Extracellular scavenging of oxygen radicals produced by polymorphonuclear leukocytes - the relevance to rheumatoid arthritis

ONE VOLUME

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SUMMARY OF THESIS

This thesis is the account of laboratory research which I undertook in the Department of Medical Biochemistry, University of Wales College of Medicine between October 1981 and September 1983, while funded by an Arthritis and Rheumatism Council Research Training Fellowship. The aim of the work was to investigate the role of oxygen radicals produced by polymorphonuclear leukocytes (PMN) in the pathogenesis of rheumatoid arthritis, and in particular to establish the availability of these reactive moieties for extracellular tissue damage.

The main objectives of the work were:-

(i) to provide direct evidence of oxygen radical production by PMN exposed to rheumatoid synovial fluid or serum in vitro

(ii) to determine whether rheumatoid synovial fluid or serum contained inhibitors or scavengers of oxygen radical production

(iii) to determine the contribution of oxygen radical scavenging to any beneficial effects of anti-inflammatory or anti-rheumatic drugs in vivo.

Luminol dependent chemiluminescence was selected as a sensitive, non toxic, continuous monitor of the rate of oxygen radical production by PMN in vitro. Luminol was known to act as a true indicator, and the superior sensitivity of the custom built luminometers available in the Department of Medical Biochemistry afforded a unique opportunity to measure oxygen radical production by 'resting' as well as stimulated PMN. The chief disadvantages of this technique at the outset of the work were the lack of known specificity of luminol's

reaction with oxygen radicals, and uncertainty about what proportion of PMN luminol chemiluminescence occurred extracellularly. In the work of this thesis a greater understanding of the mechanism of PMN luminol chemiluminescence generation developed alongside application of the technique to investigation of the role of oxygen radicals in the pathogenesis of rheumatoid arthritis.

Before using luminol chemiluminescence to investigate the effects of rheumatoid synovial fluid or serum on PMN oxygen radical production, it was first necessary to characterise the chemiluminescence responses of the cells following their exposure to 'model' phagocytic or chemotactic stimuli. The luminol chemiluminescence responses of normal human PMN exposed to unopsonised latex particles, opsonised zymosan, immune complexes and the chemotactic peptide n formyl met leu phe in vitro were thus characterised and the concentrations of stimuli required to give a half maximal chemiluminescence response were defined.

The microfiliament inhibitor cytochalasin B was found to enhance and exaggerate the biphasic chemiluminescence response of PMN stimulated by chemotactic peptide. The temporal dissociation of the second phase from cellular oxygen consumption suggested that a different radical predominated in each phase. Later experiments showed that while the first phase as preferentially inhibited by superoxide dismutase, the second phase was inhibited by the myeloperoxidase inhibitor sodium azide. It was therefore proposed that radicals in the second phase resulted from the interaction of myeloperoxidase - H_2O_2 - Cl. An isomer of luminol chemically bonded to non phagocytosable sepharose beads was used to investigate what proportion of PMN luminol chemiluminescence occurred extracellularly.

Direct evidence of oxygen radical production in the rheumatoid

disease was sought in vitro following the addition of freshly aspirated rheumatoid synovial fluid denuded of all live cells, to normal or rheumatoid PMN. The majority of synovial fluid samples tested failed to stimulate PMN luminol chemiluminescence, rather a profound inhibition of chemotactic peptide stimulated PMN chemiluminescence occurred. Rheumatoid and normal serum were similarly found to inhibit PMN luminol chemiluminescence. The possibility that serum and synovial fluid might after all contain powerful oxygen radical scavengers, sufficient to prevent oxygen radical mediated tissue damage in vivo therefore arose and required further investigation.

Persistence of the majority of the chemiluminescence inhibitor in serum following dialysis implicated a macromolecular substance, later identified as albumin. Albumin's inhibitory effect (40g/l achieved up to 80% inhibition) could not be explained by decreased cell viability, quenching of luminol excited state or by a reduction in light transmission. Its differential effect on the biphasic chemotactic peptide stimulated chemiluminescence, enhancing the first phase and inhibiiting the second indentified albumin as an oxygen radical scavenger or pathway inactivator and further suggested that the radical scavenged resulted from the interaction of myeloperoxidase - H_2O_2 - Cl^-.

Confirmation of this previously unrecognised role in the major extracellular protein was provided by evidence of covalent structural modification in the albumin molecule following exposure to oxygen radicals. Incubation with stimulated PMN resulted in up to 20% oxidation of albumin thiol groups. Up to 60% oxidation of thiol groups occurred when reduced glutathione or whole serum were exposed to the PMN. This oxidation was inhibitable by azide but not by

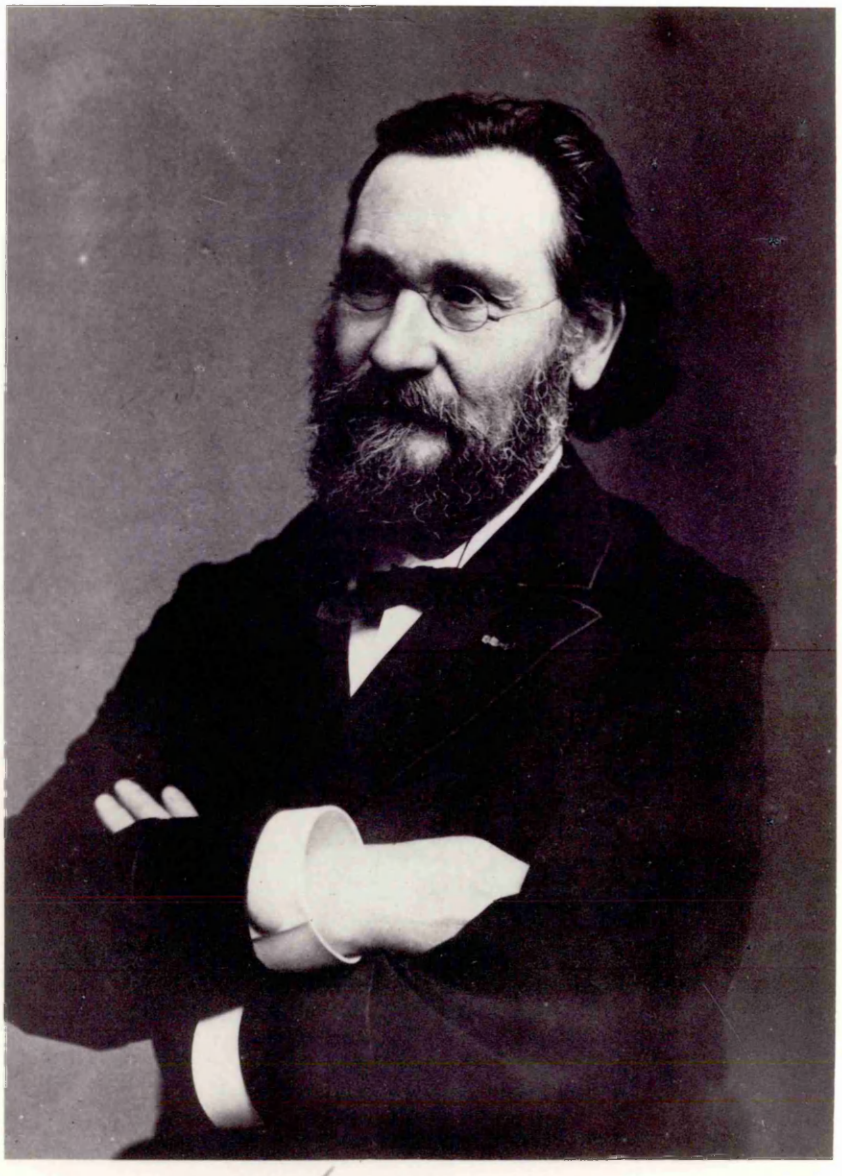
superoxide dismutase.

Demonstration of the oxygen radical scavenging ability of albumin highlighted both the potential importance of this extracellular molecule as a protector against oxygen radical mediated tissue damage in vivo, and the vulnerability of it and possibly other protein molecules to simultaneous covalent structural modification by the radicals, of potential consequence to their function. When rheumatoid synovial fluids were examined for evidence of in vivo albumin thiol oxidation, none was found. The contribution of non albumin protein (such as 1gG) to the total synovial fluid thiol levels was not, however, determined. The consequences of any oxygen radical mediated thiol oxidation on the integrity and biological function of 1gG would certainly bear further investigation.

It had previously been shown that each of the drugs known to have a 'disease modifying' or anti-rheumatic effect in the treatment of rheumatoid arthritis either contained a free thiol within their structure or was metabolised to one in vivo. Having shown the potential importance of the free thiol group on albumin to its oxygen radical scavenging it was now appropriate to use luminol chemiluminescence to compare and contrast the oxygen radical scavenging capability of anti rheumatic drugs with 'non thiol' containing anti-inflammatory drugs.

Selective inhibition of either the first or the second phases of stimulated PMN luminol chemiluminescence was taken as evidence of oxygen radical scavenging. By this means the antirheumatic drugs D-penicillamine and auranofin, and the anti-inflammatory drugs fenclofenac and indomethacin were shown to scavenge oxygen radicals. Only the effect of D-penicillamine was attributable to its thiol group.

In summary although,using luminol chemiluminescence, PMN were shown to produce oxygen radials when exposed to 'model' phagocytic and chemotactic stimuli in vitro, the ability of these moieties to cause tissue damage in rheumatoid arthritis in vivo may be restricted by commonly used drugs and by the major extracellular protein albumin which has been identified in this thesis as a potent oxygen radical scavenger.



Frontispiece - Ilya Ilyich Metchnikoff
(1845 - 1916)

(reproduced courtesy of Wellcome Institute Library, London).

Chapter 1 - Introduction

1.1. Historical Background to this Thesis

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1.1. Historical Background of Thesis

In 1883, Metchnikoff, a Russian zoologist, reported that foreign particles injected into starfish larvae were taken up by a population of 'wandering mesodermal cells' residing in interstitial tissues (Metchnikoff 1883). He was the first to postulate a crucial role in host defence for these wandering cells which he named 'phagocytes'. Metchnikoff, (1887), and later Menkin (1938), also proposed that these cells may liberate substances capable of damaging adjacent host tissues.

Subsequently, in experimental animal models, polymorphonuclear leukocyte phagocytes were found to play an important part in the pathogensis of the vasculitis of the Arthus phenomenon (Cochrane et al 1965), the arthritis of serum sickness (Krikier and Cochrane 1965), acute nephrotoxic nephritis (Umezawa and Aoyagi 1977) and immunologically induced arthritis (De Shazo et al 1972). The toxicity of the polymorphonuclear leukocyte was attributed to its extracellular release of granular proteolytic and collagenolytic enzymes (Klebanoff and Clark 1978 (d)). The finding of such enzymes in the polymorphonuclear leukocyte rich synovial effusions of rheumatoid arthritis (Smith and Hamerman 1962) similarly suggested an important role for the cell in the pathogenesis of this disease. Rheumatoid synovial fluid was, however, later shown to contain abundant protease inhibitors such as α_1 anti-trypsin and α_2 macroglobulin (Galdston, Janoff and Davis 1973, Barrett and Starkey 1973).

In 1933, Baldridge and Gerard were the first to observe the marked increase in oxygen consumption, later known as the 'respiratory burst' which accompanied the addition of sarcina lutea to dog polymorphonuclear leukocytes. This process was later linked to activation of the hexose monophosphate shunt (De Chatelet, Wang and

McCall 1972), the production of hydrogen peroxide (H_2O_2) (Paul and Sbarra 1968) and of the oxygen 'free' radical superoxide anion ($O_2^{\cdot -}$) (Babior, Kipnes and Curnette 1973). Evidence has also been provided for the production of the highly reactive hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1\Delta_g$ O_2) and the hypochlorite anion as a result of the reaction of H_2O_2 with $O_2^{\cdot -}$ or with chloride ions catalysed by myeloperoxidase released by PMN azurophil granules (Badwey and Karnovsky 1980).

In modern terminology a 'free radical' is defined as 'any atom, group of atoms or molecule in a particular state with at least one unpaired electron occupying an outer orbital' (Del Maestro 1980). The lone electron present in the outer orbital of a free radical endows it with unusual chemical reactivity and physical characteristics. The unpaired electron has a strong tendency to interact with other electrons to form an electron pair and thus a chemical bond. Most free radicals are so reactive that they normally exist only at very low concentrations from 100μM - 1nM (Pryor 1976).

Molecular oxygen (O_2) is infact the commonest free radical in biological systems, possessing two unpaired electrons with parallel electron spins in its outer orbitals (Taube 1965). The parallel electron spin arrangement prevents the direct addition of a pair of electrons (which would have one parallel and one antiparallel electron spin) and which would neccessitate an electron spin inversion before bond formation could occur (Taube 1965). Consequently oxygen in its ground state is a relatively weak oxidant and whenever energetically feasible univalent pathways of oxygen reduction (reaction of oxygen with single electrons, hydrogen atoms or other atoms or molecules containing unpaired electrons) are favoured over divalent pathways (Fridovich 1976), as in the univalent, four electron reduction of

oxygen to water (Fig 1) (Michaelis 1946).

From the time of its independent codiscovery by Joseph Priestley in 1775 and Carl Wilhelm Scheele in 1777, oxygen has been known both to sustain life and destroy it (Gilbert, 1981). It was not until 1954 however, (Gerschman et al 1954) that oxygen's toxicity was first attributed to the more reactive radicals and oxygen metabolites ($O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$) formed by its univalent reduction to water (Michaelis 1946).

The requirement for organisms which utilise oxygen to develop mechanisms which minimise the production of oxygen radicals or efficiently remove (scavenge) those whose production cannot be avoided is likely to have been a dominating evolutionary pressure (Fridovich 1976). The discovery that phagocytes such as the polymorphonuclear leukocyte purposefully generate oxygen radicals and other highly reactive oxygen metabolites, albeit for the intracellular killing of phagocytosed microorganisms, has afforded renewed meaning to Metchnikoff's proposal that PMN may liberate substances capable of damaging host tissues (Metchnikoff 1887).

Rheumatoid Arthritis has become prominent amongst the diseases in which it has been proposed that oxygen radicals produced by stimulated polymorphonuclear leukocytes may play a part (Del Maestro 1980, Greenwald 1981). Polymorphonuclear leukocytes are found at all sites of rheumatoid disease activity both extra and intra articularly, but particularly in the synovial membrane inflammatory infiltrate of early disease (Schumacher and Kitridou 1972), and the synovial fluid, articular cartilage and pannus of established disease (Krey and Bailen 1979, Ohno and Cooke 1978, Westerhellweg and Weissinhage 1981). Evidence has been provided for the stimulation of these cells by immune complexes and/or chemotactic complement components at most of

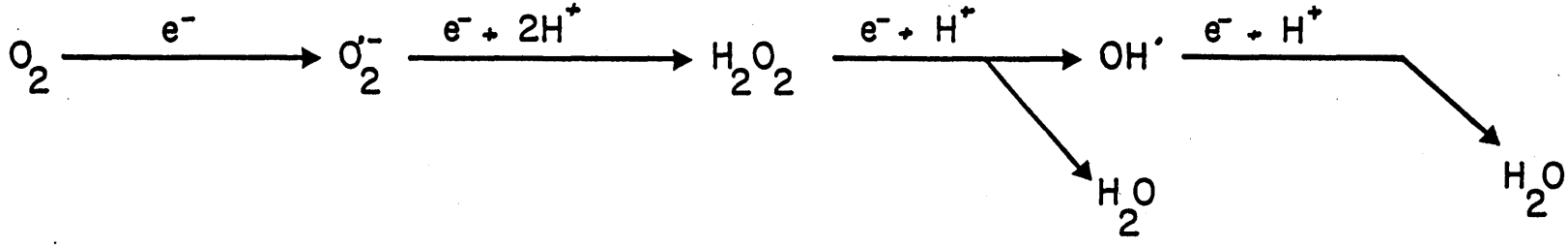

Figure 1 - The reduction of molecular oxygen (dioxygen) via the univalent pathway

these sites.

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Although the term rheumatoid arthritis was introduced by Sir Alfred Baring Garrod in 1859 (Short 1974) it was not used to describe exclusively the collections of symptoms, signs and pathological changes now recognised by that name (Ropes et al 1958) until its adoption by the British Ministry of Health in 1922 (Moll 1958). The first description of rheumatoid arthritis in the medical literature is conventionally attributed to the doctoral thesis of a French medical student, Augustin-Jacob Landré Beauvais, presented in 1800 (Wood 1976). It is likely, however, that Thomas Sydenham had already described the main clinical features of rheumatoid arthritis in 1676 (Short 1974). Attempts to establish the antiquity of the disease by perusal of early medical manuscripts, old paintings, literature and the study of paleopathology have failed to demonstrate the existence of rheumatoid arthritis as an entity distinct from gout, ankylosing spondylitis or osteoarthritis prior to the early seventeenth century (Parish 1963, Ehrlich 1967, Boyle and Buchanan 1971, Short 1974, Wood 1976, Appelboom et al 1981).

The absence of descriptions of rheumatoid arthritis from early medical writing may simply reflect a failure of its recognition. Even today the management of patients with rheumatoid arthritis is overshadowed by three major areas of ignorance. Firstly the aetiology of the diseases is unknown. Secondly no clinical or pathological parameters have been identified which serve as exclusive diagnositc features or allow quantitative comparison of the severity of individual cases. Thirdly the modes of action of the commonly used anti inflammatory and disease modifying drugs are poorly understood,

and no treatment capable of arresting rheumatoid arthritis has yet been demonstrated.

Any discussion of the pathogenesis of rheumatoid arthritis must take account of its usually chronic and progressive nature. Either the primary aetiological factor persists in the tissues and elicits a continuous response from the host, or endogenous self perpetuating mechanisms within the host replace the original stimulus, allowing progression of the disease (Harris 1981). In order to implicate any agent in the pathogenesis of rheumatoid arthritis, it must therefore be identified either as the primary initiating agent, or as a contributor to the secondary amplification mechanisms.

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Approximately one hundred years following Metchnikoff's proposal (Metchnikoff 1887), this thesis reports the results of an investigation into the role of oxygen radicals and other reactive oxygen metabolites produced by polymorphonuclear leukocytes in the pathogenesis of rheumatoid arthritis. In the remainder of this chapter the functions of the polymorphonuclear leukocyte will be examined in greater detail, as will the production of oxygen radicals, and their potential for tissue damage and disease pathogenesis. Secondly, evidence for the presence of stimulated polymorphonuclear leukocytes at sites of disease activity will be reviewed together with any additional evidence for oxygen radical activity in rheumatoid arthritis which had accumulated prior to the commencement of this work in October 1981. Finally methods of measuring oxygen radical production, and in particular luminol dependent chemiluminescence will be discussed.

1.2. The Neutrophil Polymorphonuclear Leukocyte

1.2.1 Cell morphology, kinetics and functions

Peripheral blood leukocytes can be divided into granular and non granular forms. The latter, the granulocytes, are further subdivided in man into eosinophils, basophils and neutrophils on the basis of the affinity of their granules for the acidic and basic dyes commonly employed in histological stains. All granulocytes are characterised by a many lobed nucleus, hence the term 'polymorphonuclear leukocyte'. Neutrophil polymorphonuclear leukocytes comprise the majority (40-75%, 2.5 - 7.5 x 10^9/l) and unless otherwise stated will be the variety of granulocyte referred to in the remainder of this thesis and abbreviated by the letters 'PMN'.

The mature PMN is capable of six actions:-

(a) chemotaxis

(b) target recognition

(c) phagocytosis

(d) a 'respiratory burst' of oxidative metabolism

(e) degranulation

(f) extracellular release of granule constituents.

Each of these is invoked by the PMN's participation in an inflammatory response and may result either in host defence (e.g. against bacterial infection) or in host tissue damage (e.g. in autoimmune diseases). Before discussing these actions in more detail, the natural history and morphology of the PMN will be reviewed.

