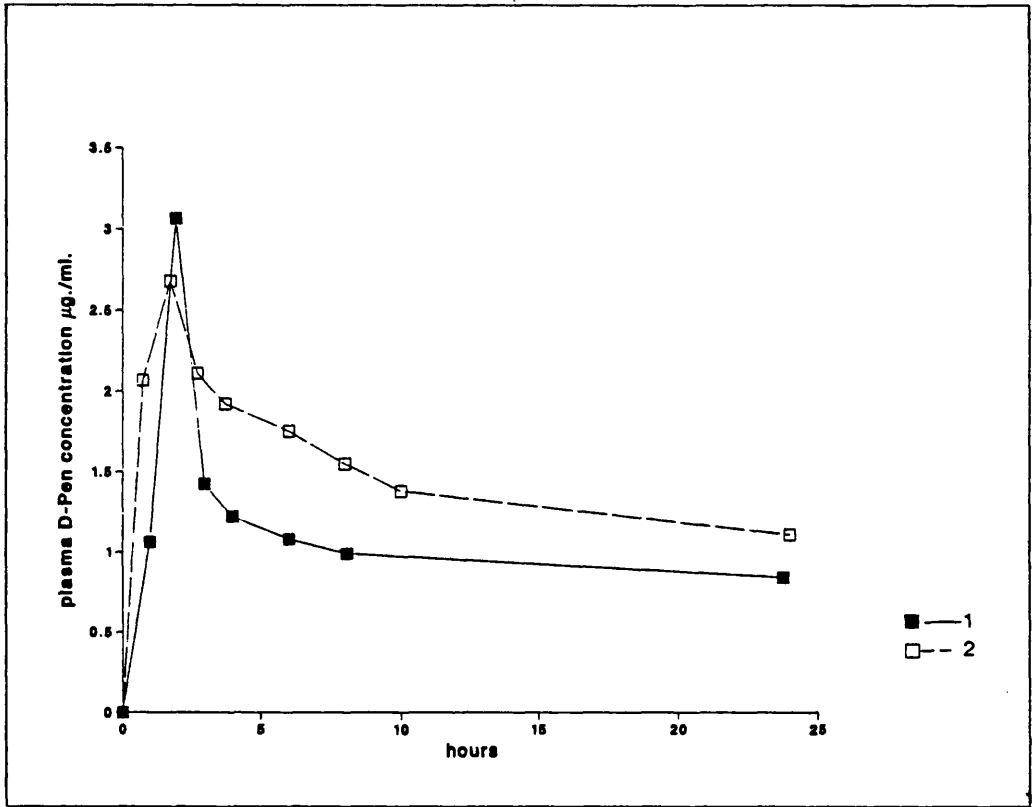
FIG.14. Pharmacokinetic profiles of two RA patients after
receiving 125 mg. D-penicillamine by mouth.
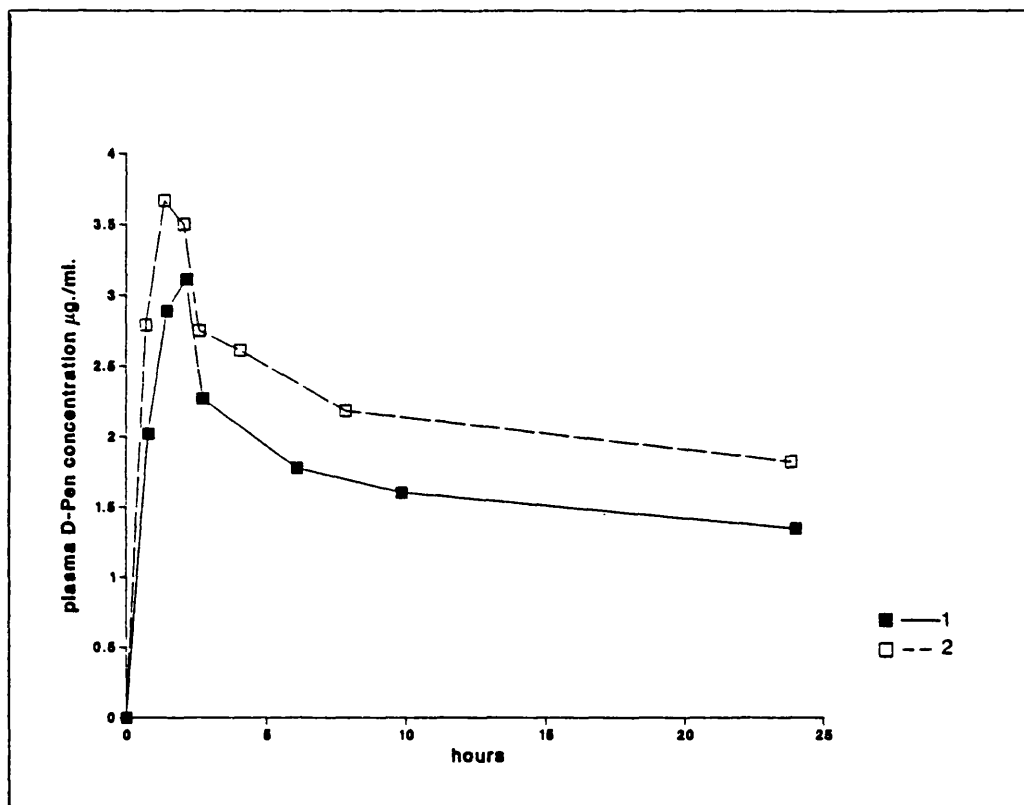
FIG.15.   Pharmacokinetic profiles of two RA patients after
            receiving 125 mg. D-penicillamine by mouth.

Chapter Four

RESULTS B

4.A. EXPERIMENTS TO FIND A REVERSIBLE SH BLOCKING AGENT

The defective accessory function displayed by monocytes in
vitro is due, at least partially, to oxidation of cell surface SH
groups (McKeown et al, 1984a). The aim of this part of the project
was to investigate the SH dependence of monocyte functions relevant
to their accessory function in the immune response.

Initial experiments were performed to find a SH-blocking agent
whose inhibitory effect upon the immune response in vitro could be
reversed by the action of 2-ME so providing a model system akin to
that seen with RA cells in which to study monocyte SH dependence.
Early investigations examined the effect of various SH blocking/oxi-
dising agents upon mitogen-stimulated mononuclear cell proliferation
and the ability of 2-ME to reverse any inhibitory effect. PHA was
used at a concentration of 1 μgml^{-1}, shown to be the optimum by pre-
liminary experiments, whilst PWM was used at a number of different
dilutions. Figure 16 shows the effect of DTNB (Ellmans Reagent,
5,5'-dithiobis (2-nitro benzoic acid) upon PHA-stimulated prolifer-
ation and the consequent effect of 2-ME. It clearly shows that DTNB
had no effect even at high concentrations (2000 μM). Furthermore,
2-ME itself inhibited proliferation almost completely at a concent-
ration of 500 μM. Similar results were achieved with PWM-stimul-
ated proliferation (data not shown). To see if the inhibition caused
by the high 2-ME concentration was due to toxicity, the viability of
cells incubated for 3 days with 2-ME was examined by trypan-blue ex-
clusion which showed >70% cell death at 500 μM 2-ME. At 100 μM
2-ME, no significant toxicity was observed by this method. The next
potential SH-oxidising agent used was oxidised glutathione, results
for which are shown in Fig. 17. Again, no inhibition was found
upon pre-incubation with this compound up to concentrations of 500 μM
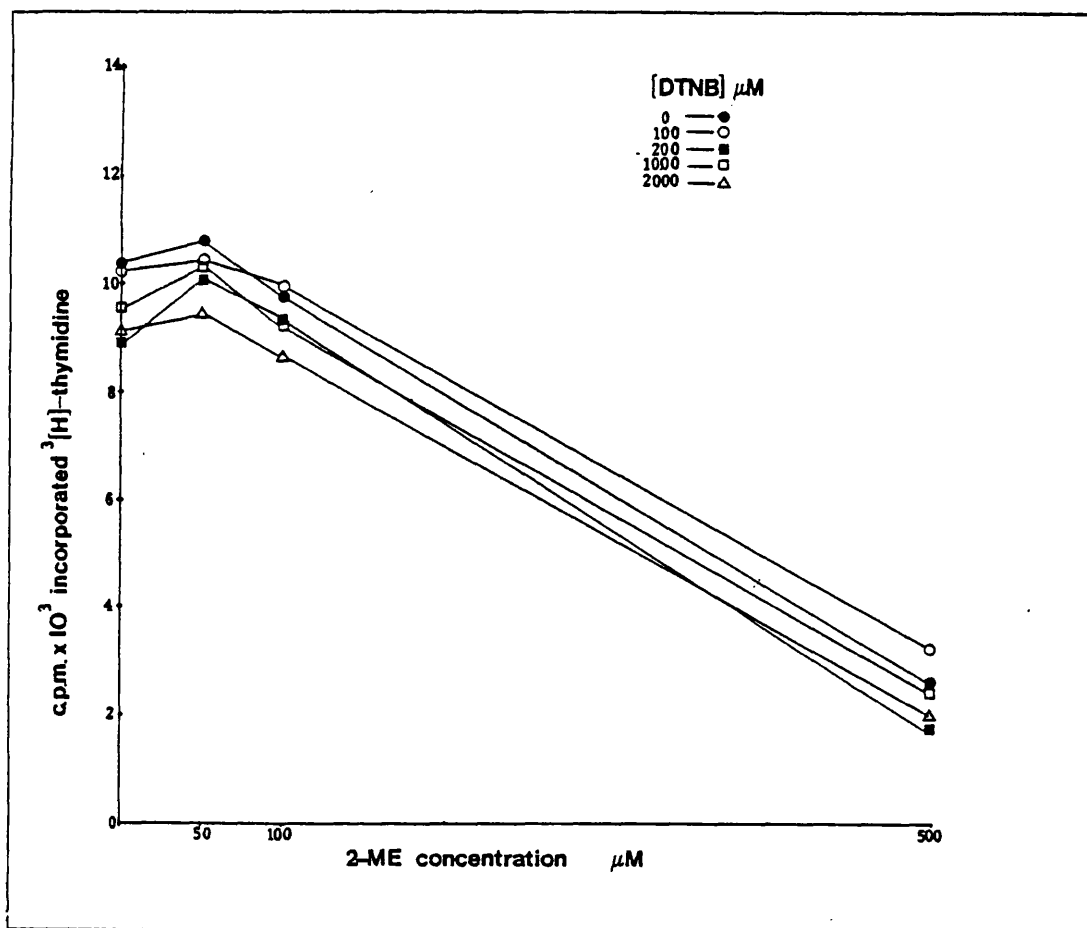
FIG.16. Effect of preincubation with DTNB on PBMC proliferation
in response to PHA (1 μgml^{-1}) in the presence and absence
of 2-ME. Each data point represents the mean value of
3 experiments (standard deviation bars omitted for clarity).

FIG.17. Effect of preincubation with oxidised glutathione on PBMC
proliferation in response to PHA $(1 \ \mu gml^{-1})$ in the presence
and absence of 2-ME. Each data point represents the mean
value of 3 experiments (standard deviation bars omitted
for clarity).

as was the case with PWM-induced proliferation (data not shown).
The toxic effect of 2-ME at 500 μM was again observed.

At this point, the proliferation assay, initially used due to
the relatively short experimental times involved, was discontinued.
Instead, PWM-induced IgG synthesis was assayed to determine if this
was more sensitive to the action of thiol-blocking agents. Initial
experiments showed a 1/200 dilution of PWM to give an optimum res-
ponse (Fig. 18) although dilutions of 1/40 and 1/1000 were also
used where sufficient numbers of cells were available. Only small
amounts of IgG were generated in the absence of PWM (< 250 ng
per million cells per ml).

The failure of DTNB and oxidised glutathione to produce in-
hibition of IgG synthesis at any significant level are shown in
Tables 8 and 9 respectively. The capacity of D-penicillamine to
form disulphides in the presence of some metal ions was the ration-
ale behind the use of D-penicillamine with Cu^{2+} ions as the next
potential reversible SH-oxidising agent. The effects of pre-incu-
bating mononuclear cells with D-penicillamine/Cu^{2+} are shown in
Fig. 19. The observed inhibition at D-penicillamine concentrations
exceeding 50 $\mu g\,ml^{-1}$ occurred irrespective of the presence of 2-ME in
culture at concentrations up to 100 $\mu g\,ml^{-1}$ Experiments performed in
the absence of copper showed no significant inhibition of IgG
synthesis.
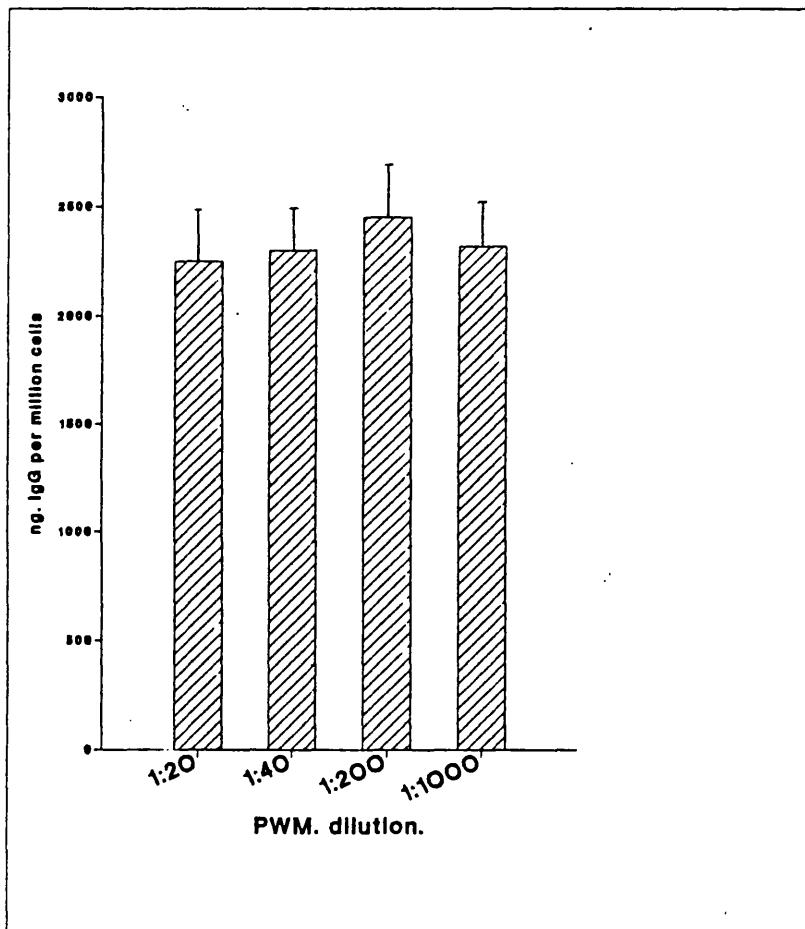
FIG.18. Effects of different dilutions of PWM upon
induction of IgG synthesis in PBMCs.