PMN natural history and morphology

PMN originate in the myeloid precursor pool of the bone marrow (Fig. 2). Myeloblasts, promyelocytes and myelocytes are all actively dividing cells. The marrow granulocyte reserve (Fig. 1) consists of

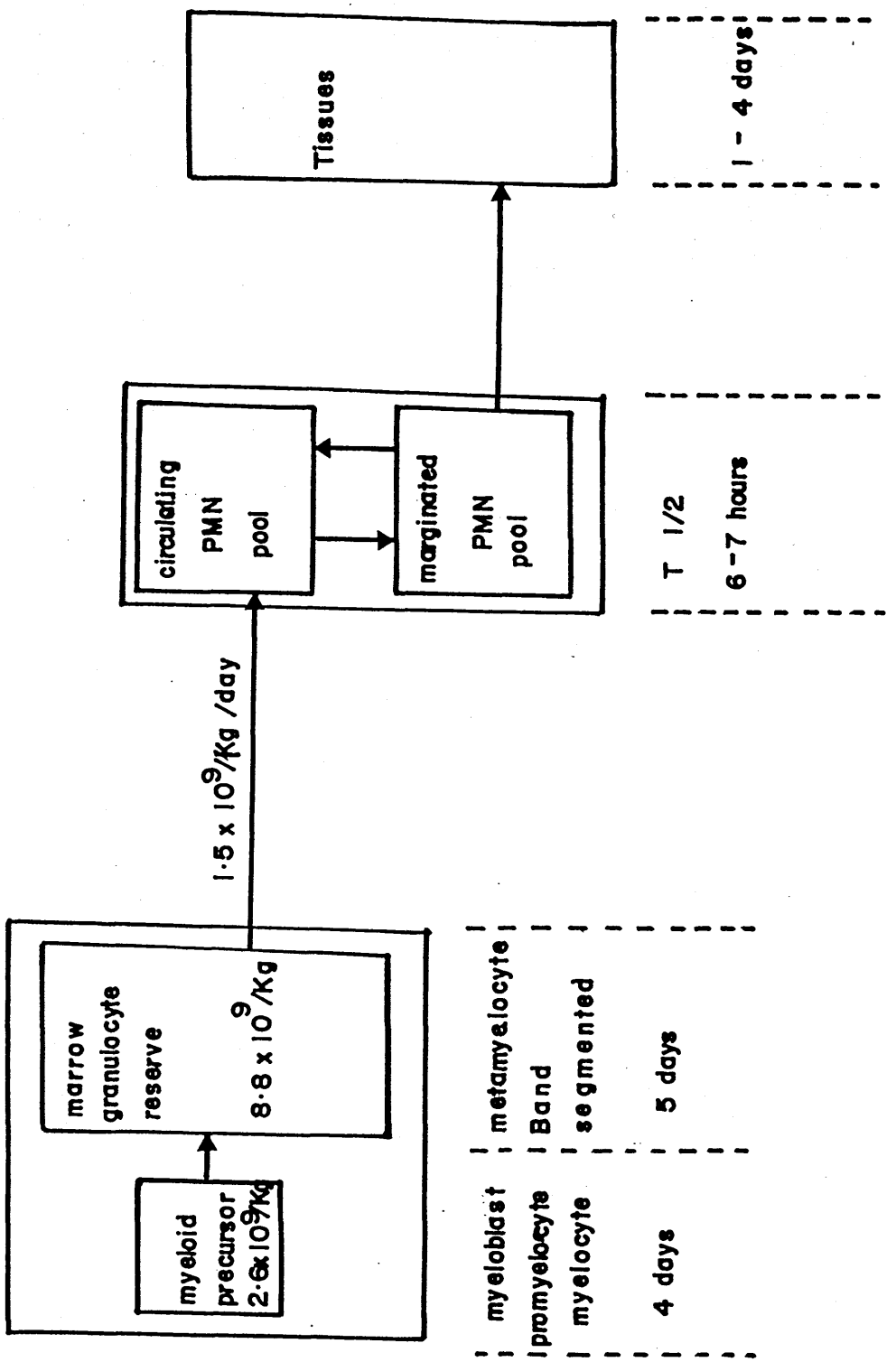


Figure 2 - Schematic representation of granulocyte kinetics (adapted from Craddock (1972)).

maturing, non dividing cells, aquiring functional capabilities, which exit from the marrow to the peripheral blood at a rate of 1.5×10^9 PMN/kg of body weight per day (Klebanoff and Clark 1978(b)). The peripheral blood PMN may be divided into the 'circulating PMN', comprising the cells sampled by venepuncture and the 'marginated PMN', consisting of cells transiently sequestered in areas of sluggish flow along endothelial walls. These pools are in equilibrium. The PMN spends a relatively short period in the vascular space (Fig. 2) before exiting via the marginated pool under the influence of chemotactic factors, to the tissues.

Although no cell division occurs beyond the myelocyte stage, PMN maturation continues (Bainton, Ullyot and Farquhar 1971) such that the nucleus becomes more indented, ultimately segmenting into distinct lobes; nucleoli disappear, the cell becomes smaller and there is a reduction in the number of cellular organelles (free ribosomes, rough surfaced endoplasmic reticulum, mitrochondria and Golgi complex). Glycogen particles accumulate in the cytoplasm, but the most distinctive morphological feature is the abundance of cytoplasmic granules, 70-90 Å in width. The granules can be divided into 'primary' (or azurophil) and 'secondary' (or specific) populations (Table 1) (Klebanoff and Clark 1978(a)). Although alkaline phosphatase is also found in the specific granules of the myelocyte (Bainton et al 1971), fractionation of mature PMN has demonstrated its presence in a membrane fraction largely devoid of granules (West et al 1974). The presence in human PMN of a distinct third type of granule population ('tertiary granules') containing acid hydrolases has also been proposed (West et al 1974).

The duration of the PMN's existence in the tissues is not known, although estimates range from 1-4 days. Some of the cells which leave

TABLE 1 Human PMN Azurophil and Specific Granule Components

(Adapted from Klebanoff and Clark, 1978)

Azurophil (primary)	Specific (secondary)
Acid hydrolases	Lysozyme
Neutral proteases	Lactoferrin
(cathepsin G Elastase collagenase*)	Collagenase*
Cationic proteins	Vitamin B_{12} binding protein
Myeloperoxidase	
Lysozyme	
Acid mucopolysaccharide	

* There is conflicting data on the location of these components

the peripheral blood are removed by the fixed macrophages of the liver and spleen (Klebanoff and Clark 1978(b)). The majority probably die within the tissues following stimulation and release of intracellular constituents toxic to the plasma membrane (Weissman, Hirschorn and Krakauer 1969, Salin and McCord 1975).

PMN chemotaxis

Unstimulated PMN migrate in a random fashion by extension of pseudopods and propagation of surface contraction waves originating from peripheral cytoplasmic microtubules and contractile microfilaments of actin and myosin (Senda et al 1975). During an inflammatory response this migration becomes directed by the influence of soluble mediators (chemotactic factors) such that PMN leave first the bone marrow and then the peripheral circulation in increased numbers for the site of tissue insult. During this directed movement or 'chemotaxis' PMN are polarised with the advancing front of the cell exhibiting a broad thin veil, while a knob like tail and trailing retraction fibres are seen at the rear (Zigmond and Hirsch 1973).

PMN chemotactic factors can be classified according to their structural properties (Goetzl 1981) as follows:-

(i) Protein fragments or complexes e.g. C5a, C567, fibrin, fragments and collagen fragements.

(ii) Enzymes e.g. plasma kallikrein.

(iii) Cellular peptides or anions e.g. most cell derived peptides and histamine, and bacterial soluble factors.

(iv) Lymphokines

(v) Lipids eg bacterial lipids, and metabolites of arachidonate lipoxygenase (HETE) and arachidonate cyclooxygenase (HHT).

Of these, complement and bacterial derived factors are probably the most important. Klebanoff and Clark (1978(b)) have proposed that

a chemotactic factor interacts with a receptor site on the cell surface triggering a complex series of events involving serine esterase activation, fluxes of calcium, sodium and potassium,. changes in transmembrane electropotential, reduced surface charge, inhibition of the respiratory burst (1.2.2) increased metabolism and granule enzyme secretion. As these events occur the receptor site is deactivated and cannot respond to a subsequent stimulus (Issekutz and Bigger 1977). Identification of chemotactic factor receptor sites on PMN has been limited by the lack of availability of pure structurally defined chemoattractants. Recent studies with synthetic formylated peptides have shown high affinity binding of [^{3}H] formyl met leu phe to human PMN (Williams et al 1977).

PMN chemotaxis is inhibited by agents which inactivate chemotactic factors (such as anaphylatoxin, lysosomal protease, and α2 macroglobulin) and those which interact directly with PMN responsiveness (such as the microtubule inhibitor colchicine and the microfilament inhibitor cytochalasin B) (Klebanoff and Clark 1978(b), Goetzl 1981). In addition PMN chemotaxis is influenced by changes in pH and temperature (Phelps and Stanislaw 1969) and osmolarity (Lotz and Harris 1956).

PMN target recognition

Before it can engulf or phagocytose the organism particle or immune complex to which it has migrated; the PMN must first recognise this target. This is achieved by stimulation of the cell's receptors for the Fc component of IgG (Messner and Jelinek 1970), and/or C3b (Henson 1969), respectively heat stable and heat labile 'opsonins' attached to the surface of the target.

The heat stable opsonin may be any antibody molecule binding with immunological specificity via its F(ab')2 region to antigens on the

surface of an organism or particle. The opsonic activity of IgG is dependent on the integrity of the Fc region and is lost by reaction with rheumatoid factor.

The heat labile opsonin, complement, can be activated directly by the particle or organism via the alternate pathway, or indirectly by immunoglobulin bound to the particle or organism, thus forming a complex which activates complement via the classical pathway. The interaction of heat labile and heat stable opsonins in turn appears to be more than additive (Klebanoff and Clark 1978(c)). The C3b receptor appears to function primarily to bind the particle to PMN and does not initiate phagocytosis, whereas reaction of the particle with the Fc receptor is required to trigger the ingestion phase but is relatively inefficient at inducing adherence (Klebanoff and Clark 1978(c)).

PMN phagocytosis

Following recognition, PMN pseudopods surround the opsonised organism or particle by a process of circumferential adherence (Griffin, Griffin and Silverstein 1976) and phagocytosis begins. Ligand attach/ment to membrane receptors on the PMN membrane is accompanied by exclusion of organelles and formation of a filamentous network in the submembrane area. Finally the pseudopods fully enclose the particle in a vacuole consisting of internalised extracellular space surrounded by plasma membrane. The opposed membranes initially form a stalk connecting the vacuolar space with the external milieu; then fusion and lysis occur and the phagosome is set free within the cell. Phagocytosis is influenced by several environmental factors including the presence of the divalent cations Ca^{2+} and Mg^{2+}, temperature, pH and various pharmacological agents (Klebanoff and Clark 1978(c)).

PMN 'respiratory burst'

This mechanism for PMN oxidative killing of ingested microorganisms will be reviewed in detail in section 1.2.2 of this thesis.

PMN degranulation

Following phagocytosis specific and azurophil granules of the PMN cytoplasm fuse with the phagocytic vacuole, the common membrane ruptures and granule contents are discharged into the vacuolar space converting the phagosome into a phagolysosome (Klebanoff and Clark 1978(d)). Since granule formation does not occur beyond the myelocyte stage of PMN development, degranulation irreversibly depletes the mature cell of these organelles. In the rabbit PMN, specific granules discharge their contents first, with alkaline phosphatase being detectable in the phagocytic vacuole as early as 30 seconds after initiation of phagocytosis, whereas myeloperoxidase appears at 1-3 minutes (Bainton 1973).

Extracellular release of PMN granule constituents

Granule constituents of the PMN can be released extracellularly under four types of circumstances (Table 2). Plasma membrane rupture leading to cell death and release of cytoplasmic contents may be caused by exposure to toxic substances (such as detergent), extremes of pH temperature and osmolarity, and membranolytic substances such as sodium urate crystals. Plasma membrane rupture may also be caused by leakage of lysosomal hydrolases from the phagolysosomes to the cytoplasm.

Regurgitation during feeding (Table 2) occurs when a phagolysosome opens to the exterior, or when degranulation occurs into a developing phagocytic vacuole, prior to its closure. Reverse endocytosis occurs when a PMN is exposed to a large membranous surface coated with immune reactants such as antigen-antibody complexes. The

TABLE 2 Mechanisms of Extracellular release of PMN granule constituents

	Mechanism			Reference
I	Membrane lysis	-	toxin	Zurier et al 1973 (a)
			environment	Klebanoff and Clark 1978(d)
			urate crystals	Rajan 1966
			lysosomal hydrolases	Spilberg 1975
II	Regurgitation during feeding	-	degranulation to developing vacuole	Klebanoff and Clark 1978(d)
III	Reverse endocytosis	-	frustrated phagocytosis	Weissmann et al 1972
			chemotaxis	Showell et al 1976
			cytochalasin B	Zigmond and Hirsch 1972(a)

cell adheres to the surface, which is too large to be engulfed, and is stimulated to release its granule enzymes. In both regurgiation during feeding and reverse endocytosis release is selective for granule and not cytoplasmic enzymes, and cell viability is maintained (Weissmann et al. 1971, Henson 1971).

Complement derived and chemotactic factors have been shown to induce extracellular discharge of PMN granule constituents (Table 2). Becker et al (1974) found that substantial enzyme release only occurred when PMN were allowed to settle on micropore filters suggesting that the chemotactic factor mediated enzyme release is a special case of reverse endocytosis.

Studies of extracellular granule content release by PMN have been greatly facilitated by the use of the fungal metabolite cytochalasin B which interferes with cytoplasmic microfilament function (Wessells et al 1971) and blocks membrane transport of sugars and nucleosides (Estensen and Plagemann 1972, Zigmond and Hirsch 1972a). PMN treated with cytochalasin B are unable to ingest opsonised particles (Zigmond and Hirsch 1972b) which none-the-less adhere to the cell surfacce. Granules merge with the cell membrane and their contents (without other cytoplasmic constituents) are released in areas of particle contact. Mechanistically this, therefore, also represents an example of reverse endocytosis.

The potential consequences of PMN extracellular degranulation are tissue injury to the host by interaction with host cells or circulatory factors (Table 3). Several lines of evidence point to the involvement of PMN granule constituents in the mediation of tissue injury in human diseases such as acute post streptococcal glomerulonephritis (Karan, Saatci and Bakkalogu, 1976), emphysema (Lieberman 1976), rheumatoid arthritis (Smith and Hamerman 1962,

TABLE 3

Potential for toxicity of PMN granule constituents released extracellularly

	Granule constituent		Toxic effect	Reference
I	Acid proteases	- Cathepsin D	Kinin generation	Greenbaum (1972)
		Cathepsin E		
II	Neutral proteases	- Elastase	Proteolysis	Janoff et al (1972)
		Collagenase	Collagenolysis	Horwitz et al (1977)
		Chymotrypsin like	Proteoglycain degradation	Janoff et al (1976)
III	Cationic proteins	-	increased vascular permeability	Burke et al (1964)
			histamine release	Ranadive and Cochrane (1971)
			cytotoxicity	Clark et al (1976)
IV	Myeloperoxidase		myeloperoxidase - H_2O_2 - halide reaction	Klebanoff and Clark (1978)(e)
V	Lysozyme		? cell membrane damage	Osserman (1975)

Bennett and Skosey 1977) and rheumatoid vasculitis (Schumacher and Agudelo 1972). Experimental tissue damage has also been produced by isolated PMN granules (Weissmann et al 1969b). Granular proteases can however be inactivated extracellularly by circulating protease inhibitors such as α_1 antitrypsin (Galdston et al 1973) and α_2 macroglobulin (Barnett and Starkey 1973). The role of extracellularly released granule proteases in the pathogenesis of rheumatoid arthritis has thus frequently been contested (Barret and Saklatvala 1981) while the case for their involvement in emphysema has been based on the known deficiency of α_1 antitrypsin in this condition (Lieberman 1976).

Consequences of the extracellular reaction of the azurophil granular protein myeloperoxidase (Table 3) with hydrogen peroxide produced during the PMN respiratory burst are discussed in the following section 1.2.2.

1.2.2 The PMN Respiratory Burst

Organisms which utilise oxygen have evolved mechanisms which minimise the production of oxygen radicals and efficiently scavenge those whose production cannot be avoided (Fridovich 1976). Most of the molecular oxygen consumed by cells is reduced to water in a single tetravalent step by the mitochondrial cytochrome c oxidase complex, thereby avoiding the generation of oxygen radicals and other reactive metabolites (Fig. 1, 1.1) (Keilin 1925). Cytochrome c oxidation is coupled to the phosphorylation of ADP to ATP and is inhibitable by carbon monoxide, cyanide, ran-tidine, hydrogen sulphide and antimycin A (Lehninger 1975). Products of the univalent reduction of oxygen are also avoided by other, non phosphorylating electron transport systems localised to the microsomal fraction of endoplasmic reticulum, and by

certain dioxygenase enzymes which are able to catalyse insertion of both atoms of the oxygen molecule into an organic substrate molecule (Lehninger 1975).

During the last twenty five years it has been shown that while the majority of cells largely avoid the production of oxygen radicals via the above mechanisms, stimulated phagocytic cells are busily involved in their production for the oxidative killing of bacteria. While the latter action is likely to benefit the host, the probability that oxygen radicals and other reactive oxygen metabolites so produced may exert a deleterious effect on host tissues and thereby cause disease is now receiving increasing study. The potential for oxygen radical involvement in disease pathogenesis will be examined in greater detail in the next section (1.2.3). In this section the production of oxygen radicals during the PMN respiratory burst is reviewed.

Historical Perspective Baldridge and Gerard (1933) were the first to observe a burst of oxygen consumption on the addition of sarcina lutea to dog leukocytes. In 1956 Stahelin, Suter and Karnovsky noted an increase in oxygen consumption and the oxidation of carbon - one - glucose to CO_2 during phagocytosis of guinea pig leukocytes. It was originally thought that the purpose of this rise in oxygen consumption was to provide energy for phagocytosis. Sbarra and Karnovsky (1959) showed, however, that phagocytosis occurred under nitrogen as well as under oxygen, and demonstrated that the increase in oxygen uptake was insensitive to inhibitors of mitochondrial respiration such as cyanide, antimycin A and dinitrophenol. The increased oxidation glucose 1-^{14}C to $^{14}CO_2$ was unaffected. Interference with glycolysis blocked the entire process (Becker et al 1958).

In 1961 Iyer, Islam and Quastel demonstrated that formate was

effectively converted to $^{14}CO_2$ by phagocytosing granulocytes. They ascribed this to a catalatic oxidation of formate due to the release of substantial amounts of peroxide during phagocytosis. This was later validated by direct observation and measurement of H_2O_2 formation (Paul and Sbarra 1968).

Iyer et al (1961) proposed that H_2O_2 was used by the phagocyte as a bactericidal agent and were the first to draw a connection between the increased PMN oxygen consumption and glucose oxidation and the microbicidal mechanisms of phagocytosis. Between 1966 and 1970 Klebanoff and co-workers (Klebanoff, Clem and Luebke 1966, Belding, Klebanoff and Ray 1970) described a powerful natural microbicidal system consisting of hydrogen peroxide, the PMN azurophil granule enzyme myeloperoxidases, and a halide, thereby linking PMN degranulation with the 'respiratory burst'. In 1967 Holmes, Page and Good showed that PMN from patients with chronic granulomatous disease do not manifest a respiratory burst and are defective with respect to bacterial killing, although they can ingest particles normally.

In 1973 Babior et al established that the phagocyte respiratory burst included not only increaed oxygen uptake, H_2O_2 production and activity of the hexose monophosphate shunt pathway, but also production of superoxide anion, From 1975, cytochemical and other techniques revealed the cellular locale of H_2O_2 and $O_2^{\cdot -}$ production as the plasmalemma and internal lining of phagosome membranes (Briggs et al 1975(b), Goldstein et al 1977).

The constituent parts of the coordinated series of metabolic events which comprise the PMN respiratory burst, their interrelation and sequelae will now be considered in greater detail.

Increased oxygen consumption

The PMN respiratory burst can be activated by perturbation of the

plasma membrane by a variety of agents, both particulate and soluble (Table 4). The up to 10 fold increase in oxygen consumption usually follows exposure of the PMN to the stimulus by 30-60 seconds (Weening, Roos and Loos, 1974, Root et al 1975) and requires neither phagocytosis nor degranulation, but simply contact of the stimulus with the cell surface (Goldstein et al 1975). In one study (De Chatelet et al 1972) ingestion of labelled bacteria was complete in 10 minutes, the burst of oxygen consumption continued for 20 minutes and the increase in hexose monophosphate shunt activity for 30-45 minutes following the additon of particles. Although the incresed oxygen consumption per se is sensitive to inhibitors of mitochondrial respiration (Sbarra and Karnovsky 1959) its activation can be blocked by inhibitors of glycolysis and mitochondrial oxidative phosphorylation (Cohen and Chovaniec 1978) and is dependent upon the extracellular presence of divalent cations calcium and magnesium (Romeo et al 1975).

The identity of the enzyme responsible for the primary oxygen consuming reaction of the respiratory burst has been a controversial issue for many years (Iyer and Quastel 1963, Rossi and Zatti 1964). Babior, Curnette, and McMurich (1976) characterised an enzyme from human PMN in the absence of manganese ions which produced $O_2^{\cdot -}$ according to the following reaction (1)

$$NADPH + 2\ O_2 \text{ ------> } 1\ O_2^{\cdot -} + NADP^+ + H^+ \text{ ------- (1)}$$

This reaction was found to employ either NADH or NADPH as electron donors, but several observations support the latter as the physiological donor (Babior 1978). Firstly the Michaelis constant for NADPH is lower than for NADH (Babior et al 1976), secondly NADPH levels fall and $NADP^+$ levels rise during PMN stimulation whereas $NADH/NAD^+$ levels are unchanged (Selvaraj and Sbarra 1967), and thirdly

TABLE 4

Stimulants of PMN Respiratory Burst

STIMULANT	REFERENCE
A. Particulate	
Opsonised bacteria	Curnette and Babior (1974)
Opsonised zymosan	Easmon et al (1980)
Unopsonised particles	Weening et al (1975)
Immune complexes	Starkebaum et al (1981)
Aggregated immunoglobulin	Goldstein et al (1975)
B. Soluble	
Immune complexes	Starkebaum et al (1981)
Complement components	Goldstein et al (1975)
N-formyl-met-leu-phe	Hatch et al (1978)
Fluroide	Curnette et al (1979)
Endotoxin	Strauss and Stetson (1960)
Calcium ionophore A23187	Hallett et al (1981)
Phorbolmyristate acetate	Repine et al (1974)
Concanavalin A	Romeo et al (1973)
Digitonin	Graham et al (1967)
Deoxycholate	Zatti and Rossi (1967)
Long chain fatty acids	Kakinuma (1974)

patients in whom NADPH levels are depleted by G-6-PD deficiency, the PMN respiratory burst is greatly diminished (Cooper et al 1972).

Experiments have shown however, that the NADPH oxidase isolated from human PMN does not exhibit sufficient activity to cover the respiratory burst of the whole cell (Gabig and Babior 1979, Badwey and Karnovsky 1980). It was therefore proposed that the activating 'enzyme' hitherto approached as a single entity is in fact a multicomponent system disrupted by cell homogenisation. Evidence for such a system was first provided by Hattori (1961) and Shinagawa et al (1966) who described b-cytochromes in horse and rabbit PMN respectively. They showed that the b-cytochrome was autoxidisable and could be reduced by NADH or NADPH under aerobic conditions. This reduction was insensitive to cyanide and antimycin A. A similar b-cytochrome was later demonstrated in the plasmalemma of human PMN, but was absent in most cases of chronic granulomatous disease (Segal and Jones 1978, Segal et al 1978). It is known that cytochromes of the b-type are initially involved in the oxidation of reduced quinones (Crane 1977). Quinones are capable of shuttling electrons across membranes, and of auto-oxidising to produce both $O_2^{\cdot -}$ and H_2O_2 (Crane 1977, Misra and Fridovich 1972). Whether quinones are present in the plasmalemma of human PMN remains to be established.

There is now general agreement that the activating enzyme complex (NADPH oxidase/b-cytochrome) is situated in the plasma membrane (Roos Homan-Muller and Weening 1976, Goldstein et al 1975, Johnston et al 1975, Salin and McCord 1974, Baehner 1975). Roos et al (1977) have proposed that the complex is membrane spanning (Fig 3) such that the reducing NADPH comes from the cell interior and O_2 from the exterior, releasing $NADP^+$ inside and $O_2^{\cdot -}$ outside. This orientation would be maintained during phagosome formation allowing release of $O_2^{\cdot -}$ into

the phagosome (Fig 3). The question arises as to which part of the PMN plasma membrane is triggered into $O_2^{\cdot -}$ production. Does PMN stimulus attachment activate all NADPH complexes in the plasma membrane, or only those at the site of attachment? Roos et al (1977) favoured the latter.

The Hexose Monophosphate Shunt

The increase in PMN glycogen degradation in the early post phagocytic period (Sbarra and Karnovsky 1959) is not associated with changes in cyclic AMP levels, nor is it influenced by epinephrine or glucagon (Stossel et al 1971). It is, however, associated with a decrease in glucose-6-phosphate levels attributed to increased degradation of this compound by the glycolytic and hexose monophosphate shunt pathways (Jemelin and Frei 1970).

In the resting PMN, less than 10% of glucose passes through the hexose monophosphate shunt. Up to a four fold increase has been observed during phagocytosis (Klebanoff and Clark 1978(e)). Glucose-6-phosphate dehydrogenase lies at the entrance of the hexose monophosphate shunt (Kahn et al 1976) and catalyses the following reaction (2)

$$\text{Glucose-6-phosphate} + NADP^+ \longrightarrow 6\ \text{phosphogluconate} + NADPH + H^+ \quad (2)$$

$NADP^+$ levels are maintained by reoxidation of NADPH. The NADPH oxidase (b-cytochrome) complex would thus appear to meet the total requirements of the PMN respiratory burst (Fig 4), namely increased oxygen consumption, superoxide anion formation, and increased NADPH oxidation to fuel the hexose monophosphate shunt. It is well accepted that $NADP^+$ is also replenished by the glutathione peroxidase - glutathione reductase system responsible for the reduction of H_2O_2 in the cytoplasm (Reed 1969).

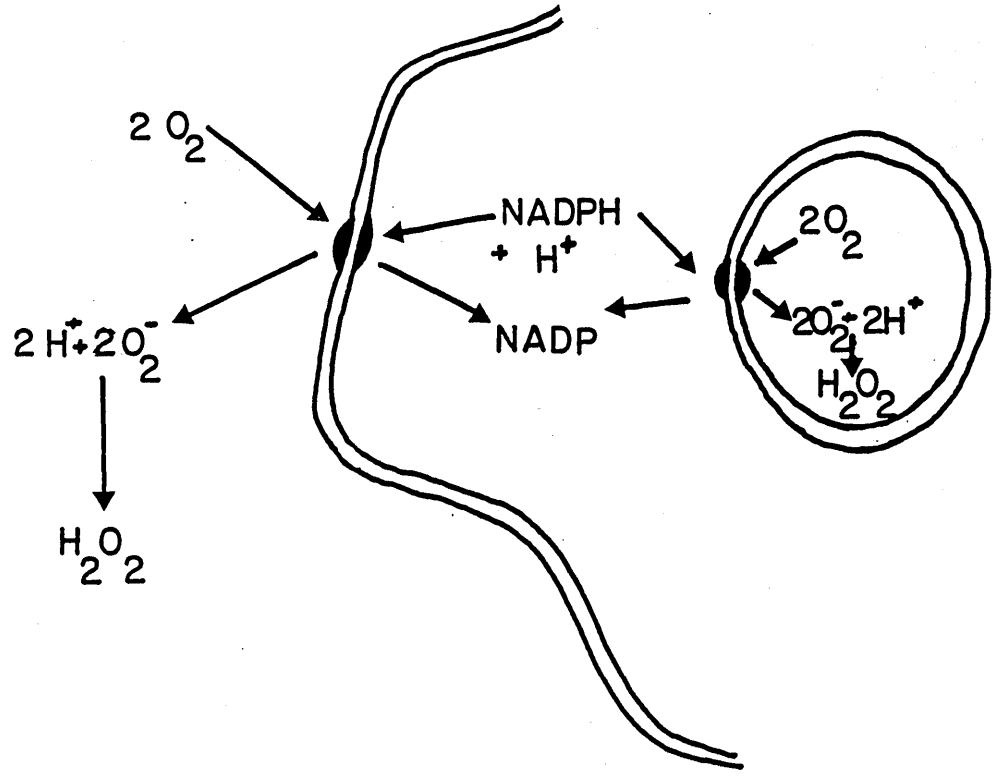


Figure 3 - A model for the localisation of the superoxide anion generating system in human polymorphonuclear leukocytes

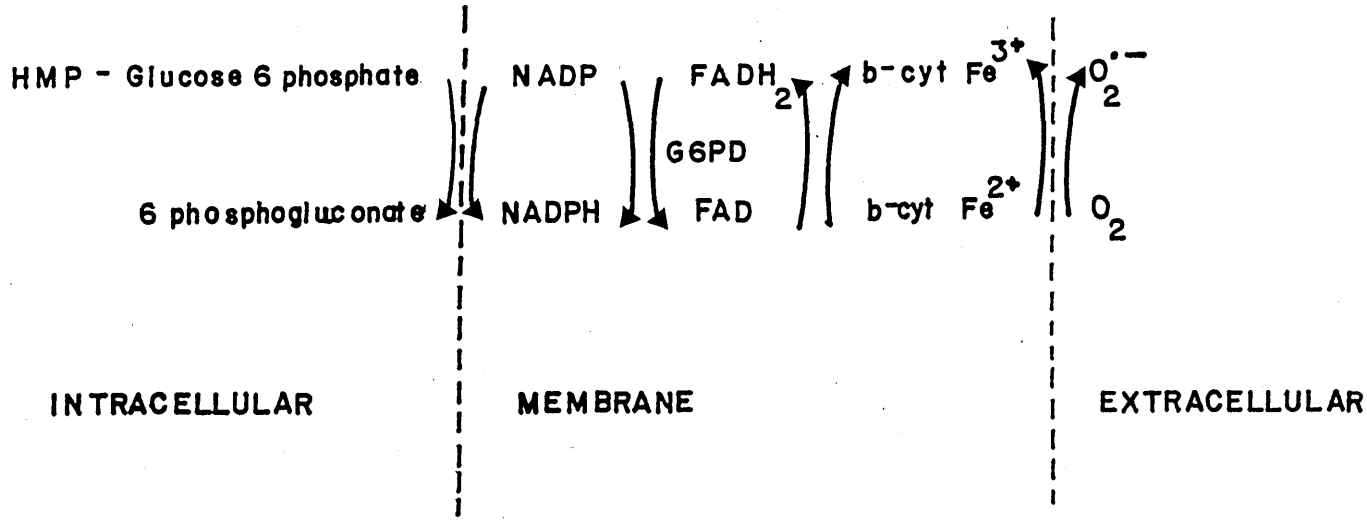


Figure 4 - The inter-relationship of the PMN hexose monophosphate shunt (HMP) and the oxygen consumption/superoxide anion generation activating complex

The production of superoxide anion and hydrogen peroxide

The generation of superoxide anion by human PMN was first suggested by Babior et al (1973) based on the reduction of ferricytochrome c by intact cells and the inhibition of this reduction by superoxide dismutase. The time course of $O_2^{\cdot -}$ production was found to be similar to that of PMN oxygen consumption (Curnette and Babior 1974) and it could be provoked by the same array of particulate and soluble stimuli, suggesting that perturbation of the plasma membrane is again an adequate stimulus (De Chatelet et al 1975, Goldstein et al 1975, Curnette and Babior 1975, Klebanoff and Clark 1978(e)).

In 1975 Weening et al demonstrated that at least 70% of the oxygen consumed by stimulated PMN could be accounted for as $O_2^{\cdot -}$. In the same year Root and Metcalf (1975) showed that at least 70% of the hydrogen peroxide produced by stimulated PMN is derived from $O_2^{\cdot -}$. Demonstration of the involvement of the NADPH oxidase enzyme in the inhibition of the respiratory burst (Babior et al 1976) provided a probable explanation for these observations. Superoxide anion was thus produced by oxidation of NADPH (reaction (1), Fig. 3) and hydrogen peroxide by 'dismutation' of superoxide anion (Babior 1977).

In the dismutation reaction two superoxide anion radicals interact in such a fashion that one anion is reduced and the other oxidised with the resultant formation of H_2O_2 and O_2 (reaction (3)).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \text{------>} H_2O_2 + O_2 \text{ ----- (3)}$$

The superoxide anion is a weak base (pKa = 4.8) (Behar et al 1970). The rate of spontaneous dismutation is therefore affected by pH and is favoured by an acid environment (Badwey and Karnovsky 1980). The acidic pH of the phagocytic vacuole was first reported by Metchnikoff (1905) who observed that litmus turned pink on ingestgion by phagocytes. Jensen and Bainton (1973) showed that the pH fell to 6.5

in three minutes and to pH 4.0 within seven to fifteen minutes of particle ingestion by PMN, thereby facilitating the spontaneous dismutation of superoxide anion to hydrogen peroxide.

Spontaneous dismutation of superoxide anion is slower at neutral pH because of electrostatic repulsion between the two superoxide anions. The reaction can, however, be catalysed by the enzyme superoxide dismutase (McCord and Fridovich 1969). This enzyme (molecular weight 33,000) exists in a cyanide sensitive copper and zinc containing form in the cytosol and a cyanide resistant manganese containing form in the mitochondria of eukaryotic cells (McCord and Fridovich 1969, Fridovich 1974). The rate constant for the interaction of the copper-zinc enzyme with $O_2^{\cdot -}$ is approximately $2x10^9 M^{-1} sec^{-1}$ over the pH range 5-9, which is close to the limit set by diffusion (McCord and Fridovich 1978).

It is not known whether superoxide dismutase is secreted by the PMN into its phagosome, althogh it can be introduced there as a component of an ingested organism (McCord et al 1971). Extracellular fluids are however deficient in superoxide dismutase, containing < 1% of the concentrations found at remarkably constant levels in the cytosol of diverse oxygen utilising cells (McCord et al 1971, Salin and McCord 1977).

Formation of hydroxyl radical and singlet oxygen

The first evidence that human PMN produce hydroxyl radical ($OH^{\cdot}$) during phagocytosis came from studies in which their bactericidal activity was inhibited not only by superoxide dismutase, but by catalase (scavenger of H_2O_2) and mannitol and benzoate, scavengers of $OH^{\cdot}$ (Johnston et al 1975). In 1934 Haber and Weiss as part of their description of the oxidation of H_2O_2 by iron salts (Fenton's reagent) had proposed the following reacton (4) in which hydrogen peroxide is

reduced by superoxide anion to form hydroxyl radical, hydroxyl anion and molecular oxygen (Haber and Weiss 1934)

$$O_2^{\cdot -} + H_2O_2 \text{ -----> } OH^{\cdot} + OH^- + O_2 \text{ ------- (4)}$$

Other investigators (McClune and Fee 1976, Halliwell 1976) concluded that physiologically relevant amounts of $OH^{\cdot}$ could not be produced by this mechanism as the rate constant for the interaction of $O_2^{\cdot -}$ and H_2O_2 (10^{-4} - 3.4 $M^{-1}sec^{-1}$) is substantially below that of the competing spontaneous dismutation of O_2^-.

To circumvent this difficulty, and provide a mechanism for the production of $OH^{\cdot}$ by PMN, McCord and Day (1978) proposed a modification to the 'Haber Weiss' reaction in which a metal (e.g. iron, copper or zinc) served as a redox catalyst. The rections are shown in (5) and (6) where the metal chelate is denoted as 'Me^{n+} chelate'.

$$Me^{n+} \text{ chelate} + O_2^- \text{ -------> } Me^{(n-1)+}\text{chelate} + O_2 \text{ -----(5)}$$

$$Me^{(n-1)+}\text{chelate} + H_2O_2 \text{ -------> } Me^{n+}\text{chelate} + OH^{\cdot} + OH^- \text{ -----(6)}$$

The most definitive evidence for the production of $OH^{\cdot}$ by stimulated human PMN was provided by Weiss et al (1978) who observed that its formation (as detected by comparison of the nonaldehyde 2 keto - 4 thio - methylbutyric acid to ethylene) was inhibited by both catalase (74%) and superoxide dismutase (44%).

The formation of <u>singlet oxygen</u> by PMN has also been proposed as a consequence of the interaction of superoxide anion, hydrogen peroxide and hydroxyl radical produced during the respiratory burst, and also as a consequence of the myeloperoxide - H_2O_2 - chloride reaction (vide infra).

Singlet oxygen is formed by the absorption of energy by molecular oxygen such that the two outer electrons occupy the same or different orbitals. In the delta form ($^1\Delta g\ O_2$) the two outer electrons occupy

the same orbital and are of opposite spin, leaving the previously occupied outer orbital empty. In the sigma form ($^1\Sigma^+_g O_2$) the two outer electrons occupy separate orbitals but are of opposite spin. Sigma singlet oxygen is more unstable and has a shorter half life (10^{-11} sec) than delta singlet oxygen (2×10^{-6} sec) which is therefore thought to be the most chemically reactive in biological systems (Foote 1976).

Production of singlet oxygen by the spontaneous dismutation of $O_2^{\cdot -}$ (reaction (7)) is controversial (Kahn 1970). The Haber Weiss reaction has however been 'modified' (reaction (8)) to allow the production of both $O_2^{\cdot -}$ and $OH^{\cdot}$. Singlet oxygen may also be formed from the interaction of $O_2^{\cdot -}$ with either hydroxyl radicals (reaction (9)) or diacyl peroxides (reaction (10)) (Harrison and Schultz 1976).

$$HO_2 + O_2^- \text{------>} (^1\Delta_g O_2) + H_2O_2 \text{-------- (7)}$$

$$O_2^{\cdot -} + H_2O_2 \text{------>} (^1\Delta_g O_2) + OH^- + OH^{\cdot} \text{-------- (8)}$$

$$O_2^{\cdot -} + OH^{\cdot} \text{------>} (^1\Delta_g O_2) + OH^- \text{------- (9)}$$

$$2O_2^{\cdot -} + \text{R-COOCC} \text{------>} 2(^1\Delta_g O_2) + 2RCO_2^- \text{------- (10)}$$

Myeloperoxidase catalysed oxygen radical reactions

The PMN azurophil granule component myeloperoxidase is released into the phagosome following phagocytosis and is also found in the supernatant fraction following centrifugation (Baehner, Karnovsky and Karnovsky 1969) indicating extracellular release of the enzyme. Myeloperoxidase has been found to catalyse both halogenation and oxidation reactions resulting in the production of oxygen radicals and other reactive oxygen metabolites implicated in bacterial killing (Klebanoff and Clark 1978(e)).

Halogenation reactions Klebanoff (1967) was the first to show that incubation of an inorganic iodide with phagocytosing PMN resulted in

part of the iodide becoming protein bound. The prominent halogen receptor group in peroxidase mediated iodination is the tyrosine residue with formation of mono- and di- iodotyrosine (Morrison and Schonbaum 1976). Histidine and thiol residues and polar and neutral lipids may also be iodinated (Fawcett 1974, Klebanoff and Clark 1978(e)).

Oxidation reactions The known products of PMN myeloperoxidase catalysed oxidation reactions comprise (i) halogens (ii) hypohalous acids (iii) chloramines (iv) aldehydes and (v) singlet oxygen.

(i) Halogens Halide ions are readily oxidised by myeloperoxidase and H_2O_2 to form iodine, chlorine or bromine (Klebanoff and Clark 1978(e)). All are germicidal (Dunn 1952) and chlorine derivatives have been shown to inhibit a variety of enzymes which depend on thiol groups for their activity (Green and Stumpf 1946, Knox et al 1948).