(DTNB) (μM)	$\pm$ 2 ME (100 μM)	IgG Production ng per 1×10^6 cells
0	–	2460 $\pm$ 255
0	+	2385 $\pm$ 310
100	–	2400 $\pm$ 290
100	+	2315 $\pm$ 205
1000	–	2240 $\pm$ 310
1000	+	2355 $\pm$ 360

TABLE 8. Effect of pre-incubation with DTNB on IgG synthesis

by PBMCs (+PWM) in the presence or absence of 100 μM

2-ME. Results shown are mean IgG productions

($\pm$ l.s.d.) (n = 3)

(ox.glut.) (μM)	$\pm$ 2-ME (100 μM)	IgG production ng per 1×10^6 cells
0	–	2395 ± 315
0	+	2320 ± 290
100	–	2270 ± 250
100	+	2355 ± 380
500	–	2230 ± 210
500	+	2265 ± 385

TABLE 9   Effect of pre-incubating with oxidised glutathione

upon IgG synthesis by PBMCs (+ PWM) in the presence

or absence of 100 μM 2-ME.   Results shown are mean

IgG productions $\pm$ l.s.d. (n = 3)
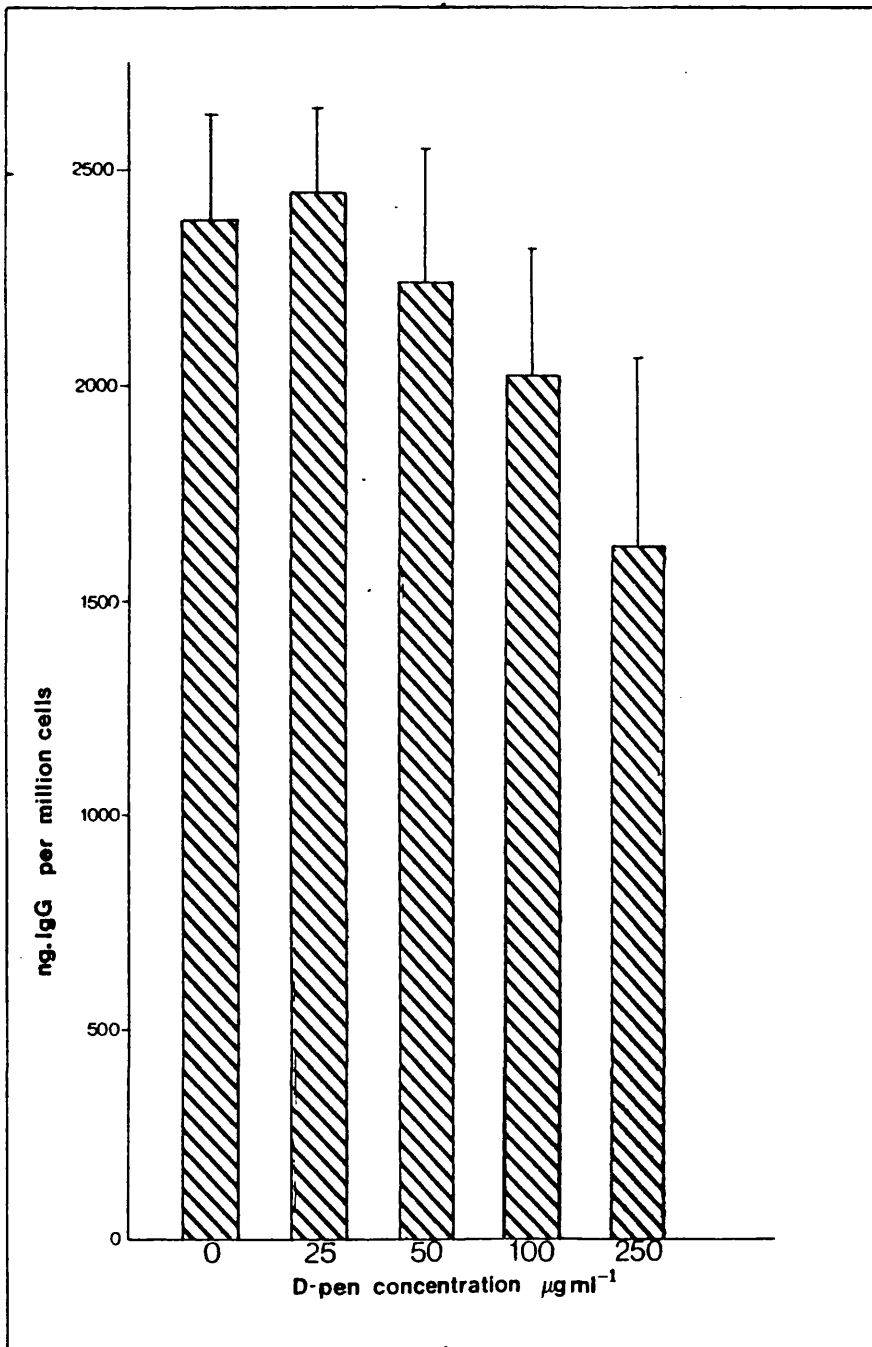
FIG. 19. Effect of D-penicillamine and Cu^{2+} ions upon
        IgG synthesis by PBMCs in response to PWM.

4.B. PRODUCTION OF H_2O_2 BY PENICILLAMINE AND Cu^{2+} IONS

The ability of D-penicillamine and Cu^{2+} ions to generate H_2O_2 was investigated using the phenol red oxidation micro-assay. Various concentrations of D-penicillamine (10 - 250 μgml^{-1}) were incubated with or without 8 μM Cu^{2+} ions (in the form of $CuSO_4$) at $37^{\circ}C$ for 5 minutes after which time phenol red buffer solutions containing HRPO were added for a further five minutes. Absorbance changes were monitored by reading the plates in a micro-ELISA reader at 610 nm. Figure 20 shows the generation of H_2O_2 by D-penicillamine in the presence of copper. The results show a biphasic effect with maximal H_2O_2 production being seen with 100 μgml^{-1} D-penicillamine. At 250 μgml^{-1} D-penicillamine there is a substantial drop in H_2O_2 production compared with 100 μgml^{-1} D-penicillamine. When copper was absent, no H_2O_2 was produced. The phenol red oxidation was shown to be due to H_2O_2 only since control experiments in which catalase (1500 Uml^{-1}) was added failed to exhibit phenol red oxidation. These results demonstrate the ability of D-penicillamine to form H_2O_2 in the presence of copper.

The failure of these studies to find a suitable reversible SH-blocker led to the use of the irreversible SH-blocking agent, pHMPSA, used in the original experiments by McKeown et al (1984a) in consequent experiments. Pre-incubation with pHMPSA results in a dose-dependent inhibition of PWM-induced IgG synthesis (Fig.21). 50% inhibition (ID_{50}) was estimated to be about 15 μM pHMPSA. No reversal of this inhibition was observed upon addition of 2ME (50 and 100 μM) (data not shown).

4.C. EFFECT OF SH BLOCKADE UPON ANTIGEN/PWM PRESENTATION BY MONOCYTES

The accessory function performed by monocytes in the immune response in vitro is complex and not fully understood. It is known that PWM-induced IgG synthesis is absolutely dependent upon intact functional monocytes. (Rosenberg and Lipsky, 1981; McKeown et al,
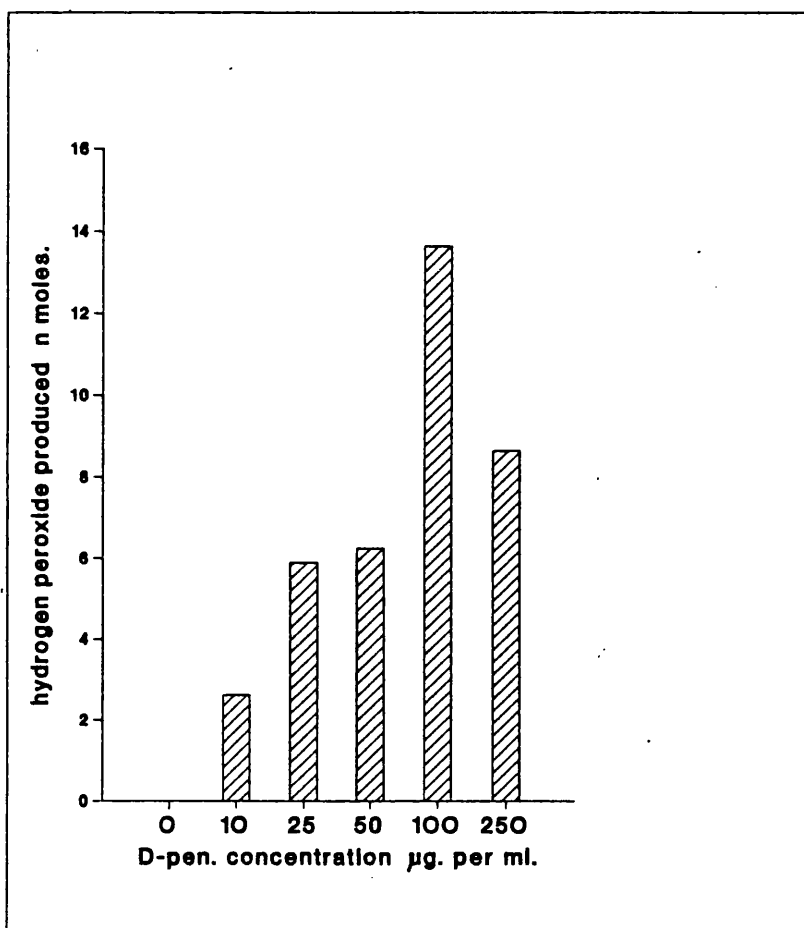
FIG. 20. The generation of H_2O_2 by D-penicillamine in the presence of Cu^{2+} ions (8 μM). Experiments where Cu^{2+} ions were omitted failed to produce H_2O_2. Results expressed are means of 4 experiments.
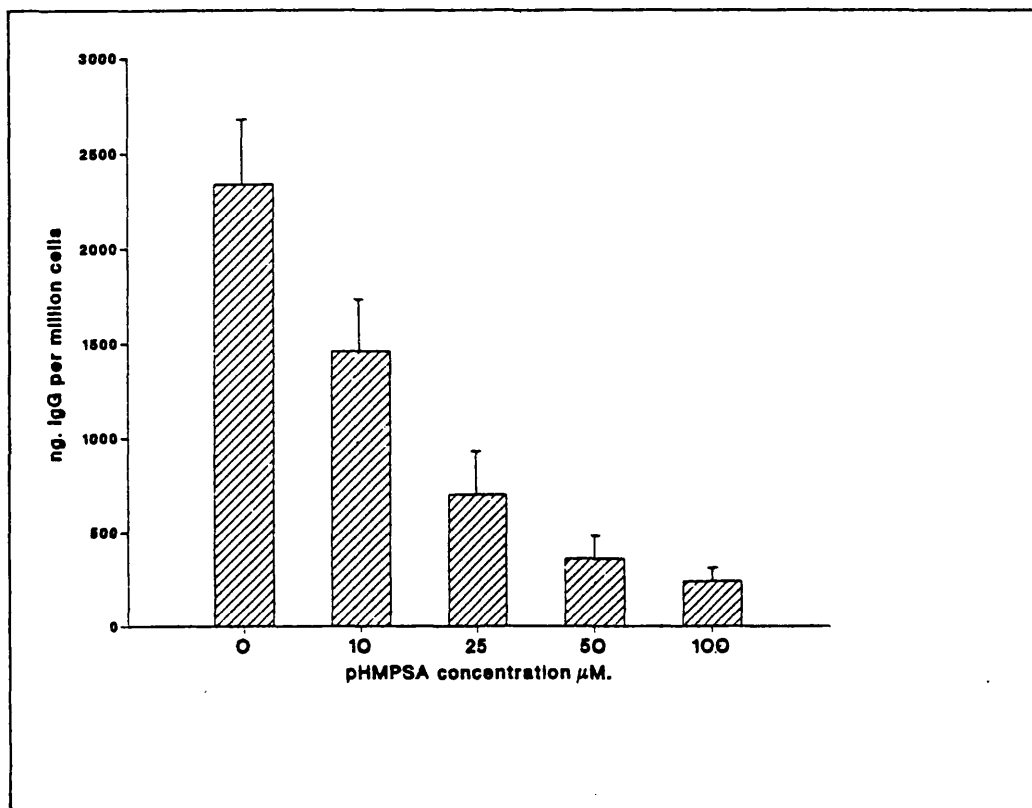
FIG.21. Effect of preincubation with pHMPSA upon PBMC IgG
synthesis. Each histogram represents the mean of
4 experiments ($\pm$ l.s.d.) ID_{50} of pHMPSA was estimated
to be 15 μM.

1984a). The ability of monocytes to present antigen, in assoc-
iation with Class II MHC antigens, and their production of the cyto-
kine, IL-1 are necessary for an effective immune response. It has
been shown that lymphocytes form characteristic clusters around mono-
cytes in response to soluble antigens in vitro (Werdelin et al,
1974; Powell et al, 1980). These clusters are thought to represent
antigen presentation by the monocytes to the lymphocytes since they
do not form in the absence of antigen. Physical interactions between
the cells can be observed by both light and electron microscopy. To
see if antigen/PWM presentation by monocytes was SH-dependent, the
ability of lymphocytes to cluster around monocytes in response to
these stimuli after pre-incubation with pHMPSA was assayed. Normal
(untreated) and SH-blocked lymphocytes were added back in reconstit-
ution experiments to blocked or unblocked monocytes which had been
pulsed with PWM. Cluster formation was assessed by light microscopy -
the results are given in Fig.22. Both these results and those of the
controls which omitted monocytes/lymphocytes ruled out an adhesive
effect. Therefore, it appears that antigen/PWM presentation by mono-
cytes is not SH-dependent. Similarly, lymphocytes do not require free
SH groups to recognise antigen/PWM on monocytes.

The production of IL-1 by monocytes was not assessed in this
work as previous studies by other workers indicated that its prod-
uction did not require free SH groups (McKeown et al, 1985b; S.F.
Chai, personal communication).

The exact biochemical events occurring at the monocyte cell
surface necessary for their accessory function in the immune res-
ponse have yet to be fully defined and the situation appears complex.
However, among these reactions which are likely to be involved are
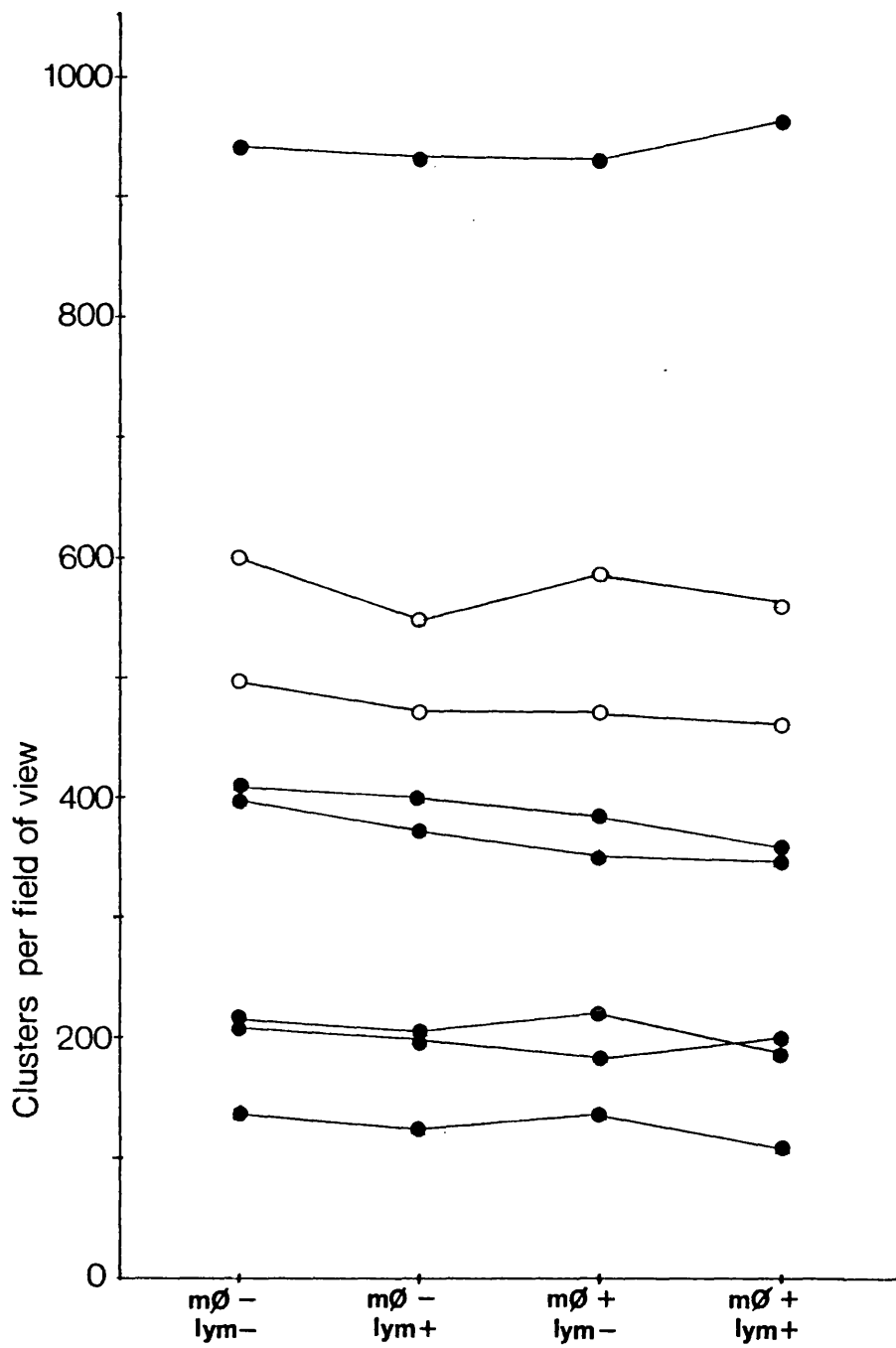
FIG.22. Cluster formation by monocytes and lymphocytes in response to PWM (●) and PPD (O). Cells were treated with pHMPSA to block surface SH groups (+) or were left untreated (-).

intracellular Ca^{2+} ion mobilisation, activation of phospholipase A_2 and the subsequent generation of arachidonic acid derived products e.g. prostaglandins and leukotrienes, and oxidative metabolism. Further experiments were performed to determine the requirement of free cell-surface SH groups in some of these monocyte functions.

4.D. EFFECT OF SH-BLOCKADE UPON Ca^{2+} ION MOBILISATION

PHA-induced Ca^{2+} ion mobilisation in mononuclear cells was monitored by use of the quinoline derivative, Quin-2 which fluoresces upon chelation of Ca^{2+} ions due to a conformational change. The experiments used whole mononuclear cell preparations due to the large numbers of cells required. Both untreated cells and those whose surface SH groups were blocked by pHMPSA showed similar responses to PHA in all cases. Fig. 23 is an actual trace obtained from one subject's cells, both blocked and unblocked. These results indicate that blockade of cell surface SH groups has no effect upon intracellular Ca^{2+} ion mobilisation.

4.E. EFFECT OF SH-BLOCKADE UPON ^{3}(H)-OLEIC ACID UPTAKE BY MONONUCLEAR CELLS.

Some arachidonic acid derived products are known to have regulatory effects upon the immune response e.g. PGE_2 (Goodwin et al, 1977; Staite and Panayi, 1982; Jellinek et al, 1985). Free arachidonic acid is generated from membrane phospholipids by the action of phospholipase A_2. One of the regulatory reactions controlling the level of free arachidonic acid available for further metabolism is its reacylation back into membrane lipids by acyl-transferase enzymes (Kroner et al, 1981). The effect of SH blockade upon this acylation capacity was determined by measuring the uptake of tritiated oleic acid into mononuclear cells. As fig. 24 shows, incorporation of ^{3}(H) oleic acid was inhibited in a dose dependent manner by pHMPSA

BLOCKED SH   FREE SH

$[Ca^{2+}]_i$

PHA

← Time
5 mins

PHA
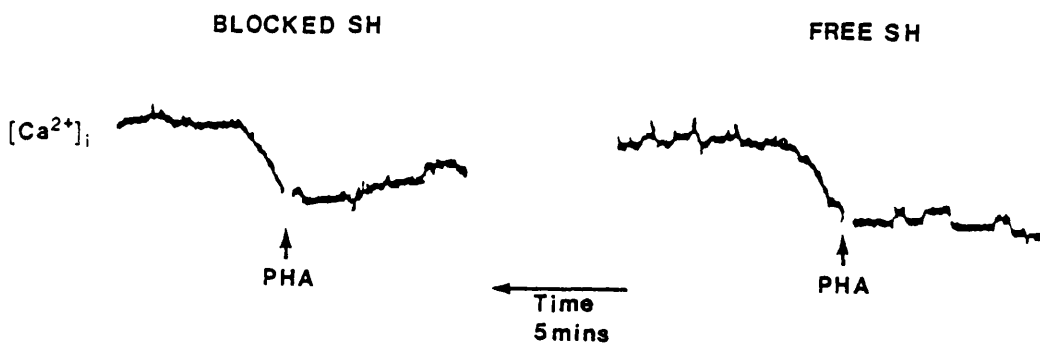
FIG.23.   Actual trace showing Ca^{2+} ion mobilisation by PHA
in normal PBMCs and PBMCs with blocked SH groups.
All subjects showed similar traces and no effect
was observed with SH blockade. Arrow represents
direction of trace for the time shown. PHA added
where indicated.
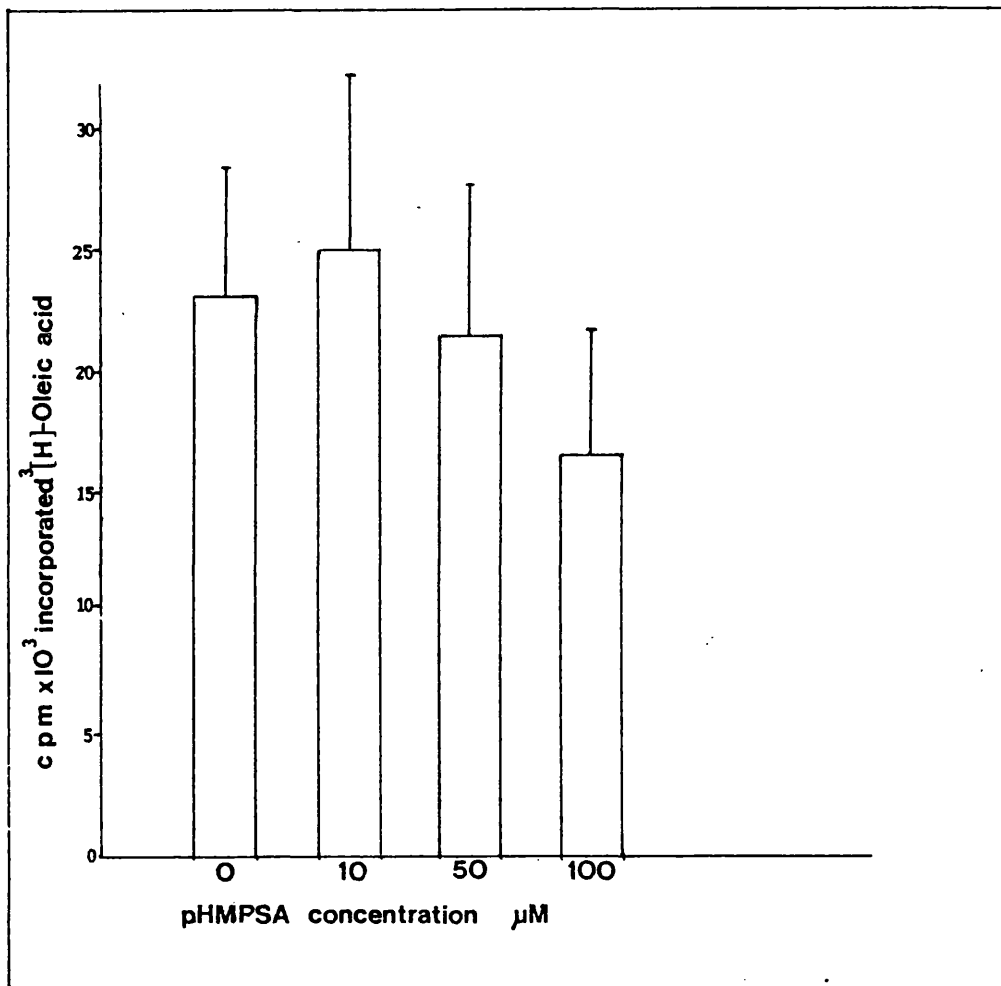
FIG.24. Effect of preincubation with pHMPSA upon
incorporation of ^{3}(H) oleic acid by PBMCs.
Each histogram represents mean $\pm$ 1 s.d.
(n = 5)

at concentrations exceeding 10 μM. At this lower concentration, however, a slight stimulation was observed. In these experiments, the cells were not washed following pre-incubation with pHMPSA. Subsequent experiments, however, in which cells were washed three times in RPMI + 1% fatty acid poor BSA after this pre-incubation was completed, failed to show inhibition by pHMPSA even at 100 μM as shown in Fig. 25. So, the ability of mononuclear cells to take up ^{3}(H)-oleic acid into their membranes by acylation is inhibited by blockade of cell surface SH groups; however, this effect is transient and can be overcome by washing the cells.

4.F. EFFECT OF NDGA UPON PWM-INDUCED IgG SYNTHESIS

There is some evidence to suggest that the lipoxygenase pathway of neutrophils might be SH-dependent (C. Maslen, PhD thesis,1985) since pHMPSA can inhibit the formation of lipoxygenase-derived hydroperoxides. Experiments were carried out to determine if lipoxygenase products e.g. LTB$_4$ are necessary for IgG synthesis by assaying the effect of a known lipoxygenase inhibitor, nor-dihydroguaiaretic acid (NDGA). The results are shown in Fig. 26. NDGA causes inhibition of PWM-induced IgG synthesis in a dose-dependent fashion with an ID$_{50}$ estimated to be about 20 μM. This was not due to a toxic effect since cell viability, as assessed by trypan blue exclusion, was not affected by incubation for 7 days with the highest dose of NDGA used. It is important to note, however, that in this concentration range of NDGA, the compound is thought to have anti-oxidant effects which might be responsible for the observed inhibition. The results provide evidence that products of the lipoxygenase pathway might be involved in regulation of the in vitro immune response.

The previous evidence raised the possibility that blockade of cell surface SH groups impaired PWM-induced IgG synthesis in vitro
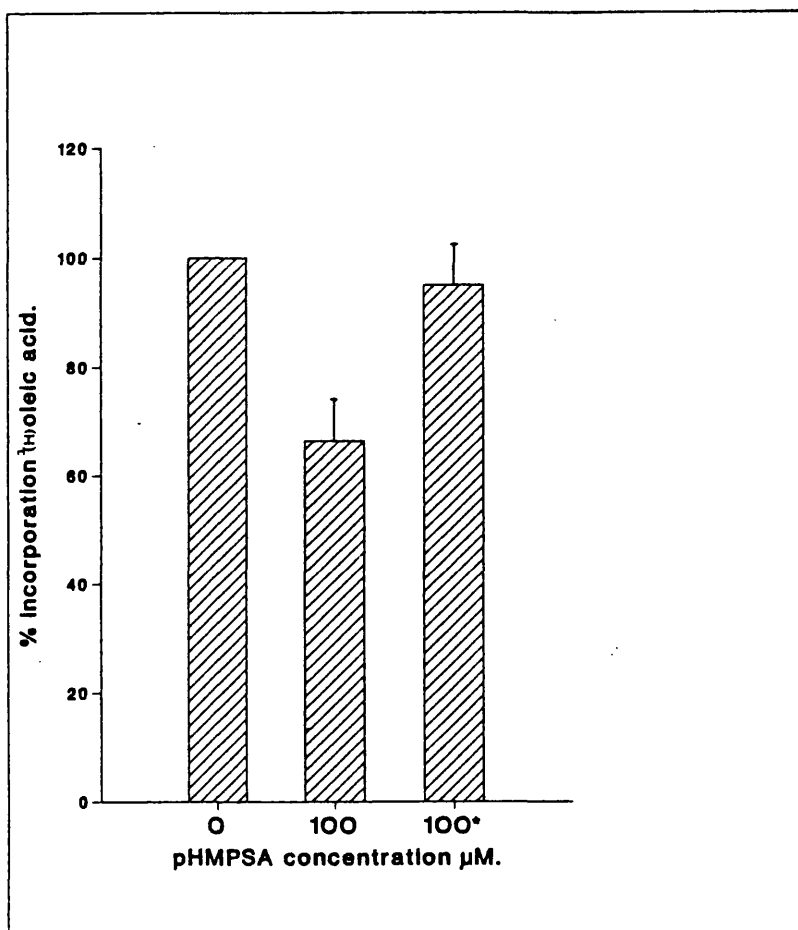
FIG.25. Effect of preincubation with pHMPSA (100 μM) upon the ability of PBMCs to incorporate [3](H) oleic acid. Cells were washed 3 times after preincubation (*) or unwashed (no asterisk). Each histogram represents mean ± l.s.d. (n = 3).
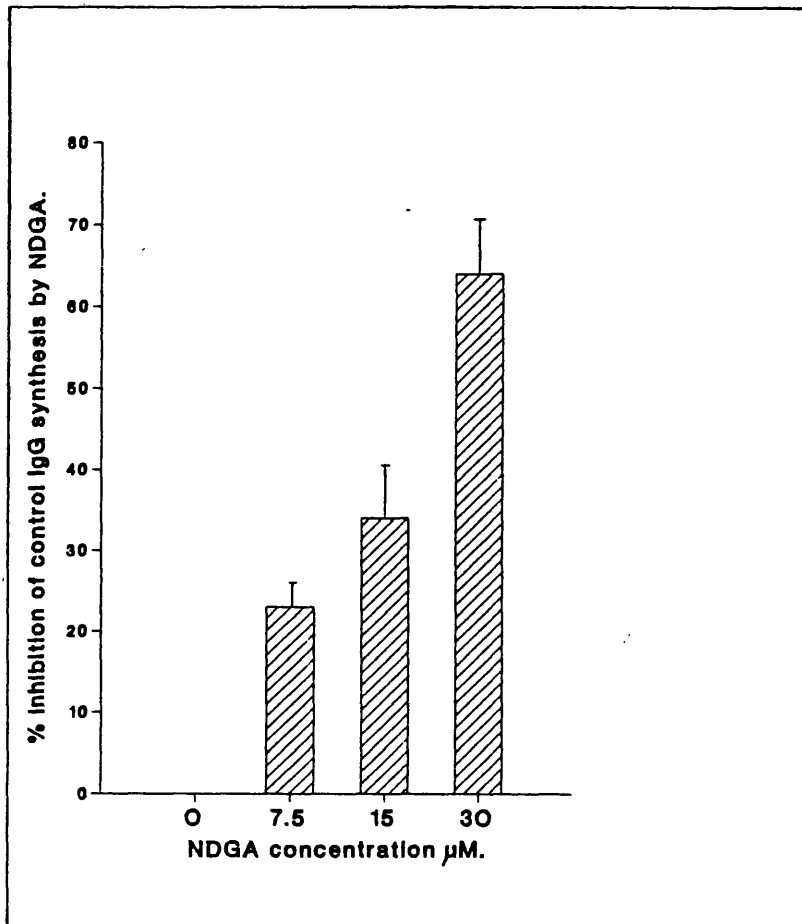
FIG.26.   The effect of Nor-dihydroguaiaretic acid (NDGA) upon