(ii) Hypohalous acids Myeloperoxidase (MPO) has two haem groups per molecule. Its catalysis of the oxidation of chloride by H_2O_2 to form free hypochlorous acid has been depicted by reaction (11) (Agner 1958, Harrison and Schultz 1976, Paul 1963). Hypochlorous acid can dissociate further to the hypochlorite anion (OCl^-) (Klebanoff and Clark 1978(e)).

(iii) Chloramines Inorganic chloramines are formed by the reaction of hypochlorous acid with amonia, and organic chloramines by its reaction with amine, amide, imine or imide groups to form an N-chloro compound (Zgliczynski et al 1971, Klebanoff and Clark 1978(e)). Chloramines hydrolyse continously with release of hypochlorous acid (Sykes 1965) and may therefore act as a store of 'available chlorine', prolonging the toxicity of the peroxidase system. while most chloramines are unstable,

$$MPO^{-Fe} + Cl^- + H_2O_2 + H^+ \longrightarrow MPO^{-Fe\,HOOH}_{Fe\,Cl} \longrightarrow MPO^{-Fe}_{Fe} + H_2O + HOCl \quad \text{-------(11)}$$

reaction of the myeloperoxidase - H_2O_2 - chloride system with the amino acid taurine, present in PMN in relatively high concentration (Iyer 1959) has been found to produce a stable chloramine (Taurine chloramine) (Zgliczynski et al 1971).

(iv) Aldehydes - Zgliczynski et al (1971) also provided evidence for the spontaneous decomposition of chloramines to form NH_3, CO_2, chloride and the corresponding aldehyde (reaction (12))

$$CH - NH - Cl + H_2O \text{ ------> } RCHO + NH_4 + CO_2 + Cl^- \text{ ---- (12)}$$

(v) Singlet oxygen Evidence for the formation of singlet oxygen from the myeloperoxidase - H_2O_2 - halide system (reaction (13)) (Klebanoff and Clark 1978(e)).

$$OCl^- + H_2O_2 \text{ ------> } Cl^- + H_2O + (^1\Delta g\ O_2) \text{ ----- (13)}$$

has comprised (a) the conversion by this system of diphenylfuran to cis - dibenzoylethylene (King, Lai, McCay 1975), (b) inhibiting this reaction by singlet oxygen quenchers and (c) its stimulation by D_2O an agent which prolongs the lifetime of singlet oxygen in solution (Rosen and Klebanoff 1977). In addition singlet oxygen scavengers have been found to have an inhibiting effect on the bactericidal effect of the myeloperoxidase system (Klebanoff 1975).

Summary

Stimulation of human PMN with particulate or soluble agents is associated with a marked increase in O_2 consumption, activation of the hexose monophosphate shunt, and production of $O_2^{\cdot -}$. The latter dismutates to H_2O_2 with which it may interact to form $OH^{\cdot}$ and $^1\Delta g\ O_2$. H_2O_2 participates in the myeloperoxidase catalysed oxidation of chloride ions with the further formation of halogens, hypohalous

acids, chloramines and aldehydes all of which are capable of damaging foreign and host tissues. The production of these active moieties consequent upon the PMN respiratory burst are summarised in Fig 5.

1.2.3 Oxygen radicals - Potential for Disease Pathogenesis

In this section the potential for oxygen radicals and other reactive oxygen metabolites to damage host tissues is investigated. Firstly, evidence that they may react adversely with cell membranes and constituents of the intra or extra cellular milieu will be reviewed. Secondly, any in vivo mechanisms which may prevent these reactions will be examined. Thirdly diseases in which it has been proposed that oxygen radicals may play a key role will be examined.

1) Adverse oxygen radical reactions

The majority of documented adverse oxygen radical reactions can occur intracellularly (Fig. 6). Most of the information on nucleic acid degradation has been derived from observation of the effects of ionising radiation which disrupts water molecules to form the hydroxyl radical (Pryor 1976). Superoxide dismutase and catalase thus protect against DNA damage (Van Hemman and Meuling 1975). Both the hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1\Delta g\ O_2$) are capable of reacting with nucleic acids (Lynch and Fridovich 1978, Cadet and Teoule 1978). It has been suggested that $OH^{\bullet}$, produced physiologically from intracellular reaction of $O_2^{\bullet -}$ and H_2O_2 may be the root cause of cellular mutagenesis and may have been part of the selection pressure for the evolution of a variety of DNA repair mechanisms.

Both intracellular and plasmalemnal cell membranes contain large amounts of polyunsaturated fatty acids (Rouser et al 1968) which can undergo peroxidative injury involving $O_2^{\bullet -}$, $OH^{\bullet}$ and $^1\Delta g\ O_2$ (Fong et

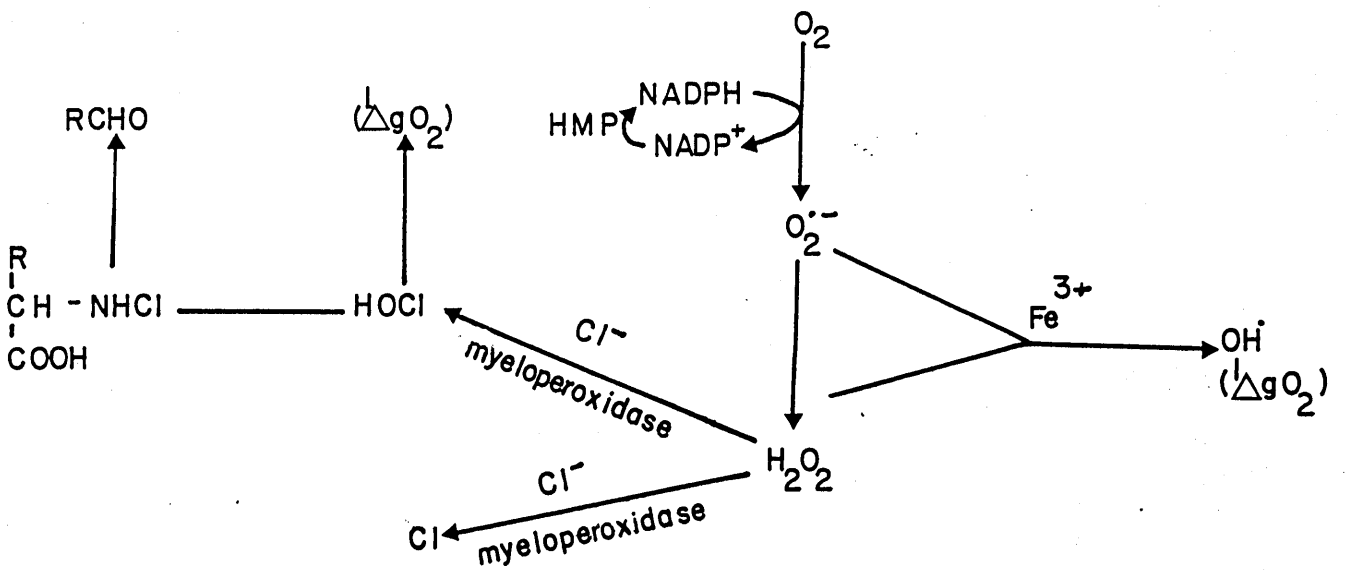


Figure 5 - Production of oxygen radicals and reactive oxygen metabolites by the PMN respiratory burst

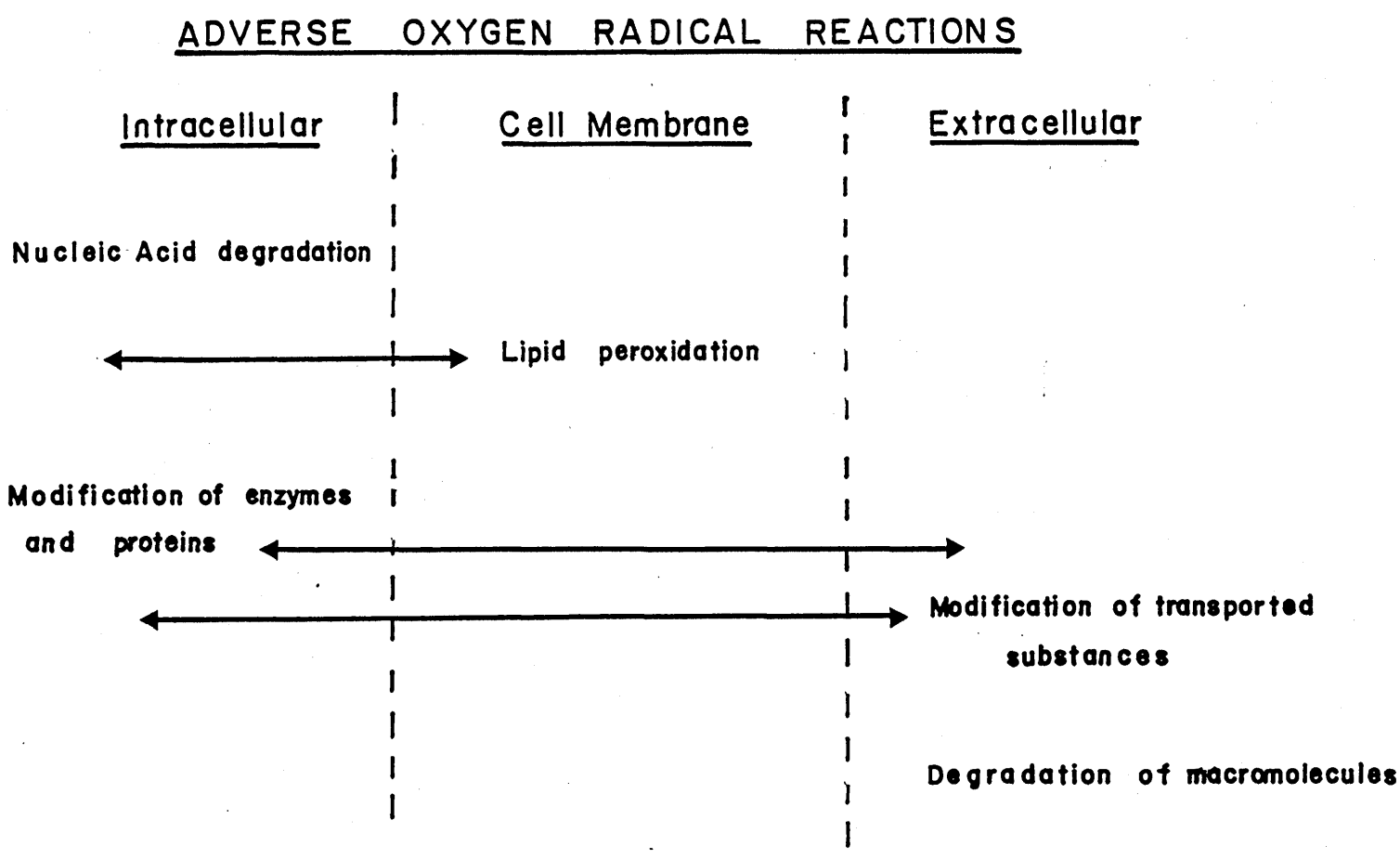


Figure 6 - Location of adverse oxygen radical reactions in vivo

al 1973, Kellogg and Fridovich 1975, Lynch and Fridovich 1978) in both non enzymatic and enzymatic mechanisms (Mead 1976).

The hydroxyl radical can initiate a lipid peroxidation chain reaction by its ability to abstract a hydrogen atom (Pryor and Tang 1978, Cram and Hammond 1959) from the allylic position of an unsaturated lipid (R_1H) thus forming an organic free radical ($R_1^{\bullet}$) (Fig. 7). Subsequent diene conjugation and incorporation of molecular oxygen leads to the formation of the corresponding lipid peroxide radical (R_1OOO) which propagates the reaction by abstracting a hydrogen atom from a second unsaturated lipid (R_2H), forming the more stable lipid hydroperoxide (R_1OOH) in the first chain and an organic free radical ($R_2^{\bullet}$) in the second chain (Fig. 7). Lipid hydroperoxide may be broken down to yield stable end products which include ethane, pentane, propane and malondialdehyde (Chance et al 1979). Singlet oxygen ($^1\Delta g\ O_2$) can react directly with polyunsaturated fatty acids to form hydroperoxides (Cram and Hammond 1959) and superoxide anion ($O_2^{\bullet -}$) can propagate lipid peroxidation by reacting with lipid hydroperoxides (ROOH) to form an alkoxy radical ($RO^{\bullet}$) which can function analagously to $OH^{\bullet}$ by abstracting a hydrogen atom from an unsaturated fatty acid leading to the generation of further lipid hydroperoxide (Pryor and Tang 1978, Cram and Hammond 1959).

The consequences of cell membrane lipid peroxidation include (i) membrane perturbation with disruption of cellular integrity (Tappel 1973, Chance et al 1979); (ii) inhibition of enzyme activity by hydroperoxides (McKnight and Hunter, 1966, Chance et al 1979), (iii) reaction of lipid hydroperoxide breakdown products such as malondialdehyde which may be oxidised or form a Schiff base with eg amino group of lysine (Chance, Sies and Boveris 1979, Tappel 1973); (iv) activation of membrane enzyme phospholipase A_2 with subsequent

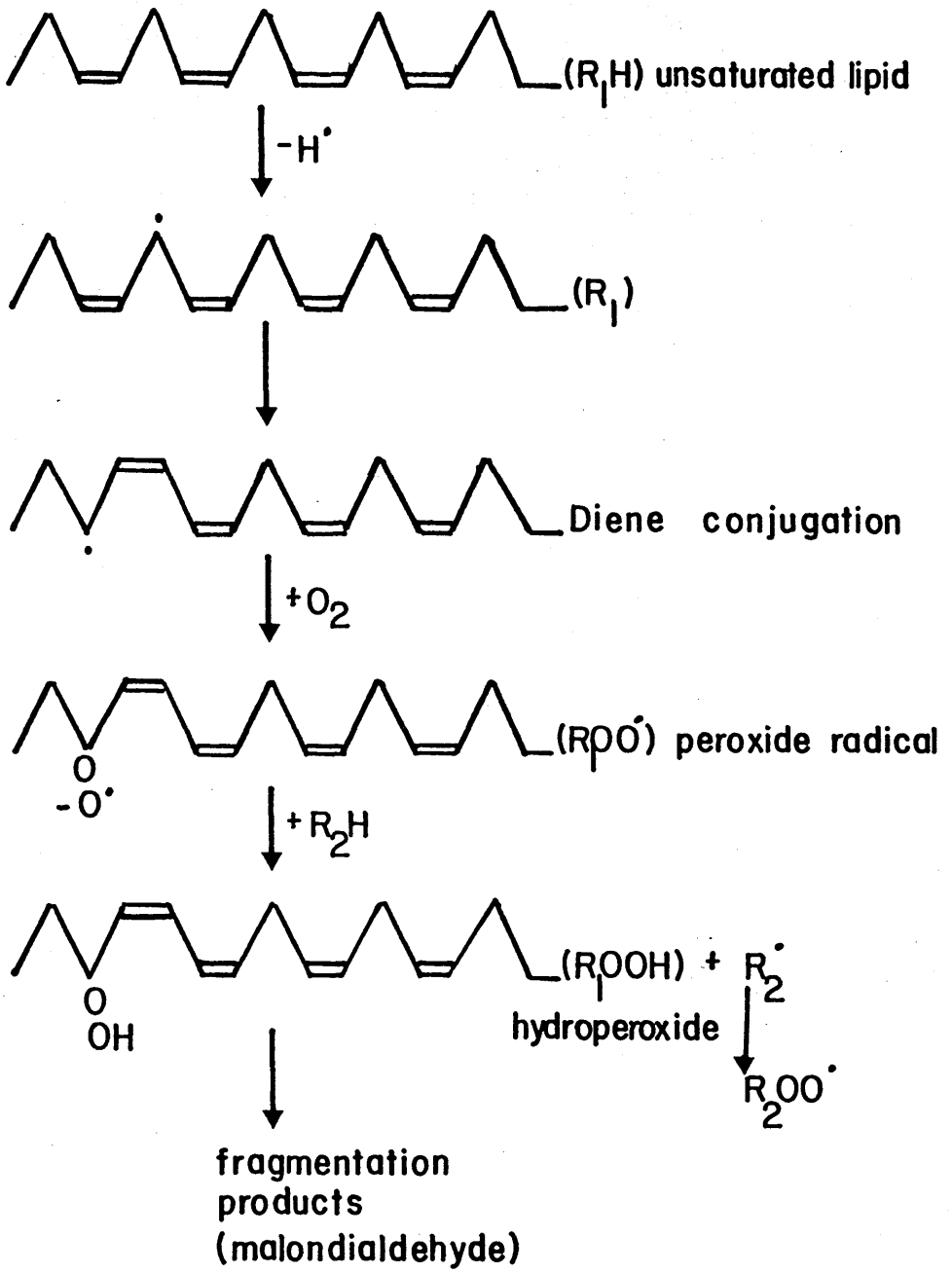


Figure 7 - Pathway of lipid peroxidation

production of arachidonic acid and prostaglandin synthesis (Del Maestro et al 1980).

Structural modificaiton of enzymes and proteins may occur both intra-and extracellularly by reaction of oxygen radicals and products of the PMN myeloperoxidase H_2O_2 - chloride system with (i) sulphydryl groups (Jocelyn 1970, Green and Stumpf 1946) to form disulphides sulphenic or sulphonic acids (Klebanoff and Clark 1978(e)) (ii) amino groups to form chloramines, nitrites, aldehydes or dichloropeptides (Pereira et al 1973) and (iii) amino acid residues tryptophane and histidine (Michelson 1973, Hodgson and Fridovich 1974), and tyrosine residues which may be halogenated (Zgliczynski and Stelmaszynska 1975).

Oxygen radicals may also react with substances (e.g. lipids) transported by large proteins. Petrone et al (1980) have reported superoxide anion dependent activation of a PMN chemotactic factor in plasma which they have localised to a chloroform extractable component bound to albumin.

The extracellular macromolecules hyaluronic acid and cartilage proteoglycans have been shown to be degradable by oxygen radicals generated acellularly in vitro (McCord 1974, Greenwald, Moy and Lazarus 1976). Superoxide anion can also impair collagen gelation in vitro (Greenwald and Moy, 1979, Venkatasubramanian and Joseph 1977).

2) In vivo protective mechanisms

These may be divided into 'structural' and 'scavenging' mechanisms. The former are intimately associated with the structural integrity of living cells, disruption of which leads rapidly to rancidification via lipid peroxidation of the membrane lipids (Dormandy 1978). The intracellular membranes localise certain oxygen radical reactions to mitochondria or peroxisomes (the latter are not

found in the PMN) where scavenging mechanisms and metal chelates occur in high concentrations.

Oxygen radical scavenging mechanisms can be divided into 'endogenous' or physiological scavengers (Fig 8) and 'exogenous' scavengers ingested in food or drugs (Slater 1972(a), Dormandy 1978). The endogenous scavengers are hydrophoboic (and thus cell membrane bound) or hydrophilic (Fig 8). The cell membrane scavengers particularly scavenge the products of lipid peroxidation. Vitamin E is the name given to a number of chemical compounds, the most active being α-tocopherol, which readily inhibit lipid peroxidation in microsomal suspensions in vitro by oxidation to tocopherolquinone. (Hochstein and Ernster 1963). In vivo, dietry deficiency of vitamin E is associated with increased lipid peroxidation (Mead 1976). The carotenes are precursors of the vitamin A. The double bond systems of all carotenoids are readily oxidised, protection being offered in vivo by vitamin E. Hove (1953) showed that β carotene was destroyed on incubation with linoleate hydroperoxide. Forbes and Taliaferro (1945) showed that a diet rich in carotenes prevented rats from carbon tetrachloride hepatotoxicity which is attributed to a free radical mechanism. It has also been proposed that α tocopherol and β carotene may quench $^1\Delta g\ O_2$ (Halliwell 1978). Ubiquinone forms part of the mitrochondrial respiratory chain and has been shown experimentally to inhibit lipid peroxidation (Slater 1972(b)) and to compare favourably in efficacy with α tocopherol (Mellors and Tappel 1966).