IgG synthesis.   Each histogram represents mean in-

hibition produced by NDGA ($\pm$ l.s.d.) (n = 4). ID_{50}

was estimated to be 20 μM.

by inhibiting the generation of essential lipoxygenase products. This hypothesis was tested by investigating the ability of exogenous leukotriene B_4 (LTB_4) to overcome inhibition of the _in vitro_ immune response caused by SH-blockade of monocytes. Monocytes, preincubated with pHMPSA, were reconstituted with untreated lymphocytes in the presence of 10 nM LTB_4 and PWM-induced IgG synthesis measured by ELISA. The results are shown graphically in Fig. 27 and they clearly show the inability of LTB_4 to overcome the inhibition. No effect upon IgG synthesis by LTB_4 was observed in normal cultures (Fig. 27). In control experiments without PWM, low levels of IgG (<300 ng per million cells per ml.) were produced both in the absence and presence of LTB_4.

4.G. EFFECT OF SH BLOCKADE UPON MONOCYTE OXIDATIVE METABOLISM

In response to various immunological and chemical stimuli, monocytes produce oxygen-derived free radicals. The effect of SH blockade upon monocyte oxidative metabolism and the possible role of this in the cell's accessory function were investigated. Monocyte superoxide anion production in response to heat-aggregated IgG (HAGG) was measured by a ferricytochrome C reduction assay. The results are given in Table 10. They clearly show that SH blockade of monocytes by pHMPSA totally abolished their ability to produce superoxide anion. Other studies by Maslen (1985) have shown H_2O_2 production by monocytes to be similarly affected by pHMPSA with an ID_{50} of about 10 μM. For these reasons, similar experiments to those conducted using exogenous LTB_4 were carried out to see if H_2O_2 was able to reverse pHMPSA-induced inhibition of the immune response. H_2O_2 (concentration range 20 nM to 2000 nM) was added to cultures with untreated monocytes and where monocytes had been blocked by pHMPSA. Figures 28 and 29 show the results respectively from culture in the presence
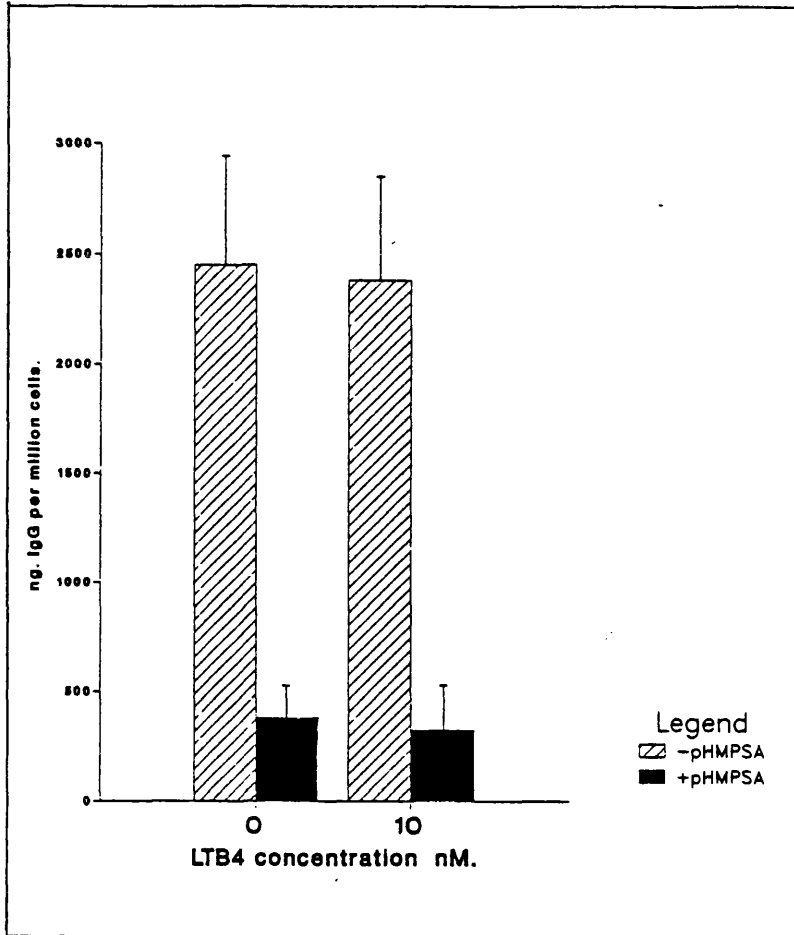
FIG.27. Effect of LTB$_4$ upon IgG synthesis by control PBMCs
with normal monocytes (-pHMPSA) and monocytes with
surface SH groups blocked (+pHMPSA) in the presence
of PWM. Each histogram represents mean IgG production
($\pm$ l.s.d.) (n = 4)

TREATMENT	n moles cyt C reduced/ 2×10^5 cells/30 mins
-pHMPSA	3.30 $\pm$ 0.9
+pHMPSA (50μM)	0.00

TABLE 10.   Effect of pHMPSA upon superoxide production

by monocytes;   (n = 3)
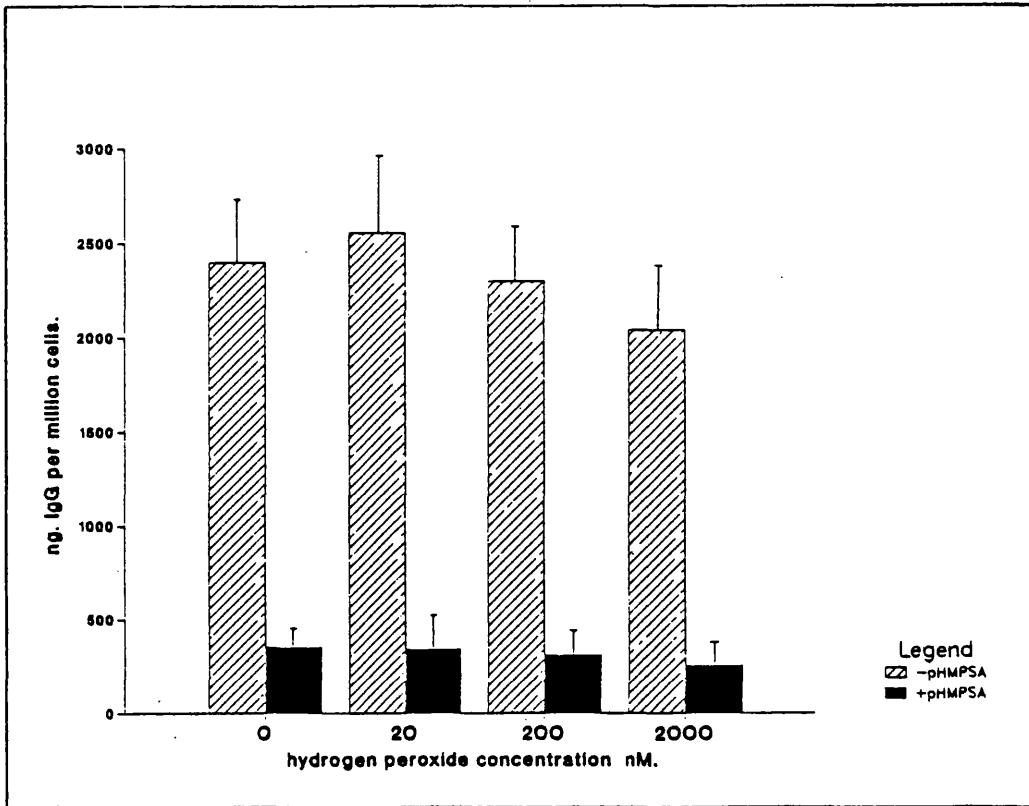
FIG.28. The effect of hydrogen peroxide upon IgG synthesis by
control PBMCs with normal monocytes (-pHMPSA) and
monocytes with surface SH groups blocked (+ pHMPSA)
in the presence of PWM. Each histogram represents
the mean IgG production ($\pm$ l.s.d.) (n = 5)
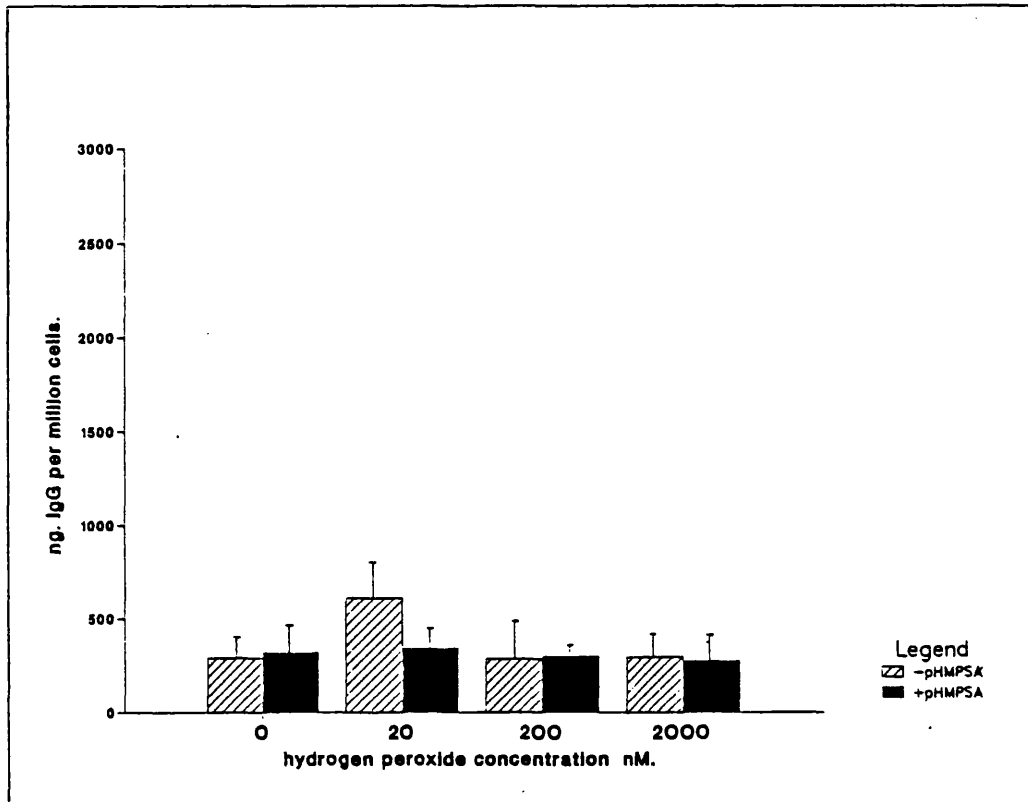
FIG.29. The effect of hydrogen peroxide upon IgG synthesis by
control PBMCs with normal monocytes (-pHMPSA) and
monocytes with surface SH groups blocked (+pHMPSA)
in the absence of PWM. Each histogram represents
the mean IgG production ($\pm$ l.s.d.) (n = 5)

and absence of PWM. The results in Fig. 28 demonstrate that H_2O_2 causes a slight enhancement of the normal PWM response at low concentrations (20 nM) whilst at 2000 nM H_2O_2 itself produces an inhibition of the _in vitro_ immune response. Evidence for the ability of H_2O_2 at low concentrations to be mitogenic itself is provided by cultures performed in the absence of PWM results in Fig. 29 which show elevated IgG production in the presence of 20 nM H_2O_2.

4.H. H_2O_2 PRODUCTION BY MONOCYTES FROM NORMAL SUBJECTS AND R.A.