The hydrophilic scavengers occur predominantly intracellularly (Fig 8). This particularly applies to the specialised scavengers superoxide dismutase and catalase (Table 5) and the glutathione peroxidase/glutathione reductase systems. Catalysis of the dismutation of superoxide by superoxide dismutase was reviewed in

Principal Locations of known physiological Oxygen Radical Scavengers

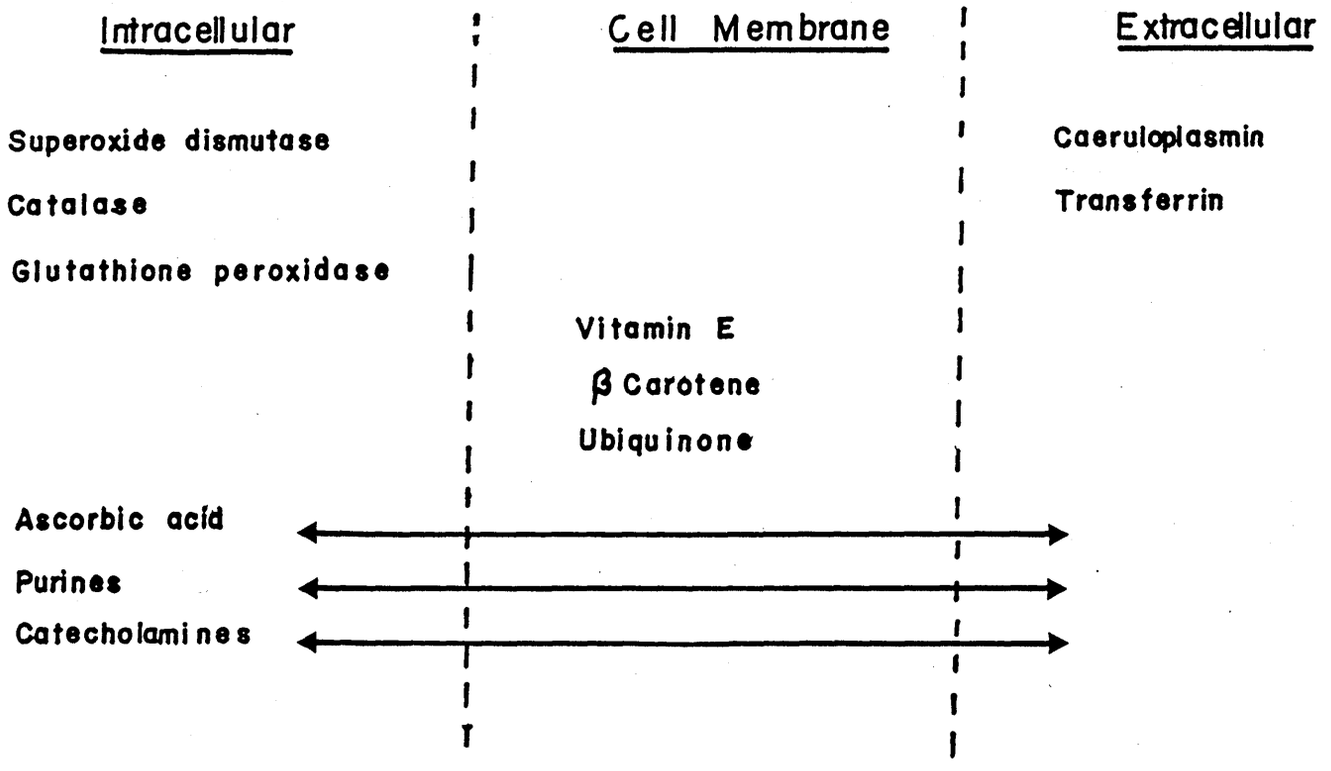


Figure 8

Table 5 Comparison of intracellular and extracellular concentrations of superoxide dismutase and catalase in human tissue

	Erythrocytes	Plasma	Synovial fluid	Cerebrospinal fluid	Aqueous humor	Reference
Superoxide dismutase mg/l	100mg/l (3μM)	0.7mg/l (0.02μM)	1.0mg/l (0.03μM)	0.23mg/l (0.007μM)	0.9mg/l (0.027μM)	Salin and McCord 1977
	58.1mg/l (1.8μM)	--	--	--	--	Michelson et al 1977
Catalase mg/l	509.5mg/l (2.1μM)	—	—	—	—	Michelson et al 1977
	--	0.8mg/l (0.003μM)	none detected	—	none detected (bovine)	McCord 1974

1.2.3. Catalase (molecular weight 240,000) is found in all mammalian cells and can exert both catalatic and peroxidatic functions by the formation of an intermediate compound I (Chance et al 1979). Reactions (14) and (15) describe the catalatic action of catalase while reactons (14) and (16) describe its peroxidatic action.

$$\text{Catalase } Fe^{3+} + H_2O_2 \text{ ------> Compound I --------- (14)}$$

$$\text{Compound I} + H_2O_2 \text{ ------> Catalase } Fe^{3+} + 2H_2O_2 + O_2 \text{ --------- (15)}$$

$$\text{Compound I} + AH_2 \text{ ------> Catalase } Fe^{3+} + 2H_2O_2 + A \text{ --------- (16)}$$

The selenoprotein glutathione peroxidase (molecular weight 46000) catalyses the reaction of H_2O_2 (and other hydroperoxides, ROOH) with reduced glutathione to form glutathione disulphide and the reduction product of the hydroperoxide (reaction (17)) (Mills 1957, Flohé et al 1977).

$$2GSH + H_2O_2 \text{ ------> } 2H_2O_2 + GSSG \text{ --------- (17)}$$

In the steady state regeneration of GSH by reduction of GSSG is provided by the NADPH dependent GSSG reductase

Extracellularly, caeruloplasmin and transferrin the plasma transporters of copper and iron respectively, have been shown also to exert an inhibitory effect on oxygen radical generation which is attributable to their blocking the catalytic action of metals in oxygen radical generation (Dormandy 1978). Caeruloplasmin, an $\alpha_2$glycoprotein of molecular weight 150,000 present in human plasma at a concentration of 300µg/ml, not only transports most plasma copper (Goldstein et al 1978), but also converts ferrous to ferric ions (Osaki et al 1966). Caeruloplasmin has been shown to inhibit lipid autoxidation (Al Timini and Dormandy 1977) and scavenge superoxide anion produced by the xanthine oxidase catalysed oxidation of hypoxanthine (Goldstein et al 1978). Transferrin (molecular weight 85,000) is fully iron saturated only in disease, and has been found to

exert an inhibiting effect on the autoxidation of lipids which is proportional to its activity in the iron free fraction alone (Dormandy 1978). It has been proposed that transferrin thereby behaves as a natural iron chelator.

A number of other molecules occurring both intracellularly and extracellularly have been shown to have oxygen radical scavenging abilities (Fig. 8). Ascorbic acid (A) is a mild reducing agent and readily undergoes oxidation to dehydroascorbic acid (DHA) in a two stage reaction with the intermediate formation of monodehydroascorbic acid radical (MDHA) (reaction (18)) (Slater 1972(a)), which is able to act as a scavenger by reaction with a second radical ($R^2H^\bullet$) to form dehydroascorbic acid and a stable compound (RH_2) (reaction (19))

$$R^1H^\bullet \text{ ------> } R^1H_2 + MDHA^\bullet \text{ --------- (18)}$$

$$R^2H^\bullet + MDHA^\bullet \text{ ------> } R^2H_2 + DHA \text{ --------- (19)}$$

Ascorbic acid, however, readily oxidises in air. This reaction is increased by the presence of metal cations with the resultant formation of $O_2H^\bullet$ radicals (Slater 1972). Ascorbic acid in the presence of metal ions can thereby stimulate lipid peroxidation.

Purine bases have been shown to exert a pronounced antioxidant effect on the autoxidation of linoleic acid (Matsushita 1963), and it has been suggested that purine bases in DNA can inhibit the peroxidative destruction of their neighbouring pyrimidine bases (Slater 1972). Of the catecholamines, both norepinephrine and epinephrine have been shown to scavenge superoxide anion which catalyses the autoxidation of the neurotoxin 6-hydroxydopamine in sympathetic nerve terminals (Cohen and Heikkla 1977). Epinephrine is oxidised by superoxide anion to form the coloured adrenochrome. This reaction is inhibitable by superoxide dismutase (McCord and Fridovich

1969).

3) Involvement of oxygen radicals in disease pathogenesis

Oxygen radicals thus have the potential not only to destroy cellular architecture (by membrane lipid peroxidation) but also to damage or inactivate molecules in the intra- or extra-cellular milieu. This potential is turned to advantage by the body in the oxidative killing of bacteria ingested by phagocytes. Oyxgen radicals may also be essential participants in certain beneficial chemical reactions (Taniguchi et al 1977). It has been proposed that oxygen radicals are involved in disease pathogenesis by virtue of their presence in vivo in increased or decreased concentrations, intracellularly or extracellularly (Table 6) (Del Maestro 1980). At present, however, direct evidence for the involvement of oxygen radicals in any disease process is conspicuously absent.

Increased intracellular oxygen radicals

In this group of diseases there may be either a deficiency of the intracellular protective mechanisms (e.g. Vitamin E or β carotene deficiency) or the mechanisms may simply be overwhelmed by an increased flux of radical species. Examples of hyperoxygenation includes the toxic effects of hyperbaric oxygen (Gerschman et al 1954). and retrolental fibroplasia of neonates (Del Maestro 1980). Hypo-oxygenation of tissues occurs in myocardial or cerebral ischaemia and may permit an increase in mitochondrial reduction of oxygen by the univalent (rather than tetravalent), pathway which may be exacerbated by reperfusion and exposure to relatively large concentrations of oxygen (Del Maestro 1980). Paraquat (methyl viologen) and certain drugs (e.g. streptonigrin and adriamycin) are among the chemicals which are known to increase intracellular superoxide anion flux (Fridovich 1978). Carbon tetrachloride causes lipid peroxidation

Table 6 Classification of diseases in which increased or decreased oxygen radical concentrations have been implicated.

	Intracellular	Extracellular
Increased oxygen radicals	1) Hyperoxygenation 2) Hypoxygenation 3) Chemicals 4) Vitamin Deficiency 5) Aging	1) Acute inflammation 2) Chronic inflammation
	1) Radiation 2) Chemical carcinogens	
Decreased oxygen radicals	1) Trisomy 21 2) Psychiatric disease	1) Chronic granulomatous disease 2) Myeloperoxidase deficiency

(Slater 1972 (c)). Aging and the laying down intracellularly of the non degradable age pigments such as lipofuschin are considered to be the universal consequence of our chronic exposure to the reduction products of molecular oxygen (Mead 1976).

Increased extracellular oxygen radicals

These diseases are characterised by inflammation which includes the chemoattraction of phagocytes (predominantly PMN) to a site of insult which may be acute in the case of infections or burns and chronic in 'inflammatory' diseases such as rheumatoid arthritis or ulcerative colitis. While the production of oxygen radicals by PMN is largely beneficial in acute inflammation, it has been proposed that their release to the extracellular milieu, where there is a relative paucity of scavenging mechanisms, may exacerbate or perpetuate chronic inflammatory conditions. An examination of the role of oxygen radicals in the pathogenesis of rheumatoid arthritis forms the remainder of this thesis.

Increased intracellular and extracellular oxygen radicals

Ultraviolet or isotopic irradiation results in the generation of oxygen radicals in the aqueous compartments both intra- and extracellularly. Both nucleic acid degradation and lipid peroxidation have been shown to result from the hydroxyl radicals (and possibly singlet oxygen) so formed. Chemical carcinogens include oxygen radicals contained within cigarette smoke (Lyons and Spence 1960).

Decreased oxygen radicals

The gene for copper-zinc superoxide dismutase is located on human chromosome 21 and individuals suffering from Down's syndrome (trisomy 21) have been found to have 50% more intracellular superoxide dismutase (Michelson et al 1977). Michelson et al (1977) found that patients with various psychoses also have increased superoxide

dismutase levels and proposed that their illness was related to a consequent decreases in the availability of superoxide anion to participate in essential chemical reactions (Taniguchi, Hirata and Hayaishi 1977).

Decreased extracellular generation

Studies over the last fifteen years have disclosed a number of inherited conditions in which there is deficiency in one or other of the enzymes involved in the oxidative killing mechanisms of phagocytes (Babior 1978). Chronic granulomatous disease which may have x linked or autosomal recessive inheritance is characterised by an absence of all features of the respiratory burst and is attributed to a deficiency either of the primary oxygen consuming reaction (catalysed by NADPH oxidase) or of the enzyme's activation (b-cytochrome, and others) (Babior 1978, Segal and Allison 1979). The condition is manifest in early childhood by an increased susceptability to pyogenic infections, particularly with staphylococci and enterobacteriaceae. Patients with myeloperoxidase deficiency have low or absent PMN myeloperoxidase. Upon stimulation the PMN demonstrate a normal or prolonged respiratory burst. Subsequent killing of bacteria is complete although markedly delayed in vitro. Patients with this deficiency are rarely troubled by infection. Other PMN oxidative killing enzyme deficiencies include glucose-6-phosphate dehydrogenese deficiency, glutathione reductase deficiency and glutathione peroxidase deficiency (Babior 1978).

1.3 Oxygen Radicals in Rheumatoid Arthritis

1.3.1 The PMN at sites of rheumatoid disease activity

Rheumatoid arthritis is a chronic inflammatory disease of unknown aetiology which affects up to 3.4% of the adult human population

worldwide (Beasley, Wilkens and Bennett 1973), females three times more commonly than males (Wood 1970). The articular features of the disease predominate over the extraarticular with attendant pain, stiffness, deformity and compromised function of the affected joints. Diagnosis is based on an aggregate of clinical, pathological and radiological features (Ropes et al 1959), the composition of which varies from patient to patient and none of which singly is specific for rheumatoid arthritis.

A comprehensive account of the variable clinical presentation, evolution and current therapy of rheumatoid arthritis does not fall within the remit of this thesis but may be found in the many standard texts (Kelley et al 1981). The intra-articular abnormalities which culminate in the erosion and destruction of cartilage are summarised in Table 7. Full discussion of the numerous immunologic abnormalities which have been recognised in association, and the evidence for involvement of infection or heredity in its aetiology are available in several contemporary reviews (Harris 1981, Bennett 1978, Brewerton 1984).

In this thesis, of necessity, attention is focussed on the polymorphonuclear leukocyte and its stimulation by chemotactic complement components and immune complexes, in order that the role of oxygen radicals produced by these cells in the pathogenesis of rheumatoid arthritis may be evaluated. Polymorphonuclear leukocyes occur particularly in synovial fluid (Krey and Bailen 1974) but may also be found in the synovitis of early disease (Schumacher and Kitridou 1972), the synovial membrane and pannus of established disease (Mohr et al 1981(a)), the articular cartilage (Ohno and Cooke 1978, Mohr and Wessinghage 1978) rheumatoid nodules (Mohr, Kohler and Wessinghage 1981) and vasculitis (Soter et al 1976). Evidence for a

TABLE 7

Articular Abnormalities in Rheumatoid Arthritis

Synovium and sub-synovial tissue	Synovial Fluid	Articular Cartilage Subchondrial Bone
Hypertrophy	Excessive production	
Hyperaemia	Increased inflammatory cells - PMN predominate	
Villus formation		
Mononuclear cell	Immune complexes	
Infiltrate	Complement	
Local IgG synthesis + complement	Lymphokines Kinins	
Pannus (vascular sub-synovial extension over articular cartilage)	enzymes enzyme inhibitors prostaglandins	Cartilage erosion by pannus
Degradative enzyme production		Subchondrial bone resorption
		Secondary Osteoarthritis
		Ankylosis

central role for these cells in the pathogenesis at least of the articular lesions can be gained from the apparent amelioration of arthritis in the neutropaenia of Felty's syndrome (rheumatoid arthritis, neutropaenia and spenomegaly) (Felty, 1924).

Evidence for the stimulation of PMN at the above sites of disease activity has been provided by the demonstration either of immunoglobulin or complement inclusions within the cells or of granular enzymes released extracellularly.

Synovial effusions in patients with established rheumatoid arthritis frequently contain more than 10,000 leukocytes per cubic millimeter, the majority of which are PMN (Krey and Bailen 1979). PMN arrive in the synovial fluid by migration through capilliary or venular walls within the synovium (Harris 1981), attracted chiefly by chemotactic components of the complement pathway, in particular (C567 and C5a (Zvaifler 1974) (1.2.1). The PMN do not leave the joint by returning to the circulation; instead they appear to be destroyed in situ within the synovial effusion (Bertino et al 1963). It has been calculated tht the half life of PMN in the rheumatoid synovial cavity is between six and seven hours (Bertino et al 1963) and that in a rheumatoid effusion containing 25,000 cells/mm^3 the rate of breakdown of cells in the synovial cavity may exceed one billion cells per day (Harris 1981). The mechanism of this postulated destruction, which has not been measured or observed directly is unknown.

Large numbers of rheumatoid synovial fluid PMN are vacuolated and extensively degranulated (Bodel and Hollingsworth 1966) and contain intracytoplasmic complexes consisting of immunoglobulins, complement and rheumatoid factor (Hollander et al 1965). It is therefore proposed that their lysosomal enzymes are released extracellulraly not only by cell death, but during the phagocytosis of

immune complexes

Numerous antibody specificities have been reported in rheumatoid synovial complexes including IgG and IgM antiglobulins, antibodies to $F(ab^1)2$, nuclear antigens and viral components, but no antigen other than IgG itself has been identified in association with the antibodies (Male et al 1980).

Rheumatoid factors, antiglobulins reacting with the Fc portion of autologous 1gG, are the most consistent immunological abnormality in patients with rheumatoid arthritis. At a dilution of serum which excludes 95% of the normal population, at least 70% of patients with rheumatoid arthritis as diagnosed by other criteria will be seropositive for IgM rheumatoid factor (Valkenburg et al 1966), the majority of the remainder being seropositive for 1gG rheumatoid factor (Torrigiani et al 1970).

Up to 50% of rheumatoid synovial membrane plasma cells make antibody to IgG (Munthe and Natvig 1972). Immune complexes are uniformly of higher concentrations and larger size in the synovial fluid than the serum (Zubler et al 1976, Gabriel and Agnello 1977). Pope, Teller and Mannik (1974) drew attention to the unique ability of IgM rheumatoid factor to self associate. It has been proposed that the high concentration of locally produced rheumatoid factor enables the formation of large, self associating aggregates of IgG anti IgG in synovial fluid, whereas in serum the abundance of normal 1gG encourages the formation of small complexes (Hay et al 1979, Male, Roitt and Hay 1980).

Several complement components are also synthesised locally in rheumatoid synovium (Ruddy et al 1969). The pattern of synovial fluid complement levels in rheumatoid arthritis is none-the-less indicative of its activation by both classical and alternate pathways (Zvalfler

1974). There is little evidence of complement activaiton in serum, however, except in the presesnce of extraarticular disease (Hunder and McDuffie 1973).

Tesar and Schmid (1970) demonstrated that the major share of complement activation by immune complexes in rheumatoid arthritis was attributable to the presence of IgM rheumatoid factor. IgM rheumatoid factor has been found in close spatial relationship to deposits of IgG and components of the complement system in the synovial membrane of patients with rheumatoid arthritis (Rodman et al 1967). It is thought that the large self associating 1gG anti IgG aggregates of rheumatoid synovial fluid are stabilised by polyvalent binders of the 1gG Fc moiety such as IgM rheumatoid factor and C1q (Male et al 1980). The hypocomplementaemia of extra articular rheumatoid disease is in turn attributable to its fixation by circulating immune complexes resulting from local extra articular antiglobulin formation (Nowoslanski and Brzoski 1967).

The synthesis of immunoglobulin and complement by rheumatoid synovial membrane, production of immune complexes, activation of synovial fluid complement, chemoattraction of PMN's and phagocytosis of immune complexes with consequent release of granular enzymes, known to be capable of degrading cartilage matrix (Barret 1978, Ohlsson 1978) appear to provide a mechanism for the cartilage erosion of rheumatoid arthritis (Table 7). The demonstration of antiproteases within the synovial fluid and the lack of correlation of proteolytic enzyme levels with erosions have however, cast doubt upon this hypothesis (Harris 1981).

More substantial evidence has been provided for involvement of granular enzymes produced by PMN within synovial membrane, pannus and articular cartilage in rheumatoid joint destruction, although the

latter has more frequently been attributed to products of synovicytes, mononuclear leukocytes and chondrocytes (Harris et al 1975, Vaes et al 1977, Dingle 1978).

PMN are, in fact, found rarely in the synovial membrane of established rheumatoid arthritis. Histochemical staining has shown that in occasional rheumatoid samples up to 2.6 PMN per 100 cells may be found (Mohr and Wessinghel 1978). Saklatvala and Barrett (1980) however showed that at least half the neutral protease activity of rheumatoid synovial membrane can be attributed to neutrophil derived cathespin G and elastase. In 1981 Mohr and co-workers demonstrated PMN within the pannus, pannus cartilage junction and articular cartilage of metacarpo- and metalarsophalangeal joints of three patients with rheumatoid arthritis. Ohno and Cooke (1978) had previously demonstrated amorphous material giving positive immunofluorescence staining for 1gG, 1gA and complement within rheumatoid articular cartilage which was also locally invaded by PMN, found on electron microscopy to be phagocytosing such aggregates. Menninger et al (1980) showed release of PMN neutrophil derived elastase into cartilage subadjacent to pannus by cytochemical and immunocytochemical techniques.

Extra articularly, PMN are found in the perivascular cellular infiltrate of patients with rheumatoid vasculitis and hypocomplementaemia (Soter et al 1976). In the vasculitis of normocomplementaemic patients with rheumatoid vasculitis lymphocytes perdominate (Soter et al 1976). Disintegrating PMN have additionally been demonstrated within necrotic areas of rheumatoid nodules (Mohr et al 1981(b)).

The PMN is thus not only present, but stimulated at important sites of disease activity in rheumatoid arthritis. PMN stimulation by

immune complexes or complement components is of course associated with initiation of the respiratory burst (1.2.2). Oxygen radicals and reactive oxygen metabolites as produced in rheumatoid arthritis may aid or exceed the tissue damaging potential of the released granular enzymes (1.2.3).

1.3.2. Biochemical evidence for oxygen radical reactions in rheumatoid arthritis

The proposal that oxygen radicals and other reactive oxygen metabolites produced by PMN may play an important role in the pathogenesis of rheumatoid arthritis rests not only on their presence in increased numbers and stimulation at sites of disease activity, but also on the paucity of known oxygen radical scavenging mechanisms in plasma and synovial fluid (1.2.3, McCord 1974, Salin and McCord 1977). Thus it has been suggested that oxygen radicals realeased extracellularly by PMN in rheumatoid arthritis are at liberty to interact with other host molecules to deleterious effect. Further some evidence has been provided for the presence of decreased intracelluar superoxide dismutase concentrations in the erythocytes and PMN of patients with adult and juvenile rheumatoid arthritis respectively (Banford et al, 1982, Rister et al 1978).

While direct evidence for the involvement of oxygen radicals in the pathogenesis of rheumatoid arthritis was conspicuously lacking, the indirect biochemical evidence which had accumulated prior to the commencement of this thesis will now be reviewed. The evidence comprised biochemical changes found in patients with rheumatoid arthritis which

(a) were attributable to oxygen radical mediated damage to cell

membranes or extracellular molecules or (b) provided a milieu which might enhance the generation and propagation of oxygen radicals (Table 8).

(a) Oxygen radical mediated cell membrane extracellular molecule damage

Cell membranes The potential for oxygen radicals to damage cell membranes via lipid peroxidation was reviewed in 1.2.3 Fluorescent lipid peroxidation products have been found in higher concentration in rheumatoid serum and synovial fluid than in normal or non rheumatoid samples (Lunec et al 1981). Synovial fluid studies showed that the initial free radical oxidation products were derived from inflamed or damaged synovial tissue (Lunec and Dormandy 1979). Plasmalemnal disruption by lipid peroxidation is known to result in the release from PMN not only of hydroperoxides, but also of arachidonic acid, lipid endoperoxides, thromboxane A_2 and other prostaglandin like metabolites, all of which have been involved in the pathogenesis of rheumatoid arthritis (1.2.3, Del Maestro 1980).

The prevention of premature cell death in phagocytosing PMN by addition of superoxide dismutase (Salin and McCord 1975) further suggested that the high rate of PMN death within rheumatoid synovial effusions (1.3.1) may be caused by oxygen radical mediated cell membrane lysis. In addition, a possible mechanism for the involvement of oxygen radicals produced by PMN in the pathogenesis of rheumatoid vasculitis was provided by the demonstration that both acellularly generated oxygen radicals and complement stimulated PMN were capable of injuring endothelial cells (Sacks et al 1978).

Extracellular molecules The decreased viscosity of synovial fluid in rheumatoid and other inflammatory arthritides has been atrributed to depolymerisation of its major glycosaminoglycans hyaluronic acid

TABLE 8

Evidence for Involvement of Oxygen Radicals in the Pathogenesis of Rheumatoid Arthritis

Biochemical changs attributable to to oxygen radical reactions	Biochemical changes which can enhance oxygen radical propagation
Cell Membranes -	Decreased synovial fluid scavengers
Lipid peroxidation products	Superoxide dismutase
PMN death	Catalase
	Ascorbic acid
Extracellular Molecules	Increased 'Free' metals
depolymerised hyaluronic acid	iron
Altered amino acid excretion	(? copper and zinc)
Hypohistidinaemia	
Decreased serum - SH levels	

(Greenwald and Moy 1980). The decrease in synovial fluid viscosity correlates strongly with the number of PMN present in the inflamed joint (Jessar 1966). PMN do not contain hyaluronidase, and hyaluronidase has not been found in rheumatoid synovial fluids (Soder et al 1970, Greenwald and Moy 1980).

In 1974 McCord demonstrated a decrease in the viscosity of bovine synovial fluid exposed to a xanthine-xanthine oxidase oxygen radical generating system which could be inhibited by either superoxide dismutase or catalase, suggesting that $OH^{\bullet}$ (produced by the Haber Weiss reaction) was the likely agent. Further support for this hypothesis was provide by Puig-Parellada and Planas (1978) who showed that the loss of synovial fluid viscosity was also inhibitable by the $OH^{\bullet}$ scavengers sodium benzoate and mannitol. Greenwald and Moy (1980) demonstrated that the decrease in human umbilical cord hyaluronic acid viscosity which occurred following exposure to stimulated PMN was likewise abolished by superoxide dismutase or mannitol. The same authors provided direct evidence for hyaluronic acid depolymerisation using sepharose chromatography.

A number of abnormalities have been identified in the urinary excretion of <u>amino acids</u> in patients with rheumatoid arthritis (viz tyrosine, (Nishimura et al 1958), tryptophan (McMillan 1960, Houpt et al 1973) and taurine, (Rylance, 1969)). The relevance of these abnormalities to the pathogenesis of rheumatoid arthritis has not been determined, but involvement of oxygen radicals in their altered metabolism (1.2.3) remains a possibility. The increased urinary excretion of taurine in rheumatoid arthritis may be of particular interest (Rylance 1969). Taurine is found in high concentration within PMN (Houpert et al 1976) and taurine chloramine produced by the myeloperoxidase - H_2O_2 - halide system (1.2.2) has been shown to be a

particularly stable chloramine (Zgliczynski et al 1971).

The only consistent abnormality found on analysis of serum amino acid levels in rheumatoid arthritis has been one of hypohistidinaemia (Gerber 1975). Histidine is capable of reacting with ($^1\Delta g\ O_2$) (Hodgson and Fidovich 1974) but it is not known whether the decreased histidine levels, if related to oxygen radical metabolism at all, result from oxygen radical reaction, or in fact represent a pre-existing deficiency in the 'scavenging' capability of rheumatoid serum. Gerber found that the decreased histidine levels correlated significantly with clinical and laboratory indices of rheumatoid disease activity and proposed that the associated low concentrations of histidine in rheumatoid synovial fluid contribute to the pathogenesis of rheumatoid arthritis by allowing sulphydryl dependent hyalunonate augmented aggregation of synovial fluid gamma globulin (vide infra) (Gerber 1975 (b), Gerber (1974)).

Oxygen radicals have been shown to react with protein bound as well as free amino acids (1.2.3). Inhibition of α_1 antiprotease activity by reaction of methionine at its active site with oxygen radicals (Clark et al 1981) is potentially of relevance to the pathogenesis of rheumatoid arthritis. Inhibition of rheumatoid synovial fluid α_1 antiprotease activity by oxygen radicals released by stimulated PMN would lead to enhanced proteolytic enzyme activity. No evidence for this occurrence has yet been provided in vivo.

Changes have been found however in protein sulphydryl groups in rheumatoid arthritis which may be related to oxygen radical activity. Serum sulphydryl levels are depressed in patients with rheumatoid arthritis, particularly during active phases of the disease (Lorber et al 1964, Haataja 1975). Serum sulphydryl groups are composed almost entirely of cysteine side chains on proteins, in particular albumin

(Thomas and Evans 1975).

Sulphydryl groups can be oxidised by $O_2^{\cdot -}$, H_2O_2 and derivatives of the myeloperoxidase - H_2O_2 - halide system to form mixed disulphides, sulphenic or sulphonic acids (1.2.3). The depression of serum sulphydryl levels and associated mixed disulphide bond formation with low molecular weight thiols (Thomas and Evans 1975) may thus result from oxygen radical mediated oxidation.

While the majority (85-90%) of serum sulphydryl groups are found on albumin, the remainder are distributed in the globulin fraction with IgG itself containing 0.2 moles sulphydryl groups per mole of IgG (Buchwald and Connell 1974). Similar oxygen radical mediated oxidation of these sulphydryl groups with resultant mixed disulphide or intra or inter molecular disulphide bridging might lead to altered antigenicity of the IgG molecule. While direct evidence for IgG sulphydryl group oxidation in vivo in rheumatoid arthritis is lacking Gerber (1974) has shown that thiol containing antirheumatic drugs D-penicillamine and aurothiomalate could inhibit the sulphydryl mediated aggregation of human IgG exposed to unphysiologically high temperatures.

In addition to their intrinsic amino acids, proteins also transport substances which may be susceptible to oxygen radical attack. The demonstration by Petrone et al (1980) of superoxide dependent activation of a PMN chemotactic factor localised to a chloroform extractable component bound to albumin suggests a further mechanism for involvement of oxygen radicals in the perpetuation of inflammatory diseases such as rheumatoid arthritis.

(b) Biochemical changes in rheumatoid arthritis which may enhance the generation and propagation of oxygen radicals

The propagation of oxygen radicals released by PMN stimulated

within rheumatoid synovial fluid, synovial membrane or articular cartilage is likely to be facilitated not only by the low extracellular concentrations of superoxide dismutase and catalase, but by the increased concentrations of free iron, copper and zinc, and decreased concentration of ascorbic acid found in the synovial fluid. Increased concentrations of synovial fluid caeruloplasmin may, however, exert a protective oxygen radical scavenging effect.

Synovial fluid iron, copper and zinc. The previously mentioned depolymerisation of hyaluronic acid in rheumatoid synovial fluid has been attributed to reaction of the acid with $OH^{\bullet}$. The formation of $OH^{\bullet}$ from interaction of $O_2^{\bullet -}$ and H_2O_2 can only occur in the presence of a metal chelate (reactions (5) and (6), 1.2.2, Haber and Weiss 1934, McCord and Day 1978). Mean concentrations of copper, iron, zinc, caeruloplasmin and ferritin have been found to be elevated in rheumatoid synovial fluid (Niedermeier and Griggs 1971, White et al 1978, Blake et al 1980). The spectroscopic and colourimetric methods used, however, would have detected both protein bound and 'free' metals simultaneously. It has been shown that, with the exception of iron saturated lactoferritin, metal-protein complexes are unable to catalyse the formation of $OH^{\bullet}$ from $O_2^{\bullet -}$ and H_2O_2 (Halliwell 1978). Micromolar concentrations of 'free' iron have, however, been measured in rheumatoid synovial fluid by Gutteridge, Rowley and Halliwell (1981), who used the dependency on Fe^{2+} of bleomycin degradation as an assay for the free metal.

White et al (1978) suggested that the increased concentrations of rheumatoid synovial fluid caeruloplasmin, (shown in vitro to scavenge $O_2^{\bullet -}$ (Goldstein et al 1978)) might be associated with a protective 'antioxidant' effect. Blake et al (1981) were however unable to detect any $O_2^{\bullet -}$ scavenging activity in their rheumatoid synovial fluid

samples.

Synovial fluid ascorbic acid. Ascorbic acid is able to scavenge oxygen radicals during its oxidation to dehydroascorbic acid (reactions (18) and (19), 1.2.3). Blake et al (1981) demonstrated depressed serum and synovial fluid ascorbic acid levels in their patients with rheumatoid arthritis, and calculated that the concentrations of reduced ascorbate present were too low to afford any significant $O_2^{\cdot -}$ scavenging activity.

1.3.3 Effects of antiinflammatory and antirheumatic drugs

Drugs currently used in the treatment of rheumatoid arthritis may be classified as (i) simple analgesic drugs (ii) non steroidal anti inflammatory drugs (iii) disease modifying drugs (anti rheumatic) (iv) immunosuppressive drugs and (v) corticosteroid drugs. Although many actions of these drugs have been identified in vitro asnd in vivo, with the exception of the simple analgesic drugs, the action(s) of greatest relevance to any beneficial effects of the drugs in the treatment of rheumatoid arthritis have not yet been identified.

In this section,evidence for actions of members of any of the above mentioned groups of drugs on (a) the responsiveness of PMN to chemotactic and phagocytic stimuli (b) the PMN respiratory burst (c) oxygen radical scavenging or (d) the occurrence of biochemical changes in rheumatoid arthritis attributable to oxygen radical scavenging, will be reviewed.

(a) Effects of drugs on PMN responsiveness

Any drug which inhibits the PMN's ability to respond to a chemotactic gradient or to phagocytose may limit the production of oxygen radicals at sites of disease activity in rheumatoid arthritis.

PMN chemotaxis has been inhibited in vitro by the antirheumatic drugs chloroquine, gold (aurothiomalate) and D-penicillamine (Ward 1966, Howat 1978), and in vivo in a rat model by chloroquine (Clarke, Vernon, Roberts and Curry 1975). After responding to a chemotactic stimulus the PMN must first adhere to and then pass through blood vessel endotheluim. The ingestion either of aspirin, the oldest non steroidal anti inflammatory drug, or of prednisolone has been shown to be accompanied by a decreased ability of PMN to adhere to nylon fibres for several hours subsequently in vitro (MacGregor, Spagnulo and Lentek 1974). The peripheral blood leukocytosis which accompanies steroid ingestion is contributed to not only by an inhibiting effect on PMN egression from the circulation, but also by an accelerated release of mature PMN from the bone marrow (Bishop et al 1968).

PMN phagocytosis has also been inhibited in vitro by chloroquine (Ward 1966). Jessop et al (1973) demonstrated an inhibition of PMN ingestion of colloidal carbon particles in vivo using a 'skin window' technique in patients with rheumatoid arthritis receiving aurothiomalate therapy. By contrast, corticosteroids appear to have no effect on PMN phagocytosis (Parrillo and Fauci 1979).

(b) Effects of drugs on PMN respiratory burst

Drugs which decrease the PMN's capacity for chemotaxis or phagocytosis need not necessarily decrease the magnitude of the respirtory burst. Membrane perturbation rather than ingestion is sufficient stimulus for the latter (Goldstein et al 1975). Certain non steroidal anti inflammatory drugs (Serge 1979) and chloroquine (Peters 1973) are, however, known to stabilise PMN lysosomal membranes. If these drugs thereby inhibit the release of myeloperoxidase, a decrease in the generation of oxygen radicals by the myeloperoxidase - H_2O_2 - halide system may occur. The release of

hydrolases from lysosomes has been shown to be unaffected by gold compounds indicating that gold does not stabilise lysosomal membranes (Ennis et al 1968).

Van Dyke et al (1979) using luminol dependent chemiluminescence as a monitor of oxygen radical production by PMN, demonstrated inhibitory effects in vitro for a number of non steroidal anti-inflammatory drugs (namely indomethacin, sodium salicylate, fenoprofen, tolmetin, naproxen and ibuprofen), all of which were noted to be prostaglandin synthetase inhibitors. Aspirin, dexamethesone and methylprednisolone did not inhibit chemiluminescence.

(c) Drugs as oxygen radical scavengers

The oxygen radical scavenger copper-zinc superoxide dismutase has been purified from bovine liver and administered in drug form (named 'orgotein') parenterally to patients with osteoarthritis or rheumatoid arthritis (Huber and Menander-Huber 1980, Goebel, Storck and Neurath 1981). The use of this drug has been largely experimental and long term benefits have not yet been reported. Of the conventional drugs, Puig-Parellada and Planas (1978) demonstrated that several non steroidal anti inflammatory drugs (including indomethacin, mefanamic acid and phenylbutazone) as well as prednisolone, dexamethasone, triamcinolone and hydrocortisone were able to scavenge oxygen radicals produced in vitro by the xanthine oxidase castalysed oxidation of hypoxanthine, thereby inhibiting synovial fluid degredation. Skosey and Chow (1981) found that gold and D-penicillamine scavenged oxygen radicals similarly generated in vitro and thereby prevented oxidation of an α_1 proteinase inhibitor.

(d) Effects of drugs on biochemical changes in rheumatoid arthritis attributable to oxygen radical activity

The biochemical changes in rheumatoid arthritis, attributable to

oxygen radical activity reviewed in 1.3.2 concerned hyaluronic acid depolymerisation, urine and serum amino acid levels and serum sulphydryl levels. Drugs currently used in the treatment of rheumatoid arthritis have been shown to favourably modify these changes either in vitro or in vivo. As above Puig-Parellada and Planas (1978) demonstrated that a number of non steroidal and steroidal anti inflammatory drugs were capable of inhibiting oxygen radical induced synovial fluid degradation in vitro. Pickup et al (1980) found that treatment of patients with rheumatoid arthritis with D-penicillamine was associated with a significant increase in their serum histidine levels, which did not occur with hydroxychloroquine treatment. The depressed serum sulphydryl levels of active rheumatoid arthritis have also been shown to correct with anti rheumatic treatment (Haataja 1975). Hall and Gillan (1979) showed that gold, D-penicillamine and levamisole therapy were associated with an enhanced rate of serum sulphydryl - disulphide exchange in vitro. Gerber (1974) showed that gold and D-penicillamine were able to inhibit the copper catalysed thermal aggregation of human gamma globulin in vitro, and Pritchard and Nuki (1978) found that treatment with these drugs resulted in a lowering of serum and synovial fluid levels of rheumatoid factor. Goebel et al (1981) found that intra articular orgotein (superoxide dismutase) therapy caused a significant fall in synovial fluid rheumatoid factor levels in their rheumatoid patients. These observations raise the possibility that a prominent mode of action of gold and D-penicillamine in the treatment of rheumatoid arthritis is to decrease the stimulus to rheumatoid factor production by inhibition of oxygen radical mediated, sulphydryl dependent aggregation of gamma globulin.

1.4 Measurement of Oxygen Radicals Produced by PMN

1.4.1 Available Methods

Despite their considerable potential for tissue destruction, the roles of oxygen radicals and reactive oxygen metabolites in disease pathogenesis have until recently been largely uninvestigated principally because of the lack of suitable systems for their measurement. On account of their extreme reactivity these agents decay rapidly in biological systems such that the half life of $O_2^{\cdot -}$ has been measured as 5 milliseconds (Brawn and Fridovich 1980) and that of delta singlet oxygen ($^1\Delta_g O_2$) as 2 micro seconds (Kasha and Khan 1970, Foote 1976). The majority of methods devised for their measurement (Table 9) are thus indirect and depend on the detection of an oxygen radical reaction product rather than of the radical itself. No method has yet been found for the direct measurement of oxygen radical production in vivo.