PATIENTS

Although the results above demonstrated the inability of H_2O_2 to overcome inhibition of IgG synthesis by monocyte SH-blockade, it was decided to assay the production of H_2O_2 by both normal and rheumatoid monocytes since H_2O_2 itself had been shown to have effects on IgG synthesis in the presence and absence of PWM. FMLP (formyl-methionyl-leucyl-phenylalanine) was chosen as the stimulus since it had been previously shown that stimulation of monocytes produces only H_2O_2 and not other peroxides (Maslen, 1985). This observation was verified in these experiments since catalase totally abolished phenol red oxidation. Figs. 30 and 31 show H_2O_2 production (with and without pre-incubation with 5×10^{-5}M 2-ME) by normal monocytes, monocytes from RA patients on NSAIDs and monocytes from RA patients established on D-penicillamine in the absence of any stimulus (Fig. 30) and in the presence of 5×10^{-5}M FMLP (Fig. 31) which preliminary experiments had shown to give maximal H_2O_2 production. Pooled data for these are given in Table 11. Stimulated H_2O_2 production values represent corrected values where unstimulated values have been subtracted from stimulated values. Pre-incubation with 5×10^{-5}M 2-ME did not significantly alter H_2O_2 production in any of the groups in either unstimulated or stimulated conditions (Wilcoxon's Ranking
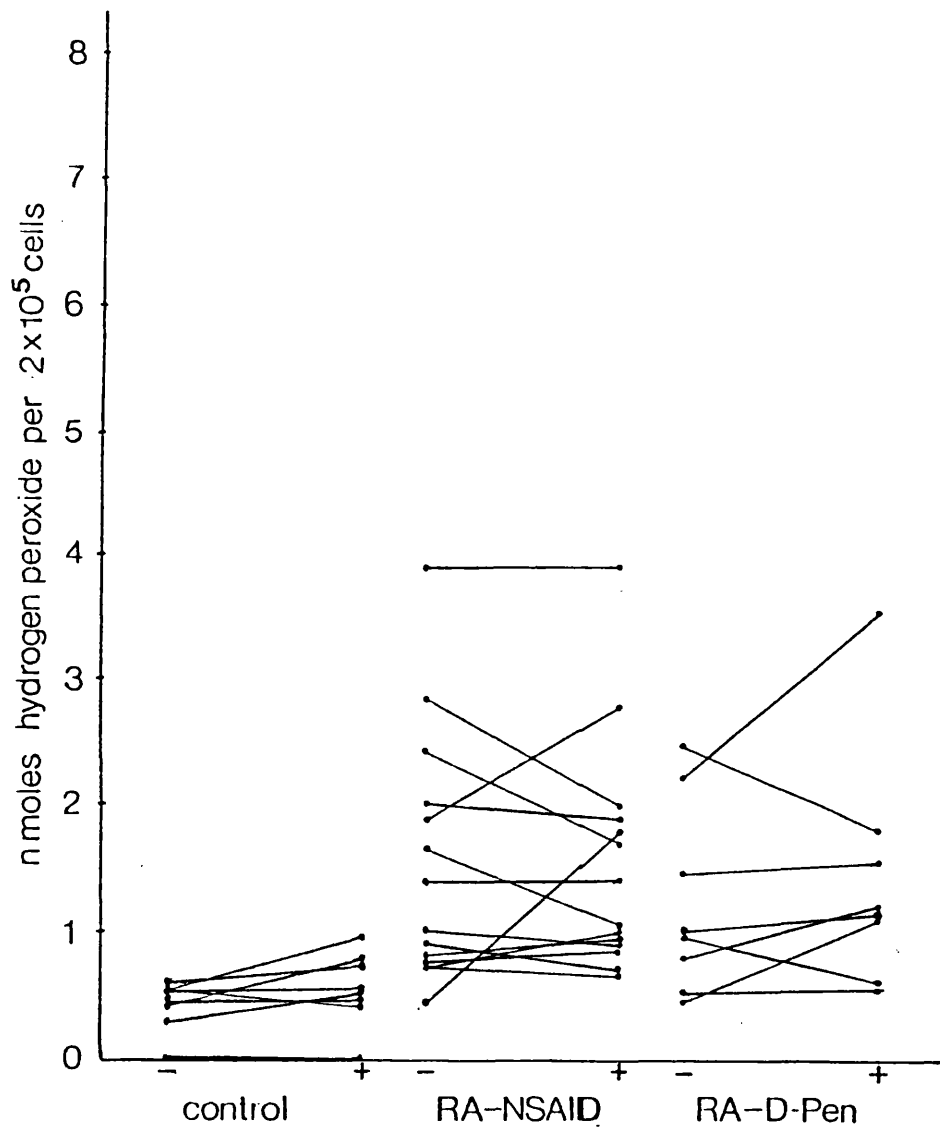
FIG.30. Spontaneous hydrogen peroxide production by monocytes
from normal healthy controls, RA patients on NSAIDs
and RA patients on D-penicillamine.in the presence (+)
and absence (-) of 5×10^{-5} M 2-ME.
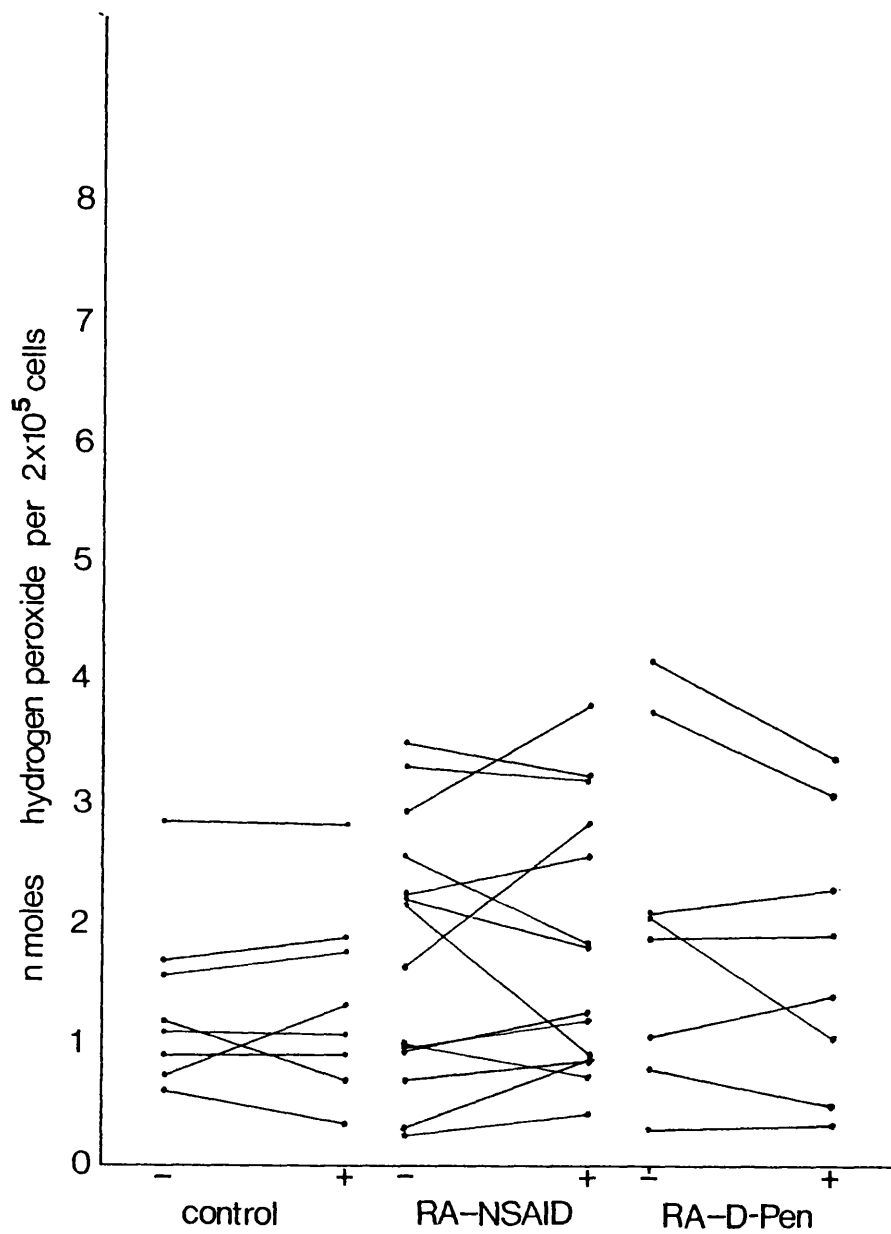
FIG.31. Hydrogen peroxide production by monocytes from normal
healthy controls, RA patients on NSAIDs and RA patients
on D-penicillamine in response to 5×10^{-5}MFMLP (corrected
for unstimulated values) in the presence (+) and absence
(-) of 5×10^{-5} M 2-ME.

GROUP	n moles H_2O_2/ 2 x 10^5 cells			
	UNSTIMULATED median (range)		STIMULATED median (range)	
	-2ME	+2ME	-2ME	+2ME
Controls (n = 8)	0.495 (0.00-0.64)	0.560 (0.00-0.96)	1.135 (0.60-2.82)	1.175 (0.32-2.78)
RA - NSAID (n = 14)	1.210 (0.45-3.90)	1.245 (0.67-3.90)	1.905 (0.25-3.50)	1.515 (0.42-3.81)
RA - D-pen (n = 8)	0.990 (0.45-2.49)	1.185 (0.54-3.52)	1.975 (0.28-4.16)	1.660 (0.32-3.38)

STATISTICAL ANALYSIS

No statastical difference was observed between $\pm$ 2ME for either unstimulated or stimulated monocytes in any group (Wilcoxons Ranking Sum test > 0.5)

s = significant

ns = non-significant

Mann-Whitney U Test

-2ME Unstimulated monocytes

RA - NSAID	vs.	controls	$p < 0.01$	(s)
RA - D-pen	vs.	controls	$p < 0.05$	(s)
RA - D-pen	vs.	RA-NSAID	$p > 0.5$	(ns)

-2ME Stimulated monocytes

RA - NSAID	vs.	controls	$p > 0.05$	(ns)
RA - D-pen	vs.	controls	$p > 0.05$	(ns)
RA - D-pen	vs.	RA-NSAID	$p > 0.5$	(ns)

+2ME Unstimulated monocytes

RA - NSAID	vs.	controls	$p < 0.01$	(s)
RA - D-pen	vs.	controls	$p < 0.05$	(s)
RA - D-pen	vs.	RA-NSAID	$p > 0.5$	(ns)

+2ME Stimulated monocytes

RA - NSAID	vs.	controls	$p > 0.5$	(ns)
RA - D-pen	vs.	controls	$p > 0.5$	(ns)
RA - D-pen	vs.	RA-NSAID	$p > 0.5$	(ns)

TABLE 11. Pooled data and statistical analysis for H_2O_2 production by monocytes from normals, patients upon NSAIDs (RA-NSAID) and patients upon D-penicillamine (RA-D-pen)

Sum Test, p $>$ 0.5). Statistical analysis comparing the different

monocyte groups was performed using the Mann-Whitney U test. Un-

stimulated monocytes from RA patients on NSAIDs (RA-NSAID) and on

D-penicillamine (RA-D-pen) produced significantly higher amounts of

H_2O_2 than healthy monocytes both with and without treatment with 2-ME.

(RA-NSAID vs. controls p$<$0.01; RA-D-pen vs.controls p $<$ 0.05).

There was no significant difference between spontaneous H_2O_2 product-

ion by RA-NSAID and RA-D-pen monocytes either with or without 2-ME

treatment (p$>$0.5). When stimulated with FMLP, both RA-NSAID and

RA-D-pen monocytes produced greater amounts of H_2O_2 than control cells;

however, these differences failed to reach significance either with-

out 2-ME (RA-NSAID vs. controls, p $>$0.05; RA-D-pen. vs. controls,

p$>$0.05), or with 2-ME (RA-NSAID vs. controls, p$>$0.5; RA-D-pen.

vs. controls, p$>$0.5).

Figures 30 and 31 show that there was considerable intersubject

variability in the production of H_2O_2 both spontaneously and upon

stimulation with FMLP with the exception of the unstimulated control

monocyte group. These data also show that the effect of 2-ME varied

quite considerably between different subjects with some showing quite

substantial increases upon 2-ME pre-incubation and others demonstrat-

ing decreased H_2O_2 production.

4.I. EFFECT OF CATALASE UPON IgG PRODUCTION

The finding that RA monocytes produced increased amounts of

H_2O_2 led to experiments to investigate the effect of catalase upon

IgG production. Peripheral blood mononuclear cells from RA patients

on NSAIDs and from control subjects were incubated for 7 days with

and without PWM either in the absence of presence of catalase

(300 Uml^{-1}). The results are shown in Fig. 32.
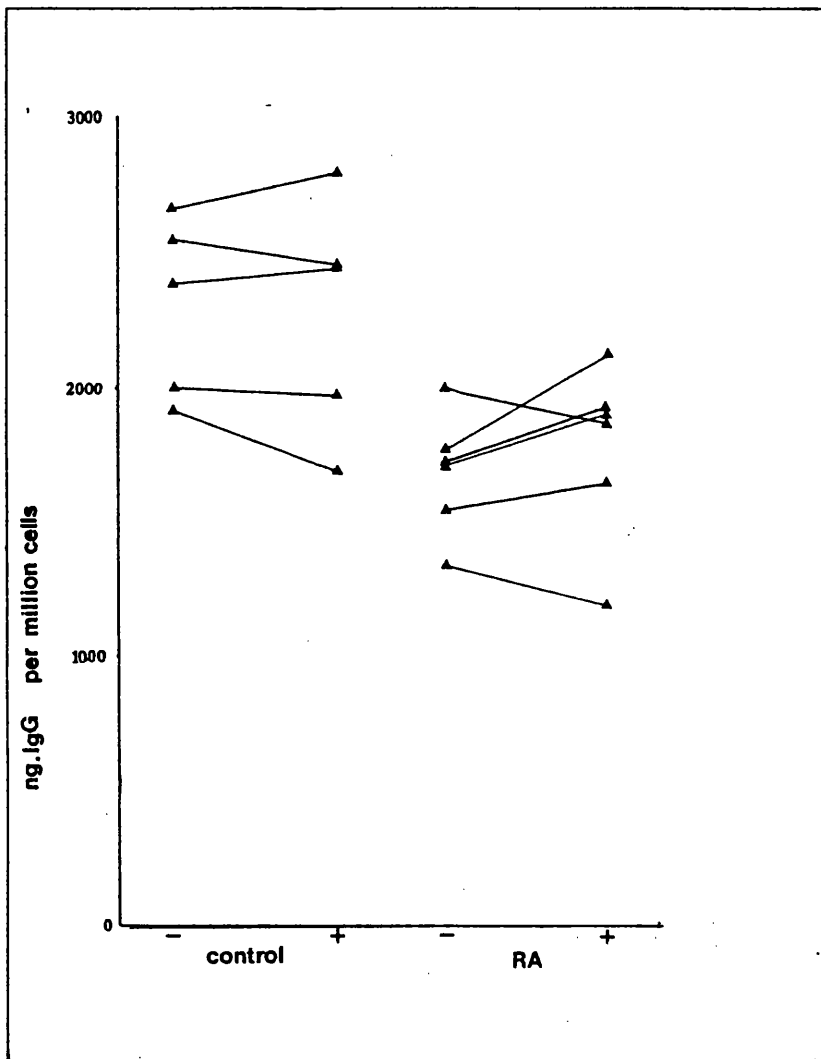
FIG.32.   Effect of adding catalase (300 Uml^{-1}) upon
          IgG synthesis by normal and rheumatoid PBMCs
          upon stimulation with PWM.
          (+) = cultures with catalase
          (-) = cultures without catalase

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Normal cells produced significantly greater amounts of IgG

than rheumatoid cells both with and without catalase (Mann-Whitney

U test, $p < 0.05$). There were no statistically significant

differences in IgG synthesis in either group in the presence of

catalase (Wilcoxons Rank Sum test $p > 0.5$).

Chapter Five

DISCUSSION

5.A.HPLC DEVELOPMENT AND PHARMACOKINETICS OF PENICILLAMINE

The analysis of penicillamine (and its metabolites) in bio-logical fluids has been a complex problem that has presented a tre-mendous challenge to workers in the field for many years. This is because:-

(i)   The penicillamine molecule lacks a chromophore so ruling

out ultra-violet spectrometry for detection.

(ii) It is very reactive i.e.

(a) strongly protein bound

(b) forms disulphides at neutral pH especially in the presence

of trace metals.

(c) forms chelates with metal ions

(d) highly water-soluble and, so, cannot be extracted into

organic solvents.

(e) decomposes when aqueous solutions are concentrated under

vacuum.

Only in recent years have procedures been adopted which have elimin-ated these problems in penicillamine analysis. These have been mainly the use of EDTA to chelate any metal ions present, so prevent-ing oxidation and chelation, and manipulation of the pH to keep the drug in its desired form (Bergstrom et al, 1980).

The analytical methods devised for penicillamine and its meta-bolites to date can be broadly divided into non-chromatographic tech-niques and chromatographic assays.

The non-chromatographic procedures basically comprise spectro-metric assays and radio-immunoassays (RIAs). The former, such as those devised by Pal (1959) and Mann and Mitchell (1979), exhibit low sensitivity and, since they utilise the drug's sulphydryl group, lack specificity because they fail to distinguish between endogenous thiols and the drug. The RIA developed by Assem and Vickers (1974),

whilst specific for penicillamine, is limited in its applications

by the need to produce specific antibodies and relative inavailab-

ility of radiolabelled drug. (For disulphide anlaysis, specific

antibodies and radiolabelled compounds are required). So, non-

chromatographic approaches have serious limitations.

Recently, most procedures for penicillamine analysis have been

of a chromatographic nature. Gas-liquid chromatographic methods have

proved unsuccessful mainly due to complex sample preparation i.e.

extraction procedures followed by derivatision due to the small size

of the molecules involved (Jellum et al, 1969; Kucharchyk and

Shahinan, 1981). A number of methods using amino-acid analysers

or ion-exchange chromatography coupled with ninhydrin detection have

been developed for determination of the drug and its metabolites. How-

ever, their application to routine analysis is limited by the long

analysis times involved which are at least 8 hours per sample (Pur-

die et al, 1968; Perrett et al,1976; Hsuing et al, 1978, Muijsers

et al, 1979).

In the last 1970s, Rabenstein and Saetre developed a HPLC- el-

ectro-chemical detection method which analysed D-penicillamine by

use of a mercury-based electrode specific for SH-containing compounds

(Rabenstein and Saetre, 1977; Saetre and Rabenstein, 1978). The

method utilised non-commerical custom-made detectors which proved to

be a drawback. However, it has provided the basis for a number of

subsequent techniques developed using the sort of detection (Berg-

strom, Kay and Wagner, 1981; Kreuzig and Frank, 1981). The methods

have been used to measure reduced penicillamine in bilogical fluids.

In this study, total plasma D-penicillamine was determined for the

following reasons. The active form of D-penicillamine in RA remains

uncertain. It appears that the vast majority of D-penicillamine in

the plasma is protein bound, mostly to albumin by means of covalent disulphide bonds (Planas-Bohne, 1981; Perrett, 1981). The ability of D-penicillamine to undergo thiol-disulphide exchange reactions means this protein-bound D-penicillamine is potentially available for its pharmacological action. Thus, it was considered appropriate to perform pharmacokinetic studies determining total levels of the drug in the plasma. The method devised in this project, as the results show, is sensitive and very reproducible. Moreover, it is fairly simple to operate and permits throughput of at least 9 samples in duplicate plus the necessary standards in the average working day due to the rapid analysis time, thus, rendering it suitable for pharmacokinetic studies such as those undertaken in this project.

Previous studies have indicated that the absorption of D-penicillamine is reduced when taken with food by about 20% in rats (Planas-Bohne, 1972) and about a third in man (Perrett, 1981). Both these studies employed indirect methods to measure the drug, the former using radio-labelled drug and the latter by measuring urinary metabolites only. The study in this thesis, using HPLC to determine plasma drug concentrations, has shown that the drug's bioavailability is significantly reduced following a light meal in agreement with the above studies. Other workers have produced similar results. Muijsers et al (1984) measured the bioavailability of total D-penicillamine similar to this study whilst some studies have measured free (reduced) drug. (Bergstrom et al, 1981; Schuna et al, 1983). Only one study has suggested that food has no effect upon penicillamine absorption (Bucknall, Rabenstein and Ng. 1982); however, this was only a single case study and, perhaps, in the light of the weight of opposing evidence ought not to be considered the normal state of affairs.

Since the time to attain maximum plasma drug concentrations in fasting and non-fasting conditions was essential the same in this investigation, this suggests that food does not inhibit the intrinsic absorption of penicillamine, but, more likely, removes the drug from the site of absorption. This conclusion was also drawn by Schuna and co-workers (1983). This is readily explained by the known chemical reactions in which penicillamine can participate e.g. chelation of metal ions, protein binding and oxidation to disulphides catalysed by metal ions. Perfusion work by Perrett (1981) suggests that formation of the internal disulphide by penicillamine during its passage through the gastro-intestinal tract would result in little absorption of the drug. The only major penicillamine compound found in the faeces is the internal disulphide (Perrett, 1981). Muijsers et al (1984) have demonstrated that simultaneous administration of oral iron preparations with D-penicillamine produces an even more dramatic reduction in absorption of the drug. Indirect evidence for this also comes from Lyle and colleagues (1977) who showed D-penicillamine-induced cupruresis was inhibited by oral iron, and by Hall et al (1981) who demonstrated a similar effect upon D-penicillamine-induced serum thiol reactivity. These studies suggest that either iron-chelation by penicillamine or iron-catalysed penicillamine disulphide formation are probably responsible for reduced D-penicillamine absorption with oral iron.

Chronic administration of the drug with food is likely to result in lower plasma concentrations at steady state compared with that obtained when taken in the fasting state. Hill (1979) has reported that patients receiving penicillamine with meals had a lower incidence of side effects and withdrawals due to adverse reactions than patients taking the drug without food. In both cases, clinical

efficacy was equivalent. This might suggest that patients who received the drug whilst fasting attained higher plasma drug concentrations which perhaps exceeded a "toxicity threshold" i.e. perhaps plasma levels may contribute to penicillamine toxicity. Unfortunately, no monitoring of drug levels was performed in this study presumably due to the lack of an appropriate analytical method.

Many RA patients take iron supplements for the anaemia seen in the disease; in these patients simultaneous administration of such preparations with penicillamine may result in plasma drug concentrations insufficient to produce a clinical effect. Thus, oral iron preparations should not be given to RA patients taking penicillamine or, at least, not given at the same time.

The pharmacokinetics of penicillamine in both normal volunteers and RA patients have been studied. The observation that peak plasma concentrations occur at about 2 hours agrees well with the results obtained by other workers (Patzschke et al, 1977; Saetre and Rabenstein, 1978; Wiesner et al, 1981; Bergstrom et al, 1981 Butler et al, 1982; Kukovetz et al, 1983; Muijsers et al, 1984). A number of pharmacokinetic studies by these workers have measured free, reduced D-penicillamine only (Saetre and Rabenstein, 1978, Wiesner et al, 1981; Bergstrom et al, 1981; Butler et al, 1982; Kukovetz et al, 1983; Schuna et al, 1983). In this study, total plasma D-penicillamine concentrations have been determined for the reasons previously expounded. One other group of workers have also directly measured total D-penicllamine in the plasma (Muijsers et al, 1979). In their studies they have shown that penicillamine exhibits a biphasic decay in the plasma with values of 1.6 - 3 hours for the rapid elimination phase followed by a slower decline of about 4 - 6 days (Muijsers et al, 1979; Vander Korst et al, 1981;

Muijsers et al, 1984). Similar results were found in a study

using ^{14}C-labelled D-penicillamine (Patzsche et al, 1977). The

values obtained by these workers do not vary greatly from those ob-

tained in this study. Although the slow elimination phase reported

here is shorter than those in the previously mentioned studies, the

value found here, as outlined in the results section, can be criti-

cised and may well be shorter than the actual value.

Those workers measuring free, reduced penicillamine have also

found rapid initial half-lives for D-penicillamine varying from

1 to 5 hours (Russell et al, 1979, Bergstrom et al, 1981, Wiesner

et al, 1981; Butler et al, 1982; Kukovetz et al, 1983). In these

studies, no slow elimination phase was reported.

In those studies where total D-penicillamine was measured,

including this one, the observation of two half-lives suggests the

presence of at least 2 pools, one rapid pool with a half-life of a

few hours and a slow pool with a half-life in the order of several

days. This slow pool may be due to sequestering of the drug in tis-

sues from which the plasma pool is constantly refilled. The initial

half-life observed for free-drug is similar to total drug although

perhaps slightly more rapid. This may be because this half-life

is probably the oxidation of the free reduced drug as opposed to

actual loss from the plasma compartment.

The observed peak plasma concentrations showed considerable

intersubject variability. This has been found in most of the other

studies whether measuring total or free drug. Van der Korst et al,

(1981) measuring total D-penicillamine found a peak plasma drug

level of about 5 μgml^{-1} for an oral dose of 250 mg. whilst for a

dose of 500 mg. it was approximately 10 μgml^{-1}. When compared to

the values obtained in this work of 2.3 μgml^{-1} for a 125 mg. dose

(RA patients) and about 8 μgml^{-1} for 500 mg. (volunteers, fasting state), the two sets of data seem quite compatible. Other studies measuring free penicillamine have shown peak plasma levels lower than these as expected. Wiesner et al, (1981) found that an 800 mg. oral dose gave a peak value of about 4 μgml^{-1} in normal volunteers. Kukovetz and associates (1893) found an average peak concentration in plasma of 0.9 μgml^{-1} for 250 mg. penicillamine whilst Schuna et al, (1983) reported a value of 3 μgml^{-1} for a 500 mg. oral dose in normal subjects. Bergstrom et al (1981) found peak levels ranging between 3 μgml^{-1} and 5 μgml^{-1} for a 500 mg. oral dose in 5 healthy subjects. A two subject study by Russell and colleagues (1979) found values of 0.5 μgml^{-1} for 250 mg. and 2 μgml^{-1} for 750 mg. penicillamine.

Taken altogether, these results indicate that free penicillamine in plasma constitutes between 10% and 50% of the total drug when administered initially with a mean value of about 25%. In his review on penicillamine metabolism, Perrett (1981) has suggested that free penicillamine constitutes about 6% of the total plasma penicillamine. This figure is derived mainly from studies measuring drug levels in RA patients and cystinurics who were stabilised on the drug and not receiving it for the first time. The inherent reactivity of penicillamine may explain this apparent discrepancy i.e. as the drug initially enters the plasma, a considerable amount of it i.e. about 25% remains in the free form, but, as more drug enters the plasma and the longer it remains there, more of it oxidises causing a drop in the level of free drug to a steady basal level e.g. 6%.

Another explanation is also possible. In the studies measuring free drug, acid precipitation was used to obtain a non-protein

fraction in which to measure free-drug. This assumes that penicillamine is protein-bound by means of covalent disulphide bridges unaffected by acid treatment. However, if some drug is ionically bonded to proteins, such treatment would release the drug and keep it in the reduced form due to the acidic pH, so giving falsely higher values for free drug. Perhaps, a more preferable way to obtain a non-protein fraction is by ultrafiltration. Indeed, when this method was employed by Abounassif and Jefferies (1983) to measure free drug,very low levels of free drug were found (<1%). It should prove interesting to compare values for free drug from the same samples obtained by ultrafiltration and acid precipitation to see if differences exist.

The identity of the pharmacologically active form of D-penicillamine is still unknown although most authors suggest either the free thiol form or a copper-penicillamine complex is responsible for its action in RA. The reported levels for free drug are low even in patients stabilised on high doses e.g. Brooks et al, (1984) found a mean value of 3.7 μgml^{-1} free D-penicillamine in patients taking 750 mg. after one year. It seems unlikely that such low concentrations can provide the therapeutic effects of the drug. The ability of D-penicillamine to participate in thiol-disulphide exchanges suggests that plasma protein-bound drug is potentially available for its pharmacological actions. The disulphides that D-penicillamine forms with itself and cysteine etc. may be active forms per se although there is no evidence to support this idea.

Both this study and those by Muijsers' group (Muijsers et al, 1979; Van der Korst et al,1981; Muijsers et al, 1984) have shown the existence of two pools of D-penicillamine. This slow pool does not reflect binding to plasma albumin since total D-penicillamine

was measured, but, is probably due to sequestration in tissues perhaps by protein binding. Since this is reversible then even this tissue pool is available for pharmacological actions. Muijsers and colleagues have shown that the deep pool is not easily saturated and suggest that, perhaps, therapeutic benefit is derived upon saturation of this pool - this would account for slow onset of clinical effect of the drug. There is no direct evidence that D-penicillamine accumulates in the connective tissues in humans although Planas-Bohne (1981) has shown that in rats, D-penicillamine is taken up by tissues especially of those with high collagen contents.

The method devised in this project has been used to investigate the initial pharmacokinetics of D-penicillamine in both normal volunteers and RA patients. Work is currently in progress using the method to follow up the total D-penicillamine plasma concentrations of RA patients to steady state kinetics to see if a correlation exists between plasma levels of the drug and its clinical efficacy/toxicity. Table 12 (courtesy of Dr. L.J. Notarianni) shows total D-penicillamine plasma concentrations at steady state. Patients whose dose was increased at intervals of less than 7 days did not achieve steady state conditions on a particular dose (Notarianni, personal communication). This observation is compatible with the calculated slow half-life from the initial pharmacokinetic study presented in this thesis since it is generally accepted that steady-state conditions are achieved after 5 terminal half-lives.

Thus, clinicians who prescribe D-penicillamine for RA patients should wait at least 7 days and probably much longer before increasing the prescribed dose in order to see if toxic side effects are manifest at a particular dose. Clinical efficacy and observed toxicity are now being related to these steady-state drug levels. Two

Daily Dose D-Penicillamine	Plasma Concentration at SS (Range) μg/ml
125 mg.	5.12 ± 0.24 (1.72 - 8.44) n=53
250 mg.	8.55 ± 0.59 (5.41 - 14.10) n=24
375 mg.	9.89 ± 0.10 (5.28 - 16.41) n=10
500 mg.	12.94 ± 1.18 (8.60 - 17.17) n= 7

TABLE 12 .   Total D-Penicillamine Plasma Concentrations at Steady State (n values represent number of observations at each dose)

very recent studies, one measuring free drug (Brooks et al, 1984) and one measuring total drug (Muijsers et al, 1984) have failed to find any correlation between D-penicillamine levels and efficacy/ toxicity. This would seem to exclude the possibility that lack of clinical response is due to defective intestinal absorption of the drug. Both these studies emphasised that some of the most common side effects observed with D-penicillamine therapy, proteinuria and thrombocytopenia, were not connected to high drug levels. An earlier preliminary observation by Van der Korst et al, (1981) showing that a correlation existed between serum cysteine depletion and D-penicillamine induced side effects has been made marginal by their latest study of a larger group of patients (Muijsers et al, 1984).