In order to select a system for monitoring the production of oxygen radicals by resting and stimulated PMN in vitro, which would allow further investigation of their role in the pathogenesis of rheumatoid arthritis, the available methods (Table 9) were evaluated according to the following criteria:-

(a) Is the method compatible with (i.e. not toxic to) living cells?

(b) Is it sufficiently sensitive to measure the production of oxygen radicals in the micromolar to nanomolar range?

(c) Is the method specific for the detection of any individual radical(s), and if so which?

(d) Can it continuously monitor the time course of oxygen radical production by PMN?

TABLE 9

Methods for detecting oxygen radicals and reactive oxygen metabolites in biological systems

Method	Reference
1. Electron paramagnetic resonance	Bolton et al (1972)
2. Electron spin resonance	Rosen and Raukman (1984)
3. Ultraviolet light absorption	Czapski (1971)
4. Oxidation/reduction reactions	Babior et al (1973)
5. Endogenous cell chemiluminescence	Allen et al (1972)
6. Probe dependent cell chemiluminescence	Easmon et al (1980)
7. Detection of naturally occurring products of oxygen radical reactions	Gutteridge et al (1974)

(e) Does the method act as a true indicator?

Measurements of electron paramagnetic resonance (EPR) detect the magnetic moment of the unpaired electron of a free radical, the practical lower limit of detection being 1μM (Borg 1976). The method is not specific for any one radical, but the high reactivity of hydroxyl radical prevents it from reaching sufficient concentration to be measured by EPR. Even for the detection of other less reactive oxygen radicals by this system, their rate of disappearance has to be slowed by rapid freezing or lyophilization. The technique is further limited by the concentration of radical present before freezing and the length of time required to freeze the sample (Bolton, Borg and Swartz 1972). While this method can act as a true indicator of oxygen radicals it cannot be used for continuous monitoring of their production by PMN.

Some of these problems can be overcome by electron spin resonence (ESR) in which the necessity for freezing is overcome by use of a 'spin-trap' - a compound which forms a stable free radical by reacting covalently with an unstable free radical. Nitrones are the spin traps usually used to detect $O_2^{\cdot -}$ and $OH^{\cdot}$, with which they react at room temperature to produce nitroxide spin adducts (Wargon and Williams, 1973). A potential problem with spin trapping in biological systems is the reduction of the nitroxide to its hydroxyl-amine (which cannot be detected by EPR) by sulphydryl groups, ascorbic acid or mitochondrial electron transport chains (Rosen and Raukman, 1984).

While there is a great deal of controversy about the intensity of the absorption, there is no doubt that $O_2^{\cdot -}$ does absorb light maximally in the ultraviolet 240-260nm range. There are conflicting reports in the literature as to whether superoxide ($O_2^{\cdot -}$) and

perhydroxy (HO_2) ions have different spectra or wavelengths and as to whether superoxide possesses an absorption band in the visible region (Czapski, 1971). Ultraviolet absorption is of course only useful in the absence of interfering chromophores such as proteins and is therefore not suitable for the detection of oxygen radicals in a whole cell system.

In the detection of oxygen radicals by oxidation/reduction reactions, the product of the reaction between the radical and an added chemical is measured rather than the radical itself. What is gained by specificity of the reacton is frequently lost by the chemicals 'scavenging' of the oxygen radical in question with resultant distortion of the PMN oxygen radical generating pathways. These systems of oxygen radical detection thus do not employ 'true indicators' and furthermore the requirement for detection of the reaction product spectroscopically in cell free supernatant prevents that use as continuous monitor of oxygen radical production. Nevertheless included under this heading are the three methods which have been used most commonly to detect the production of reactive oxygen metabolites by stimulated PMN, namely detection of $O_2^{\bullet -}$ by nitro blue tetrazoluim reduction (Beauchamp and Fridovich 1971) or by ferri cytochrome c reduction (Land and Swallow 1971) and detection of H_2O_2 by oxidation of scopoletin (Root et al 1975). In addition $OH^{\bullet}$ can be detected following its reaction with methional or ketomethylthiobutyric acid to form ethylene (Tauber and Babior, 1977) or by its reaction with dimethyl sulphoxide to form methane (Repine et al 1974).

As a consequence of the difficulties inherent in attempting to measure oxygen radicals in vivo, a variety of assays have been devised to measure stable, naturally occurring products of oxygen radical

reactions such as those resulting from lipid peroxidation (1.2.3), measured by their reaction with thiobarbituric acid (Gutteridge et al 1974) or diene conjugation absorbance detected by visible or ultra violet fluorescence (Lunec et al 1981).

Chemiluminescence is the emission of light during the course of a chemical rection and implies the presence of energy rich molecular states (such as reactive oxygen metabolites) in which electrons occupying orbitals of higher than ground state energy emitt light upon relaxation.

Endogenous cellular chemiluminescence refers to the 'low level', 'dim' or 'ultra weak' chemiluminescence produced by respiring higher plant and animal cells and is distinguished from the more effective luciferin/luciferase systems found in the specialised organs of luminescent organisms such as the firefly (Seliger 1975). In 1961 Tarusov and co-workers demonstrated that mouse liver in situ produced a very weak light emission, not visible to the human eye but detectable with a sensitive photomultiplier connected to a photon counting device. These observations were later extended to brain, muscle, intestine, tissue homogenates and lipid extracts (Tarusov 1962). Following the demonstration by Allen, Stjernholm and Steele in 1972 that phagocytosing PMN also chemiluminesce , these cells have been the most widely studied source although monocytes, macrophages, eosinophils, platelets, seminal vesicles, mitrochondria and submitochondrial particles have been shown to chemiluminesce (Nelson et al 1976, Mills et al 1978, Jowa, Fisher and Forman 1980, Cadenas et al 1980, Marnett, Wlodawer and Samuelsson 1974).

The endogenous chemiluminescence of stimulated PMN can be correlated with the increased oxygen consumption and hexose monophosphate shunt activation of the respiratory burst (Allen et al

1972, Stevens and Young 1977). The chemiluminescence is oxygen dependent and is partially inhibited by superoxide dismutase, catalase or azide (Rosen and Klebanoff 1976) indicating a dependence on both superoxide anion and myeloperoxidase related oxygen metabolite production.

It is not known, however, whether PMN endogenous chemiluminescence arises from the presence of oxygen radicals and other reactive oxygen metabolites per se (and if so, which?), or from the reaction of these species with available organic substrates to form electronically excited molecules. The original suggestion of Allen et al (1972) that PMN chemiluminescence resulted from the relaxation of singlet oxygen to triplet oxygen, or from the formation of excited carbonyls cleaved from dioxetanes generated by reaction of singlet oxygen with carbon dioxide bonds, has since been partly discredited on both spectroscopic (Cheson et al 1976) and chemical grounds (Johnston and Lehmeyer 1977).

The broad spectrum of emitted light (Cheson et al 1976) does however support the hypothesis that PMN endogenous chemiluminescence may result from reaction of $O_2^{\cdot -}$ and excitable organic substrates (possibly contained within the stimulating particle) to form an excited intermediate (such as an epoxide) which emitts light on decaying (Hamman and Seliger 1976, Cheson et al 1976). Stauff, Saunder and Jaeschke (1973) found that the weak blue-green chemiluminescence observed in some systems was greatly decreased by exclusion of carbon dioxide, it was subsequently proposed that $OH^{\cdot}$ formed by the interaction of $O_2^{\cdot -}$ and H_2O_2 (1.2.2) may react with carbonates to form carbonate radicals which then dimerise to yield electronically excited species (Hodgson and Fridovich 1976).

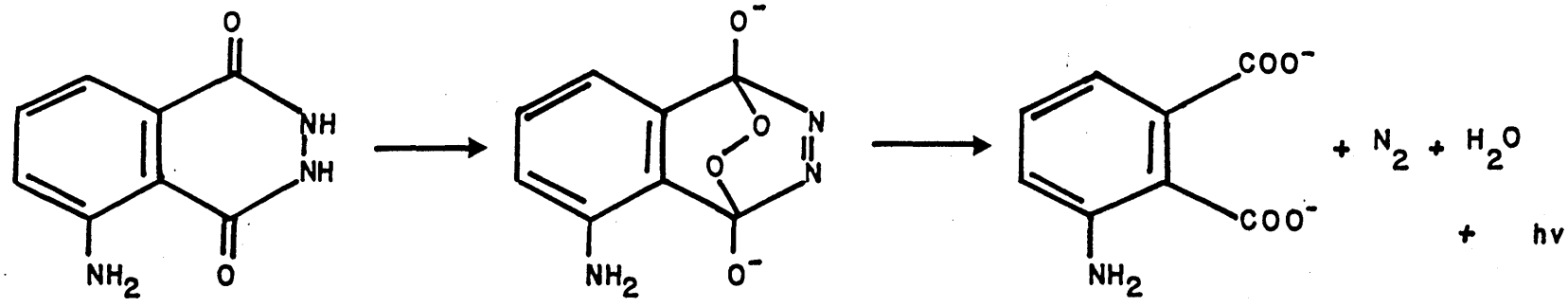
Whilst measurement of PMN endogenous chemiluminescence cannot,

therefore, be used to monitor the production of specific oxygen radicals, it can provide a continuous monitor of the rate of production of oxygen radicals which is sensitive and not toxic. The sensitivity can be increased 1000 fold by the use of a chemiluminescence probe such luminol (5 - amino - 2,3 - dihydro - 1,4 - phthalazinedione) or lucigenin (10, 10′ - dimethyl - 9,9" - biacridinum dinitrate) which emitt light upon reaction with products of the PMN respiratory burst. In the concentrations required these substances are not toxic to cells and their greater sensitivity allows the use of smaller numbers of PMN in assay systems. Luminol dependent chemiluminescence was selected as the method of choice for investigation of the scope of oxygen radical production in this thesis.

1.4.2 PMN luminol dependent chemiluminescence

Light emission resulting from oxidation of the phthalazine dione, luminol in alkaline solutions of H_2O_2 was first described by Albrecht (1928). The mechanism of its dioxygenation via a cyclic peroxide to the electronically excited aminophthallate anion with accompanying light emission (Fig 9) was determined subsequently (White and Roswell 1970, Gunderman 1974). As an oxidative process the chemiluminescence of luminol requires the presence of either O_2 or H_2O_2 and can be provoked by a variety of radicals including superoxide anion, hydroxyl radical, carbonate, formate, hypochlorite and persulphate (Hodgson and Fridovich, 1973, Puget and Michelson 1976, Henry and Michelson 1977). Maximum light yields generally occur in the presence of a peroxidase catalyst and at alkaline pH.

Despite the neutral pH of the PMN cytoplasm and the acidic pH of its phagolysosome, luminol dependent chemiluminescence can be measured in PMN stimulated by both particulate and soluble stimuli (Easmon et



(luminol) (luminol peroxide) (aminophthallate) (light)

Figure 9 THE DIOXYGENATION OF LUMINOL

al 1980, Hallett, Luzio and Campbell 1981). As with endogenous chemiluminescence, luminol dependent PMN chemiluminescence is partially inhibited by superoxide dismutase and azide (Hatch, Gardner and Menzel 1978) and it is not known which reactive oxygen metabolites produced as a consequence of the PMN respiratory burst are responsible for its oxidation. Luminol (molecular weight 177.2) can diffuse readily across cell membranes (Allred, Margetts and Hill, 1980)and be taken up during phagocytosis. The proportion of PMN luminol dependent chemiluminescence provoked by oxygen radicals released extracellularly by PMN is therefore not known.

The chief advantage of luminol dependent PMN chemiluminescence over endogenous chemiluminescence is its increased sensitivity. Further, its low quantum yield (Lee and Seliger 1965) allows luminol to act as a true indicator, thus preventing significant distortion of the reactive oxygen metabolite population produced by stimulated PMN (Campbell et al 1985).

Four of the original five criteria (1.4.1) for a satisfactory monitor of oxygen radical production by stimulated PMN are therefore provided by luminol dependent chemiluminescence which offers a non toxic, sensitive, continuous method in which luminol acts as a true indicator. The work of this thesis was commenced in October 1981, however, in the sanguine knowledge that interpretation of PMN luminol dependent chemiluminescence would be complicated not only by its lack of specificity but also by its susceptibility to a large number of environmental variables (Table 10).

The collective experience of PMN luminol dependent chemiluminescence did not extend back more than seven years. Early experiments had been performed with PMN suspended in dark adapted vials at room temperature with light emission detected by the

TABLE 10

PMN Luminol dependent chemiluminescence

Environmental Variables

Type of Variable	Particular Variable	Reference
1. Detector	Scintillation counter	Webb et al (1974)
		Rosen and Klebanoff (1976)
	Commercial luminometer	Easmon et al (1980)
	Home built luminometer	Hallett et al (1981)
2. PMN preparation	Source and species	Harvath et al (1978)
	Conditions of isolation	Andersen and Amirault (1979)
	Purity	Easmon et al (1980)
		Andersen and Brendzel (1978)
		Van Dyke et al (1978)
		Easmon et al (1980)
	Cell Storage	Easmon et al (1980)
3. Chemiluminescence assay conditions	Cell-stimulus ratio	Nelson et al (1977) Easmon et al (1980)
	Temperature Oxygenation and mixing	Andersen and Brendzel (1978)
	Medium - phenol red	Nelson et al (1977)
	pH	Hastings et al (1982)
	Ca^{2+} +Mg^{2+}	Andersen and Amirault (1979) Hallett et al (1981)
	Glucose	Jowa et al (1980)
	Protein	Andersen and Amirault (1979)
	Amino acids	Nelson et al (1977)

photomultiplier tube of a liquid scintillation counter set in the 'out of coincidence' mode (Webb et al 1974, Rosen and Klebanoff 1976). Measurements were necessarily interrupted, often separated by several minutes, and the magnitude of luminol dependent chemiluminescence was decreased by the unphysiologically low temperatures. Caution was therefore required in the interpretation of data obtained from these experiments when compared with that gained by later workers who used commercially available or home built 'luminometers' (Andersen and Amerault 1979, Easmon et al 1980, Hallett et al 1981) attached to chart recorders which were often computer linked, allowing continuous monitoring of PMN chemiluminescencce at 37^{o}C.

There was thus a need not only for standardisation of the type of apparatus used but also of the concentrations of the numerous medium variables which had been shown to enhance or inhibit PMN luminol dependent chemiluminescencce (Table 10). It would be important to determine whether such variables exerted their effect on the production of oxygen radicals by PMN per se, on the oxidation of luminol, or on the aminophthallate anion.

1.5 The Problems

Before the work of this thesis began the putative role for oxygen radicals in the pathogenesis of rheumatoid arthritis hinged upon the availability of these highly reactive moieties for extracellular tissue damage. Any extracellular presence of oxygen radicals in the disease must, however, be a function not only of their release by stimulated phagocytes but of their scavenging by physiological or pharmacological agents. As reviewed in the preceding sections,

evidence had been provided for:-

(a) the production of oxygen radicals and other reactive oxygen metabolites by stimulated PMN (1.2.2, 1.2.3).

(b) the presence of PMN and known stimuli of the PMN 'respiratory burst' at intra- and extra-articular sites of disease activity in rheumatoid arthritis (1.3.1, 1.2.2).

(c) the occurrence in patients with rheumatoid arthritis of biochemical and morphological changes to cell walls and extracellular molecules compatible with in vivo oxygen radical damage (1.2.3, 1.3.2).

and

(d) possible prevention of generation or propagation of oxygen radicals by anti-inflammatory and antirheumatic drugs (1.3.3).

Three barriers to the establishment of oxygen radicals as important mediators of tissue damage in patients with rheumatoid arthritis remained:-

(a) there had been no <u>direct</u> demonstration of oxygen radical production or release by stimulated PMN in rheumatoid synovial fluid or blood in vivo or in vitro.

(b) the existence and potency of physiological oxygen radical scavengers in serum and synovial fluid had not been fully investigated.

(c) the contribution of oxygen radical scavenging to any beneficial effects of anti inflammatory or anti rheumatic drugs in vivo had not been defined.

1.6 <u>Aims of this Thesis</u>

The <u>overall</u> aim of this thesis was to provide direct evidence of oxygen radical production by polymorphonuclear leukocytes in

rheumatoid arthritis and to identify any major physiological and pharmacological scavengers.

The specific aims were thus:-

(a) to determine what proportion of oxygen radicals generated by stimulated PMN occur extracellularly and may thus be available to damage the surrounding tissue matrix

(b) to examine rheumatoid serum and synovial fluid for the presence of (i) stimuli of PMN oxygen radical production

(ii) naturally occurring oxygen radical scavengers

(c) to investigate the chemical basis of the principal physiological extracellular oxygen radical scavengers

(d) to examine the effects of commonly used anti inflammatory and anti rheumatic drugs on the generation and scavenging of oxygen radicals produced by stimulated PMN

In order to achieve these aims luminol dependent chemiluminescence was chosen as a sensitive, continuous monitor of oxygen radicals by intact PMN preparation (1.4.2). To commence the work it was first necessary

(a) to develop methods for the isolation of PMN from whole blood and synovial fluid

(b) to characterise the luminol chemiluminescence of normal human PMN following their exposure in vitro to soluble and insoluble phagocytic and chemotactic stimuli.

Chapter 2 - General Methods

2.11.2. Relationship to oxygen consumption

2.11.3. Dependence on oxygen radical production

2.12 Standardisation of PMN luminol chemiluminescence

2.12.1 Parameters of measurement

2.12.2 Conditions of assay

2.1 Materials

2.1.1 Radiochemicals: from Amersham International Limited, Amersham, Buckinghamshire, U.K.

$[^3H]$ Inulin

Specific activity 904 mCi/mmol

2.1.2 Enzymes: from Sigma Chemical Company Limited, Poole, Dorset, U.K.

Xanthine Oxidase (xanthine: oxygen oxidoreductase EC 1.2.3.2) One unit will convert 1μmole of xanthine to uric acid per minute at pH 7.5 at 25°C. Grade IV from milk suspension in 2.3M$(NH_4)_2SO_4$ containing sodium salicylate activity approximately 0.1 unit per mg protein.

Microperoxidase MP 11 sodium salt from equine heart cytochrome c.

Superoxide dismutase (superoxide: superoxide oxidoreductase EC 1.15.1.1) 3200 units/mg (assayed per McCord and Fridovich 1969).

Catalase (H_2O_2:H_2O_2 oxidoreductase EC 1.11.1.6). One Sigma unit will decompose 1μmole H_2O_2 per minute at pH 7.0 at 25°C while the H_2O_2 concentration falls from 10.3 to 9.2 μmoles per ml of reaction mix.

2.1.3 Chemicals

(a) From Sigma Chemical Company Limited, Poole, Dorset, U.K.

adenosine 5′ - triphosphate (ATP)

nicotinamide adenine dinucleotide (reduced form) (NADH)

cytochalasin B from Helminthosporium dematiodeum dextran (produced by leuconostoc mesenteroides) approximate average molecular weight 70,000.

dithiobis 2 nitrobenzoic acid (DTNB)

firefly lantern extract

human gamma globulin (Cohn fraction II)

luminol (5 amino, 2, 3 dihydro 1, 4, phthalazinedione)

N-formyl-l-methionyl-l-leucyl-l-phenylalanine (Chemotactic peptide)

sodium pyruvate

xanthine (2,6-dihydroxypurine)

zymosan A (from s.cerevisiae yeast)

(b) from Armour Pharmaceuticals Limited, Eastbourne, Sussex, U.K.

bovine serum albumin - fraction V from bovine plasma

(c) from BDH Chemicals Limited, Poole, Dorset, U.K.