It is highly desirable to obtain assays for metabolites of D-penicillamine (see Fig.33). Such assays would permit full metabolic profiles for RA patients to be determined and correlation studies may shed light on the mechanisms by which D-penicillamine exerts its clinical benefit and produces side effects e.g. patients who respond to D-penicillamine may show elevated levels of cysteine–D-penicillamine disulphide suggesting perhaps that this metabolite is an active form. The only truly "metabolised" form of D-penicillamine, as yet identified, is the S-methyl derivative which has been detected in the urine of cystinurics taking D-penicllamine (Perrett, Sneddon and Stephens, 1976). The site of methylation is thought to be the liver (Perrett, 1981). No satisfactory analytical procedure has been developed to measure this metabolite although its presence in plasma has been detected (Muijsers et al, 1979).

A recent study has tested the ability of RA patients on comparable doses of D-penicillamine to oxidise S-carboxymethyl
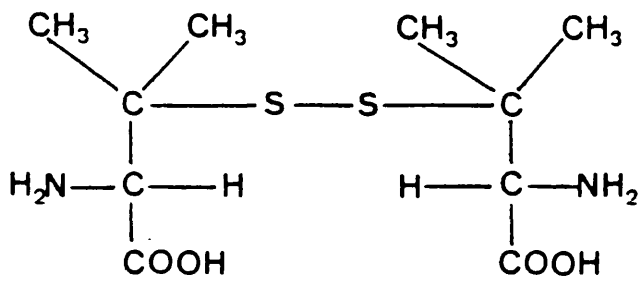
PENICILLAMINE
DISULPHIDE
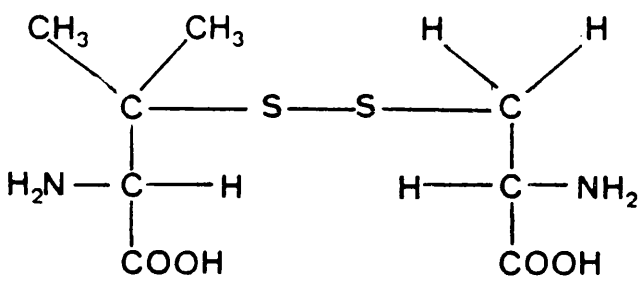
CYSTEINE-
PENICILLAMINE
DISULPHIDE
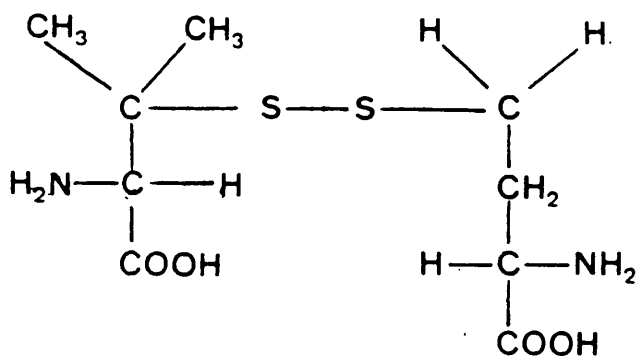
HOMOCYSTEINE-
PENICILLAMINE
DISULPHIDE
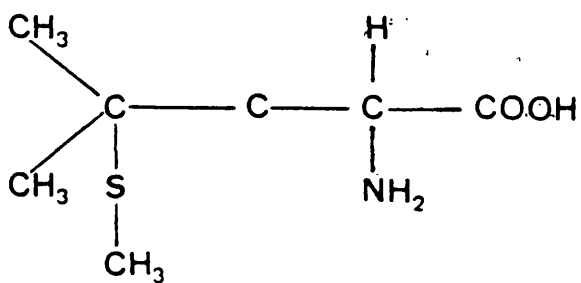
S-METHYL D-
PENICILLAMINE

FIG.33.   Chemical structures of known penicillamine
metabolites.

L-cysteine (carbocysteine). (Emery et al, 1984). Patients were classified as either poor "sulphoxidisers" or extensive "sulphoxidisers". There was a significant association betwen impaired sulphoxidation and the manifestation of toxic side effects to D-penicillamine. Although these workers point out the structural similarity between D-penicillamine and carbocysteine, the similarity between the latter and S-methyl D-penicillamine is more marked. If this metabolite is responsible for toxicity, patients unable to oxidise it may produce side effects. Should this be true, then increased levels of S-methyl D-penicillamine in the plasma might usefully predict the development of toxicity as, indeed, would classification of a patient's sulphoxidation status.

Patients with Wilson's disease taking higher doses of penicillamine than RA patients do not show a significantly enhanced incidence of toxicity. Since the majority of D-penicillamine presumably forms the copper chelate in Wilson's disease, it might suggest that this form of the drug does not contribute greatly, if at all, to the toxic side effects caused by the drug. One study, however, has shown that copper supplements given to RA patients are not prophalactic against toxicity (Andrews et al, 1973).

Finally, many studies attempting to elucidate the mechanism(s) by which D-penicillamine acts in RA have used in vitro model systems, particularly investigations on the immunological effects of the drug. A considerable number of these have reported effects with D-penicillamine at concentrations which, as these results and others show, are far in excess of those attained in vivo and extreme caution is warranted in the interpretation of such results.

5.B.   SH-DEPENDENT REACTIONS OF MONOCYTES

McKeown et al (1984) have shown that the defective accessory function exhibited by rheumatoid monocytes when compared to normal monocytes is corrected in vitro by the addition of 2-ME whilst in vivo a similar improvement is achieved by successful treatment with D-penicillamine. These results suggest that the defect may be due to cell surface SH-oxidation. Attempts to find a reversible SH blocking agent to mimic the situation with rheumatoid monocytes, in order to study SH-dependent functions relevant to accessory function as possible targets for D-penicillamine therapy, proved unsuccessful. DTNB failed to produce significant inhibition upon pre-incubation with PBMCs. Although this contrasts with the observations of Chaplin and Wedner,(1978), these workers added the compound directly to culture. Indeed, an additional experiment performed in which DTNB was directly added produced total inhibition of PBMC proliferation at 1000 μM (data not shown). The possibility that these differences could be explained by prevention of lectin binding by DTNB itself cannot be ruled out. However, Chaplin and Wedner have shown that another inhibitory SH-blocking agent, diamide, does not inhibit lectin-binding. Other conceivable explanations include reversal of DTNB binding to the cells during the pre-incubation period before the addition of lectin.

Oxidised glutathione has been reported to inhibit mitogen-induced PBMC proliferation upon direct addition to culture due to the oxidation of cell surface SH groups (Johnson, 1980). However, as with DTNB no marked inhibition was seen with this reagent. Again, reversal of glutathione binding might account for this result.

D-penicillamine can act synergistically with copper ions

to inhibit both mitogen-stimulated mononuclear cell proliferation

and PWM-induced IgG synthesis in vitro as shown in this study and

those by Lipsky (1981, 1984). Lipsky (1984) has proposed that this

inhibition is due to suppression of T-helper lymphocyte function

by hydrogen peroxide generated by the interaction of D-penicillamine

with the Cu^{2+}. However, Cu^{2+} ions are potent catalysts of di-

sulphide formation raising the possibility that Cu^{2+} catalysed di-

sulphide formation between D-penicillamine and cell-surface SH

groups may be responsible for the inhibition. This was the rationale

behind the experiments performed here to use D-penicillamine/Cu^{2+}

as a potential reversible SH-oxidising agent. No reversal of the

inhibition of PWM-induced IgG synthesis was achieved with 2-ME

perhaps suggesting that disulphide formation is not the underlying

mechanism behind the inhibition. Thus, D-penicillamine/Cu^{2+} was

not suitable for use as a reversible SH-blocking agent.

A number of different workers have claimed D-penicillamine is

immunosuppressive in RA. This results from experiments showing

inhibition of the in vitro immune response either with copper (Lipsky,

1981; 1984) or without copper (Room et al,1979; Lewins et al,1982).

However, the concentrations found to be inhibitory have been gener-

ally far in excess of those attained in vivo. In this study, marked

inhibition of PWM-induced IgG synthesis was achieved at high con-

centrations (100 μgml^{-1} and 250 μgml^{-1}) only. Pharmacokinetic

studies measuring total drug show drug levels only approaching

20 μgml^{-1} after months of treatment (Muijsers et al, 1979; Muijsers

et al,1984; Notarianni and Ferrie, personal communication using

the method developed here). Further still, it is important to note

that D-penicillamine is highly protein bound in vivo (> 80%) and

if free drug only is available for pharmacological purposes, then the drug concentrations used to produce in vitro inhibition are totally unrealistic when compared to the situation in vivo. The ability of D-penicillamine to generate hydrogen peroxide in vitro in synergy with Cu^{2+} ions is apparent from the results presented here. (fig.20). This occurs at all concentrations with a peak occurring at 100 μgml^{-1}. At 250 μgml^{-1}, there is a drop in apparent H_2O_2 production but one reason for this may be actual scavenging of H_2O_2 by possibly an association with D-penicillamine Cu^{2+} ions. A similar scavenging of H_2O_2 by D-penicillamine has been demonstrated by Staite and colleagues (1984). The relevance of such scavenging to the mechanism of D-penicillamine in RA is called into doubt by the high concentrations needed to achieve such an effect. However, the relatively poor sensitivity of the assay may not monitor scavenging effects at lower concentrations of drug. Also, D-penicillamine (with copper) may act as a superoxide dismutase which may be relevant to its action in RA.

The failure to find a reversible SH-blocking agent led to the use of the irreversible blocking agent, para-hydroxymercuri-phenyl-sulphonic acid (pHMPSA) which was found to cause marked inhibition of PWM-induced IgG synthesis upon pre-incubation with the cells in agreement with the results of McKeown et al (1984a).

The immune response to most, if not all antigens requires processing and presentation of the antigens by specialised antigen-presenting cells (APCs) which in peripheral blood mononuclear cell cultures are probably the monocytes. The PWM response in vitro is absolutely dependent upon the presence of monocytes which suggests that PWM is probably presented by these cells in a fashion akin to antigens. Presentation of antigen to reactive T-lymphocytes by APCs occurs in association with antigenic protein determinants

encoded by genes in the HLA-D region of the major histocompati-

bility complex in man (Moller,1978). Antigen presentation appears

to involve an actual physical interaction between monocytes/macro-

phages and lymphocytes resulting in the formation of clusters in

which several lymphocytes are attached to single monocytes in man

(Werdelin et al, 1974; Powell et al, 1980). The finding that mono-

cytes and lymphocytes both require free cell surface SH groups for

an effective immune response in vitro (McKeown et al, 1984a) and

that D-penicillamine in association with copper apparently inhibits

PWM-induced cluster formation (Hawkes and Kennedy,1984) led to spec-

ulation that antigen presentation and, hence, cluster formation might

involve cell surface SH groups on both types of cell e.g. by for-

mation of disulphide bridges between them. However, the results

presented in fig.22 clearly show that pHMPSA failed to inhibit clust-

er formation thereby excluding this hypothesis.

Maintenance of free surface SH groups during early stages of

culture is essential for cellular activation in response to lectins

(Chaplin and Wedner, 1978). The biochemical events occurring at the

membrane upon stimulation appear to be complex and their precise

nature is unclear. Among the early events likely to be involved are

Ca^{2+} ion mobilisation, phospholipase activation and subsequent

arachidonic acid metabolism, and oxidative metabolism.

Ca^{2+} ion mobilisation within the cytoplasm appears to be

important in the activation of many cell types, for example neutro-

phils (Smolen et al, 1982). The results in this study show clearly

that free surface SH groups are not necessary for Ca^{2+} mobilisation

in response to PHA in mononuclear cells. It is unclear whether

the increase in free cytoplasmic Ca^{2+} concentration occurs as a

result of fluxes across the membrane from the outside or from mobil-

isation of accumulated intracellular Ca^{2+} ion stores. Should the latter be the case, then, the observation that free surface SH groups are not necessary is perhaps not surprising.

The role of arachidonic acid derived metabolites as regulators of the immune response is uncertain; however, a number of studies have demonstrated that PGE_2, for example, can be immunomodulatory at least in vitro (Goodwin et al, 1977; Rappaport and Dodge, 1982; Jellinek et al, 1985). Corrigall and Panayi (1979) have shown that the depressed proliferative response exhibited by rheumatoid mono-nuclear cells in vitro could be partially restored by the addition of indomethacin to the cultures. This suggests that excessive PGE_2 production by monocytes may be responsible for the defective in vitro mononuclear cell function. Such a situation has been observed with murine cells (Metzger et al, 1980). The most direct approach to studying the sulphydryl-dependence of cyclo oxygenase activity would have been to assay prostaglandin production by monocytes with and without pHMPSA treatment. However, a number of practical cons-traints precluded such a study. Prostaglandin production requires the liberation of arachidonic acid from membrane phospholipids by action of lipase enzymes (Irvine, 1982). The mechanisms of arach-idonate release and its regulation remain contentious (Irvine, 1982). Kroner et al (1981), from studies on bone marrow macrophages, have suggested that reacylation of liberated fatty acid back into the membrane lipid pool is an important control step in regulating free arachidonate levels. In that study, they demonstrated that para-chloromercuribenzoate (pCMB), a compound the same as pHMPSA in solution, inhibited the reacylation capacity suggesting perhaps that the acyl-transferase responsible is sulphydryl dependent. In this study the SH-dependence of acyltransferase enzymes was exam-

ined by assaying the uptake of ^{3}(H)-oleic acid. pHMPSA treatment

was found to inhibit acylation in agreement with Kroner et al (1981).

Furthermore, as with that study, the observed inhibition was trans-

ient and could be totally abrogated by thorough washing of the

cells. The reason for this transient effect is not immediately

clear. The acyltransferase is dependent upon co-enzyme A. Thus

if the inhibition is due to its interaction with co-enzyme's free

SH group, turnover of co-enzyme during washing might cancel out the

inhibition. It may be that control of levels of free arachidonic

acid might be aberrant in rheumatoid monocytes in vivo where pres-

umably there is continual SH-oxidation. This could lead to in-

creased cyclo-oxygenase and lipoxygenase activity and consequently

perturbed immunoregulation. Such a hypothesis to explain depressed

in vitro immune responses by rheumatoid PBMCs, however, is ruled

out by the inability of washing alone to alleviate defective rheum-

atoid cell function. In vivo, the actions of NSAIDs should effect-

ively inhibit cyclo-oxygenase activity which would seem to exclude

a role for increased prostaglandin production in altered immuno-

regulation in rheumatoid patients. The effect of NSAIDs upon cyclo-

oxygenase in rheumatoid cells would not be apparent in in vitro

experiments since washing of the cells probably removes the drug.

Experiments to assay the SH-dependence of phospholipase A_2 activity

per se were attempted but suffered from poor reproducibility making

it impossible to draw any firm conclusions; however, Kroner and

colleagues (1981) have demonstrated that in bone marrow macrophages,

at least, phospholipase A_2 activity was unaffected by pCMB treat-

ment.

Little is known about the role of lipoxygenase products in

immunoregulation. Kelly et al (1979) demonstrated the ability of

nor-dihydroguaiaretic acid (NDGA) and eicosatetraynoic acid

(ETYA) to inhibit lectin-stimulated lymphocyte proliferation. These

compounds are inhibitors of the lipoxygenase pathway suggesting

that products of that pathway might be involved in production of

proliferation. It is important to note that the inhibitory effects

of NDGA and ETYA might also be mediated by their ability to inhibit

thromboxane synthesis. However, the possible role of thromboxanes

as immunoregulators is very poorly defined. Moreover, Kelly and

colelagues have reported that inhibition of mitogenesis is more sens-

itive than thromboxane synthesis inhibition. The results in fig.26

show that NDGA can inhibit IgG synthesis again suggesting the im-

portance of lipoxygenase products in immunoregulation. Caution is

indicated in interpreting these results since not only can NDGA

inhibit thromboxane production but at the concentration used in this

study it may also have anti-oxidant activity. ETYA was not used

in these experiments due to solubility problems. Maslen (1985) has

shown that the lipoxygenase pathway might be sulphydryl dependent

since pHMPSA blocks the production of lipoxygenase-derived perox-

ide(s). LTB_4, the most stable lipoxygenase product, failed to re-

store the ability to synthesise IgG upon addition to cultures in

which monocytes had been treated with pHMPSA. Although the inability

to reverse inhibition might rule out the possibility that SH-block-

ade inhibits the immune response by affecting lipoxygenase activ-

ity, it may be that other lipoxygenase products might achieve re-

versal of the inhibition. Alternatively, other concentrations of

LTB_4 might have been effective. Problems with obtaining adequate

numbers of cells prevented use of more LTB_4 concentrations Lipoxy-

genase activity in rheumatoid monocytes has not been measured and

so it is unclear whether an abnormality exists which might con-

tribute to the aberrant in vitro immune response

As previously stated, NDGA inhibition of IgG synthesis might be due to its anti-oxidant activity. The contribution of oxygen derived radicals produced by monocytes to their interaction with lymphocytes is contentious. Novogrodsky et al (1982) have shown that hydroxyl radical scavengers inhibit lymphocyte mitogenesis particularly when induced by phorbol myristate acetate (PMA), a monocyte-dependent mitogen. Gallagher and Curtis (1984) have shown that superoxide can enhance mitogenesis produced by some stimuli and can be mitogenic per se. Hydrogen peroxide has been shown to have both inhibitory and stimulatory effects upon mononuclear cell responses. Metzger and colleagues (1980) demonstrated the ability of macrophage derived H_2O_2 and PGE_2 to suppress murine cell proliferation. Lipsky (1984) has postulated that the immunosuppression exerted by D-penicillamine with copper ions in vitro is due to H_2O_2 suppression of T-lymphocyte activity. In contrast, Finazzo-Agro et al (1980) have shown H_2O_2 can be mitogenic itself at low concentrations ($<$ 50 nM) in agreement with the results produced in this study (fig.29). Similarly,Finazzi-Agro and colleagues showed that higher concentrations of H_2O_2 ($>$500 nM) are inhibitory again agreeing with the results here.

The possibility that enhanced H_2O_2 production by rheumatoid monocytes was responsible for depressed in vitro IgG synthesis was investigated by the addition of catalase to the cell cultures. Whilst catalase enhanced IgG synthesis in more patients than normal subjects, this was not significant. Unfortunately, no parallel experiments were performed measuring the monocyte H_2O_2 production. Panayi (personal communication) from studies involving larger numbers of patients and controls has demonstrated that catalase

can significantly increase lymphocyte proliferation in RA patients compared with controls.

Superoxide generation by monocytes is inhibited following SH blockade by pHMPSA (Table 10). This compound has previously been demonstrated to abolish superoxide production by neutrophils due to its interaction with surface SH groups (Tsan et al, 1976). It is possible that the inhibition is caused by interaction with the super-oxide producing enzyme, NADPH oxidase which is known to be inhibited by high concentrations of pCMB in mouse macrophages (Berton et al, 1982). Maslen (1985) has shown that H_2O_2 production by both neutro-phils and monocytes is abolished by treatment with pHMPSA. However, Tsan (1983) has indicated that whilst superoxide production is in-hibited by pHMPSA, H_2O_2 production is apparently unaffected. Since most of the H_2O_2 is thought to be produced by dismutation of super-oxide, this finding is somewhat surprising though it may be that H_2O_2 is produced by a mechanism independent of superoxide dismut-ation, in response to some stimuli, which is unaffected by SH-blockade.

Hydrogen peroxide was not able to reverse SH-blockade induced inhibition of IgG synthesis. As with the LTB_4 experiments, it may be that H_2O_2 is not the sole requirement needed to regenerate a normal response. Another possible reason for this inability is that H_2O_2 may need to be specifically localised perhaps at a monocyte-lymphocyte "junction" which is not achieved by addition of exogen-ous H_2O_2. This explanation similarly applies to the experiments where exogenous LTB_4 was added to blocked cultures. Some experi-ments were performed in which potassium superoxide was added in the same fashion as H_2O_2. Although the superoxide, as with H_2O_2, failed to reverse pHMPSA-induced inhibition (data not shown), too

much emphasis should not be placed on the results because of the short half-life of the exogenous superoxide in neutral buffers. In contrast to Gallagher and Curtis (1984) no stimulation of IgG synthesis by superoxide was observed either in mitogen-free or mitogen-induced cultures. Again, the short half-life of exogenous superoxide might explain this difference.

The finding that monocyte oxidative metabolism is dependent upon free cell surface SH groups raised the possibility that defective rheumatoid monocyte accessory function might be due to impaired oxidative metabolism due to surface SH-oxidation. However, monocytes from rheumatoid patients both on NSAIDs and on D-penicillamine produced higher amounts of H_2O_2 than control cells under unstimulated conditions and when stimulated with FMLP (although only unstimulated H_2O_2 production was significantly different under the Wilcoxon's ranking Sum test). The inability of 2-ME to alter H_2O_2 production by rheumatoid cells significantly suggests that excessive H_2O_2 production is independent of surface SH oxidation, a paradoxical conclusion perhaps in view of the inhibition produced by pHMPSA. Inhibition of oxidative metabolism by pHMPSA is probably mediated via interaction between the Hg moiety and SH groups whereas rheumatoid cell surface SH oxidation is due to disulphide formation probably with cysteine (Thomas and Evans, 1975). It is possible that mercury reacts with those SH groups involved in oxidative metabolism which are inaccessible to cysteine.

No significant difference was observed between H_2O_2 production by cells from patients on NSAIDs and those on D-penicillamine in this study. Hurst et al (1984) have shown that immunologically stimulated monocytes from rheumatoid patients treated with D-penicillamine show significantly enhanced rates of superoxide production compared

to cells from patients on NSAIDs. Both groups of cells exhibited

increased rates in comparison with normal cells when activated

by immunological stimuli but not when unstimulated or when acti-

vated by biochemical stimuli. This latter observation, they sugg-

est, might indicate an effect of second-line drugs on expression

of receptors such as Fc receptors. These results, together with

other data they have produced (Hurst et al, 1982) indicate that

D-penicillamine and sodium aurothiomalate treatment leads to en-

hanced monocyte responsiveness. The discrepancies between these

results and those produced in this study where no differences in

stimulated RA-NSAID and RA-D-penicillamine monocyte H_2O_2 product-

ion were noted might be explained by the differences in stimuli

used to elicit the oxidative response. FMLP stimulation is not

mediated via Fc or C3b receptors but more likely via specific

receptors which might be unaffected by D-penicillamine therapy.

Another possible explanation may be dissimilarities in the dis-

ease activity of the patient groups selected in each study. En-

hanced spontaneous H_2O_2 production by rheumatoid monocytes com-

pared to normals was not mirrored by increased rates of superoxide

production by unstimulated rheumatoid monocytes in the studies by

Hurst and colleagues. Disparity between patient selection might

again account for this difference; however, it may suggest a

superoxide independent H_2O_2-producing mecnahism in monocytes which

is activated in rheumatoid disease.

It is unclear from the results produced in this study whether

H_2O_2 production is progressively enhanced by D-penicillamine ther-

apy since H_2O_2 production was not assayed before commencement of

therapy. It would be interesting to initiate a larger study to

see if this was the case and to investigate whether responders

and non-responders exhibited different oxidative metabolism profiles

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which might prove useful as a prognostic indicator for drug therapy.

The improvement in monocyte accessory function in rheumatoid patients successfully treated with D-penicillamine appears to be mediated, at least partially, by the SH-reducing properties of the drug. Antigen presentation and Ca^{2+} ion mobilisation by monocytes are not affected by SH-blockade by pHMPSA. Rheumatoid monocytes display activated oxidative metabolism and it may be that D-penicillamine and other second line agents such as sodium aurothiomalate enhance this capacity still further. However, such an effect appears to be independent of the direct SH-reducing properties of the drug. Altered thiol expression may lead to abnormal regulation of free arachidonate levels and a direct effect upon cyclo-oxygenase and lipoxygenase activities cannot be ruled out. It is unlikely though that prostaglandins themselves are responsible for perturbed immuno-regulation in rheumatoid arthritis since treatment with NSAIDs fails to correct the abnormality. Therefore it is probable that D-penicillamine itself does not exert its beneficial effects via a cyclo-oxygenase inhibitory mechanism. Other possible thiol dependent reactions may include protein phosphorylation and control of the level of cyclic nucleotides and these may be targets for the action of D-penicillamine and other thiol-containing second line agents in rheumatoid arthritis. Further studies are indicated to investigate these possibilities.

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APPENDICES

A.C. (M) 42 FASTING		L.J.N.(F) 30 FASTING		J.F.(F) 34 FASTING		A.J.W.(M) 22 FASTING		M.O. (M) 23 FASTING	
hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml
0	0	0	0	0	0	0	0	0	0
1.08	2.34	1.08	3.62	1.00	3.15	0.75	2.20	1.00	3.69
2.00	8.02	2.45	7.96	2.00	7.43	1.91	4.44	2.00	7.50
3.00	6.60	3.05	7.10	3.00	5.93	2.91	9.32	4.00	5.91
4.08	5.08	6.08	4.66	4.00	5.22	4.08	4.80	6.00	4.97
6.20	3.62	6.08	3.68	6.16	4.63	6.08	4.03	8.00	3.22
7.91	3.39	7.91	3.40	8.16	3.51	8.00	3.54	24.00	2.64
24.00	2.31	24.00	2.66	24.00	2.76	24.08	2.67		

NON-FASTING		NON-FASTING		NON-FASTING		NON-FASTING		NON-FASTING	
hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml	hrs	[D-pen] μg/ml
0	0	0	0	0	0	0	0	0	0
1.00	3.00	1.08	3.32	1.00	2.94	1.03	2.43	1.10	2.50
2.00	6.00	2.08	5.74	2.00	5.33	2.00	6.56	2.00	4.04
3.00	5.37	3.05	4.14	3.00	4.08	3.33	5.77	3.06	3.69
4.00	4.60	3.95	3.77	4.00	3.67	4.08	4.44	4.00	2.98
6.16	3.59	6.05	3.37	6.00	3.38	6.00	3.55	8.00	2.56
8.00	3.15	7.75	3.17	8.00	3.14	8.00	3.03	24.50	2.32
24.00	2.21	24.00	2.53	24.00	2.66	24.00	2.49		

APPENDIX 1.   Penicillamine plasma concentrations for normal volunteers (at times shown) in fasting and non-fasting conditions.

M.C.(F) 55		B.D.(F) 43		E.S.(F) 74		J.S.(M) 60		B.H.(F) 60	
hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml
0	0	0	0	0	0	0	0	0	0
0.75	2.07	1.00	1.94	1.00	2.11	1.00	1.06	0.97	1.83
1.75	2.69	2.00	1.86	2.00	2.54	1.91	3.07	2.66	1.83
2.75	2.11	3.12	1.68	3.05	2.00	3.05	1.42	3.66	1.65
3.75	1.91	4.13	1.58	4.04	1.89	4.00	1.22	4.50	1.45
6.00	1.75	6.00	1.32	6.04	1.47	6.00	1.08	5.92	1.11
8.00	1.55	8.60	1.09	8.00	1.35	8.08	0.99	8.10	0.87
10.00	1.38	23.75	0.98	24.04*	1.60	23.75	0.84	23.92	0.73
24.00	1.11								

P.N.(F) 62		J.H.(M) 43		O,S.(F) 72		P.H.(F) 69		J.C.(M) 55	
hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml	hrs	(D-pen) μg/ml
0	0	0	0	0	0	0	0	0	0
0.7	2.79	1.00	2.49	0.78	2.02	1.50	3.27	1.03	1.25
1.33	3.67	2.00	2.50	1.45	2.89	2.50	2.84	2.00	2.18
2.03	3.50	3.00	2.21	2.13	3.11	4.50	2.00	4.05	1.75
2.58	2.76	4.66	1.87	2.70	2.27	9.30	1.31	6.04	1.39
4.05	2.61	6.00	1.58	6.08	1.78	24.00	1.08	7.75	1.31
7.83	2.18	9.00	1.52	9.87	1.59			23.80	1.04
23.84	1.83	10.00	1.50	24.00	1.34				
		23.84	1.21						

* 2nd 125mg. tablet administered
at 23.45

APPENDIX 2.   Penicillamine plasma concentrations for
RA patients (at times shown).

O.T.(F) 62		E.J.(F) 77		H.H. (M) 68		L.H. (F) 57		O.G.(F) 45	
hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml
0	0	0	0	0	0	0	0	0	0
1.00	0.95	1.00	1.30	1.00	ND	1.00	1.35	1.00	0.76
3.00	1.54	2.75	1.84	2.00	1.07	3.75	1.47	4.00	1.50
4.00	1.30	4.00	1.63	4.00	0.91	9.00	1.32	8.00	1.22
9.00	1.19	7.00	1.18	8.00	0.60	26.00**	41.58	24.00	0.75
24.00	0.94	23.58	0.94	12.00	0.50				
				24.00*	1.37				

E.S.(F)									
hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml	hrs	(D-pen) µg/ml
0	0								
0.75	0.76								
1.70	1.05								
2.50	1.18								
4.00	1.20								
6.00	0.84								
8.08	0.84								
24.00	0.47								

ND = none detected
* = 2nd tablet administered at 12.00
** = 2nd tablet administered at 24.00

APPENDIX 2. continued.