N_2-hydroxy-ethylpiperazine N'2 ethanol sulphonic acid (HEPES)

Ethylene diamino tetra acetate disodium salt (EDTA)

Dimethyl sulphoxide (DMSO); p-Bis(2-(5-phenyloxazolyl)-benzine(POPOP)

N-Tris(hydroxymethyl)methyl glycine (tricine)

(d) from Hoechst, Hounslow, U.K.

human serum albumin

(e) from Leo Laboratories Limited, Hayes, Middlesex, U.K.

Heparin (mucous) injection B.P. (1000 1u/ml, with preservative).

(f) from Pharmacia Limited, Hounslow, Middlesex, U.K.

Ficoll-Paque (each 100ml containing 5.7g Ficoll 400, 9.0g Diatrizoate sodium, edetate calcium disodium).

(g) Amino-butyl-ethyl-iso-luminol (ABEI) was synthesised by Mr. C. J. Davies, Research Fellow, Department of Medical Biochemistry.

(h) from Dow Chemicals, Indianapolis, U.S.A. 1.01μm latex particles.

(i) from Nuclear Enterprises Limited, Edinburgh, Scotland

2,5-Diphenyloxazole (PPO)

(j) General laboratory chemicals were analar grade and were obtained either from B.D.H. Chemicals Limited, or from Sigma Chemical Company Limited. All water was double distilled.

2.1.4 Drugs

Sodium ibruprofen dihydrate

from the Boots Company, PLC, Nottingham, U.K.

Sodium indomethacin trihydrate

from Merck Sharp and Dohme Research Laboratories, Hoddesdon, Hertfordshire, U.K.

Fenclofenac

from Reckitt and Colman, Kingston upon Hull, U.K.

D-penicillamine

from Lilly Research Centre Limited, Windlesham, Surrey.

Myocrisin (Disodium aurothiomalate)

from May and Baker Limited, Dagenham, Essex.

Auranofin (2,3,4,6-Tetra-o-acetyl-1-thio- -D-glucopyranosato-s) (Triethylphosphine) Gold.

from Smith, Kline and French Laboratories Limited, Welwyn Garden City, Hertfordshire, U.K.

2.1.5 Equipment

LP_3 tubes	Luckham Limited, Burgess Hill, Sussex, U.K.
10ml test tubes(plastic)	Brunswick, Ballymoney, N.Ireland
Sterile Universal containers	Alpha Laboratories, Eastleigh, Hampshire, U.K.

Fluorimeter tubes	Samco, Old Woking, Surrey, U.K.
Eppendorf tubes and Spectophotometric cuvettes	Sarstedt (UK) Limited, Leicester.
Precision optical cells, 1m cuvettes	Thermal Syndicate Limited, Wallsend, Tyne and Wear.

2.2 Media, Buffers and Stock Solutions

Media

$A_{2/1}$	NaCl	140mM
	KCl	5mM
	$MgCl_2$	2mM
	$CaCl_2$	1mM
	Tes	10mM

adjusted with 2MNaOH to pH 7.4

Krebs Ringer HEPES (hereafter called 'Krebs Medium')

NaCl	120.0mM
K Cl	4.8mM
KH_2PO_4	1.2mM
$MgSO_2$	1.2mM
$CaCl_2$	1.3mM
HEPES	25.0mM

adjusted with 2M NaOH to pH 7.4

Buffers

Phosphate Buffered Saline

Dulbecco 'A' Phosphate Buffered Saline from Oxoid Limited, Basingstoke, Hampshire.

Stock Solutions

Luminol (5 amino 2,3, dihydro 1,4 phthalazinedione) 10mM in DMSO, stored stably in the dark for up to four weeks, was diluted on the day of experiment in Krebs medium pH 7.4 to a final concentration of 10μM (0.1% DMSO (v/v)) for PMN chemiluminescence and 100pM for acellular chemiluminescence.

Chemotactic peptide (N formyl met leu phe) 1mM in DMSO, stored stably at -20°C for up to six months, was diluted on the day of experiment in Krebs medium pH 7.4 to a final concentration of 0.1-1μM (0.01 - 1% DMSO (v/v)) for PMN chemiluminescence experiments.

2.3 Statistics

(a) Standard Deviation (S.D.) was calculated according to the formula:-

$$SD = \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$$

where x = the observation

$\bar{x}$ = the mean of observation

n = the number of observations

(b) Standard Error of Mean (S.E.M.) was calculated according to the formula

$$S.E.M. = \frac{S.D.}{\sqrt{n}}$$

(c) Students t tests were used as indicated to determine whether means of two sets of observations were significantly different assuming unequal variance, (Swinscow 1982).

(d) The correlation coefficient (r) was calculated according to the formula (for two populations x and y)

$$r = \frac{\Sigma (x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma (x-\bar{x})^2 \Sigma (y-\bar{y})^2}}$$

2.4 Origins of whole blood and synovial fluid samples

Whole blood 10-30ml normal blood was venesected on repeated occasions from myself and from 8 healthy volunteer male and 3 healthy volunteer female colleagues from the Department of Medical Biochemistry Research Laboratory, Univeristy of Wales College of Medicine, and the Department of Rheumatology, University Hospital of Wales. The mean age for male subjects was 29.6 ± 3.9 years (± SD, n=8) and was 27.3 ± 2.5 yrs (±SD n=4) for female subjects. Blood was not withdrawn for a minimum of eight days from any subject who had sustained a viral or bacterial infection, or who had received drug therapy of any description.

Following their informed consent 10-30ml whole blood was also venesected from in-patients and out-patients of the Department of Rheumatology who had rheumatoid arthritis or other arthropathies as indicated.

Synovial fluid

With their informed consent, synovial fluid was obtained from patients with rheumatoid arthritis or other arthropathies, attending the Department of Rheumatology, who were undergoing knee joint arthrocentesis for therapeutic purposes.

Ethical Approval for the above was granted by the Division of Medicine

Ethical Committee, University Hospital of Wales.

2.5 Opsonisation of Zymosan

The method used adopted the optimal conditions for opsonisation of zymosan defined by Easmon et al (1980). 20-50mg of zymosan was suspended in phosphate buffered saline pH 7.4 at 20mg/ml in a plastic 10ml test tube, diameter 14mm and centrifuged at 2500rpm (1000g) for 5 min. The supernatant was removed and the pellet resuspended at 20mg/ml in 50% (v/v) normal human serum in phosphate buffered saline pH 7.4. The test tube was sealed and placed in a gently shaking water bath at 37°C for 30 min, after which the suspension was centrifuged at 2500 rpm for 5 min and supernatant removed. The zymosan pellet was then washed in phosphate buffered saline and centrifuged at 2500rpm (1000g) for 5 min on three occasions before final resuspension at 20mg/ml.

The opsonised zymosan suspension was stored for up to five days at +4°C and resuspended in Krebs medium pH 7.4, 20mg/ml, immediately prior to use in PMN experiments. The serum for opsonisation was always obtained from the same donor (M.E.H.). 20-40ml venous blood was allowed to clot at room temperature (22°C) for 45 min and then centrifuged at 2500 rpm (1000g) for 5 min. The serum supernatant was removed and stored in 1ml aliquots at -70°C for up to 4 months prior to use in the opsonisation of zymosan.

2.6 Assay for Lactate Dehydrogenase

This assay was used to assess extracellular release of lactate dehydrogenase as an estimate of the proportion of non-viable cells in PMN preparations. The method, based on Keiding et al (1974), employed

the following reaction; (20).

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{lactate dehydrogenase}]{} \text{L-lactate} + NAD^+ \qquad (20)$$

Three stock solutions were required:

Solution A 56mM Tris, 5.6mM EDTA, pH adjusted to 7.4 stored at 4°C.

Solution B 13mg NADH in 100ml Solution A.

Solution C 13.5mM sodium pyruvate.

100µl of cell sample was centrifuged at 10,000rpm in an Eppendorf Centrifuge for 2 min. Cell pellet and supernatant were separated and the cell pellet was lysed by vigorous resuspension in 10µl cold double distilled water. 50µl lysed cell pellet or supernatant was added to 1ml solution B in a plastic spectrophotometer cuvette. Reaction was initiated by addition of 100µl solution C and continuous monitoring of the change in absorption at 340nm reflected consumption of NADH in the presence of lactate dehydrogenase. The percentage lactate dehydrogenese in the supernatant (i.e extracellular) was calculated by the following formula

$$\frac{\Delta \text{ Supernatant}}{\Delta \text{ (pellet + supernatant)}} \times 100\%$$

where Δ = change in absorption at 340nm per minute. Cell preparations were considered viable if the percentage of lactate dehydrogenese in supernatant increased at less than 5% per hour.

2.7 Assay for Adenosine Tri Phosphate

The method which was based on that of Wettermark and Stymne (1975) allowed estimation of ATP in the range 10-10,000pmoles, and was used as an assay of PMN viability. 100µl aliquots of PMN in Krebs

medium pH 7.4 (0.3 - $1x10^6$PMN) were protein precipitated by addition of 20µl 1.8N Per chloracetic acid, 10µl 3M K_2CO_3 and 10µl phosphate buffer pH 7.5 and stored for a minimum of 12 hours at -70°C prior to assay.

Stock solutions (I) 40mM $MgCl_2$, pH 7.4 and (II) 200mM sodium arsenate, pH 7.4 were prepared and stored at +4°C. On the day of experiment 5ml solution (I) and 10ml solution (II) were diluted to 100ml to give solution (III) (2mM $MgCl_2$, 20mM sodium arsenate). Approximately 10-20mg of firefly lantern luciferase extract was added to 5ml double distilled water, mixed vigorously and then centrifuged at 1000g for 5 minutes. The supernatant, added to solution (III) formed the 'luciferase solution'. To measure ATP 10µl of supernatant from the protein precipitated cell solution was placed on the wall of an LP_3 tube containing 1ml 'luciferase solution'. A stopwatch was started, the contents of the LP_3 tube was placed in the sample housing of luminometer 'A' or 'B' (see next section) and rotated in front of the photomultiplier tube aperture as the stopwatch registered 10 seconds. Chemiluminescence counts over the subsequent 10 sec were then recorded. The assay was calibrated using 10µl aliquots of an ATP solution of known concentration (1mM ATP in double distilled water diluted 1:10, 1:100, 1:1000) with results plotted on a log:log scale (Fig. 10).

2.8 Apparatus for measurement of chemiluminescence

2.8.1 Specification of luminometers

Luminol dependent and endogenous PMN chemiluminescence were measured in one of three custom build luminometers, 'A', 'B' and 'C', designed and developed in the Department of Medical Biochemistry, University of Wales College of Medicine, by Mr. M. E. T. Ryall.

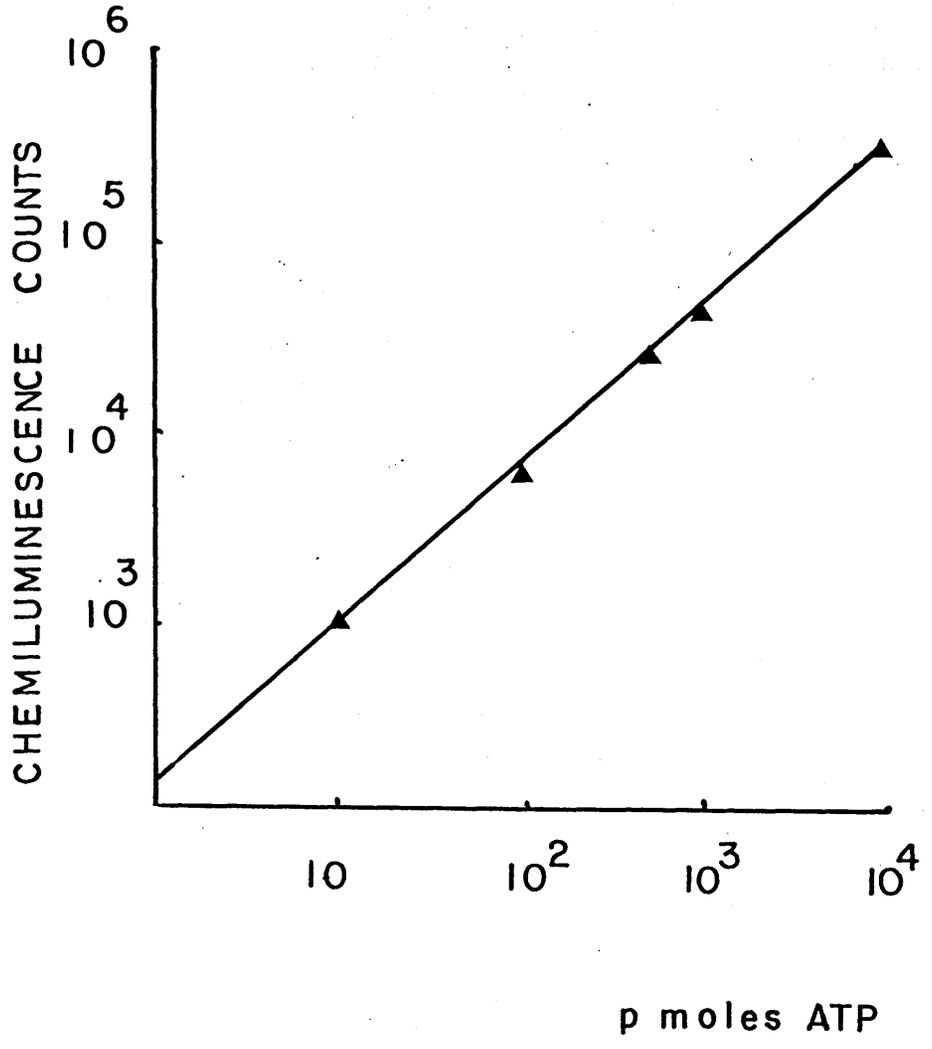


Figure 10 Calibration of ATP assay

Total chemiluminescence counts measured between 10 and 20 seconds following the addition of 10ul ($10 - 10^4$ p moles) ATP to 1ml 'luciferase solution' (firefly luciferase in 2mM $MgCl_2$, 20mM sodium arsenate) pH 7.4, room temperature.

Luminometer 'A' comprised a brass light tight cell housing adjacent to a highly sensitive low dark current photomulitplier tube (Centronics P4232B, from Twentieth Century Electronics Limited, Centronics Works, New Addington, Croydon, CR9 0BG, U.K., dark current 0.07nA at 1190V, overall sensitivity 200Å/lumen). The cell housing was warmed to 37°C by water pumped from a bath, and contained an inner drum allowing rotation of the cell sample to the photomultiplier tube where stimulus could be injected via a needle hole in the roof of the cell housing. The photomultiplier was connected to a scalar (Ekco Electronics Limited, Maidenhead, Berks, U.K. M5060A) which in turn was connected to a rate meter (Ekco Electronics M5190). The rate meter was connected to a chart recorder (26000 X-Y, from Bryans Limited, Mitcham, Surrey, CR4 4W, U.K.) which had a response time of 100ms for full scale deflection.

Luminometer 'B' (Fig II) also comprised a brass light tight cell housing thermostatically controlled at 37°C by a thermocouple, joined to a highly sensitive low dark current (< 10nÅ) photomultiplier tube (EM19757AM) mounted in a cooled housing (Products for Research) which maintained the photomultiplier tube temperature at - 20°C. The electrical output from the photomultiplier was processed by a fast amplitude discriminator, and the output connected to an eight decade counter with a maximum rate of 10^8 counts/s. The counter was interfaced to a Digital Equipment Corporation LSI II computer system running on an RT II operating system programmed in BASIC to read the instantaneous value of counts at 10 second intervals. This data was stored for real time analysis in memory, and for archival purposes on a floppy disc. At the end of each sample analysis of the cell background (unstimulated or resting) chemiluminescence value was printed followed by a graphical representation of the reaction time

Figure 11 - The author and Luminometer 'B'

course, (Fig 12). The value and time of peak height as well as the integral count to peak height were also estimated and printed.

Luminometer 'C' The low level of light of the endogenous chemiluminescence, produced by PMN stimulated in the absence of luminol was measured using the following apparatus (Fig. 13). A photomultiplier tube (EMI 9757AM) (connected as above to a discriminator, counter and computer) was placed vertically in a darkened room. A light tight black wooden box with a sliding roof was placed over the light detecting end, enabling the positioning of a small glass vessel containing water at 37°C directly on top of the photomultiplier tube. An LP_3 tube containing the PMN sample was placed in the glass water vessel. Stimulus was injected throught the roof of the black box via a needle hole which was rapidly closed before activation of the photomultiplier tube and measurement of chemiluminescence.

2.8.2 Standardisation of Luminometers

In order to allow quantitative comparison of PMN luminol dependent chemiluminescence counts/s measurements made either in the two different luminometers ('A' and 'B'), or in the same luminometer sequentially, over the two year period of this work, it was necessary, for both pieces of apparatus:

1. to calibrate and determine the stability of light detection, using a series of standard photon emitters.
2. to determine the effect of changes in volume or height of the light emitting source on the efficiency of light detection.

Tritiated Inulin Standards

To provide a series of standard photon emitters, 12 quenched

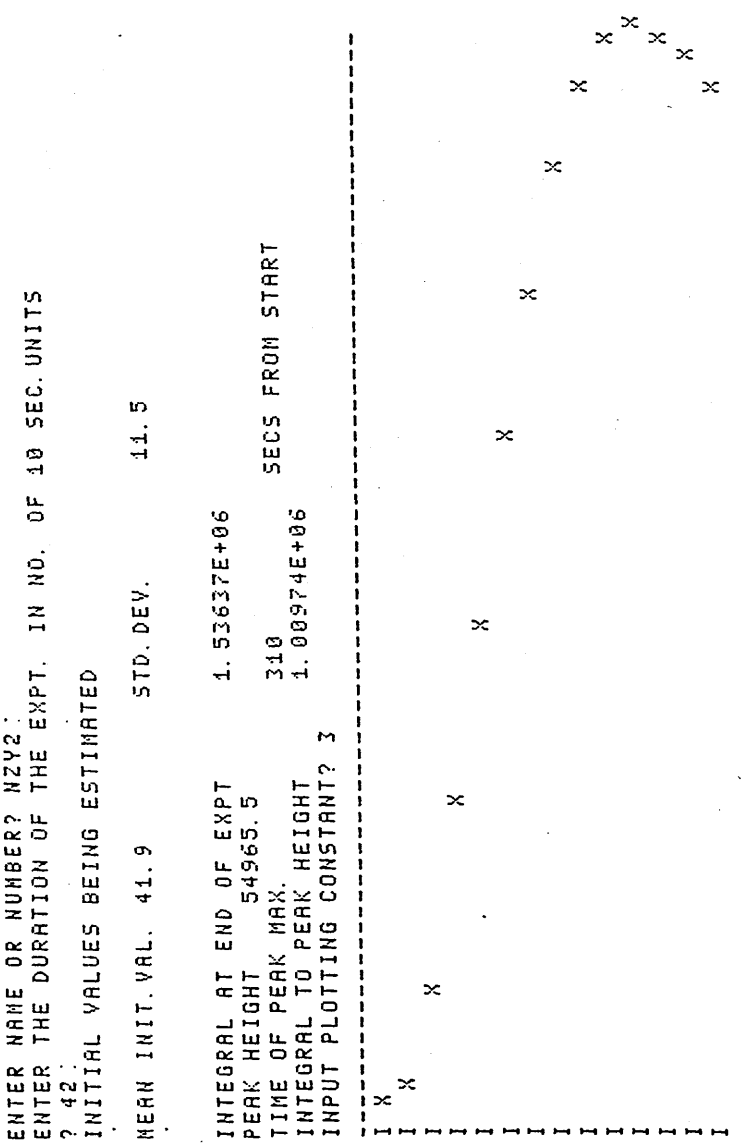


Figure 12 - Sample Computer Printout from Luminometer 'B'

5×10^5 PMN/ml were stimulated by 2mg/ml opsonised zymosan in the presence of 10μM luminol (37°C, pH 7.4)

'Mean Init Val' = background/resting PMN chemiluminescence counts/s

'Peak Height' = maximum chemiluminescence counts/10s achieved following stimulation.

With an input plotting constant of '3' the value of chemiluminescence (counts/10s) at the end of each 30s interval following stimulation was presented graphically.

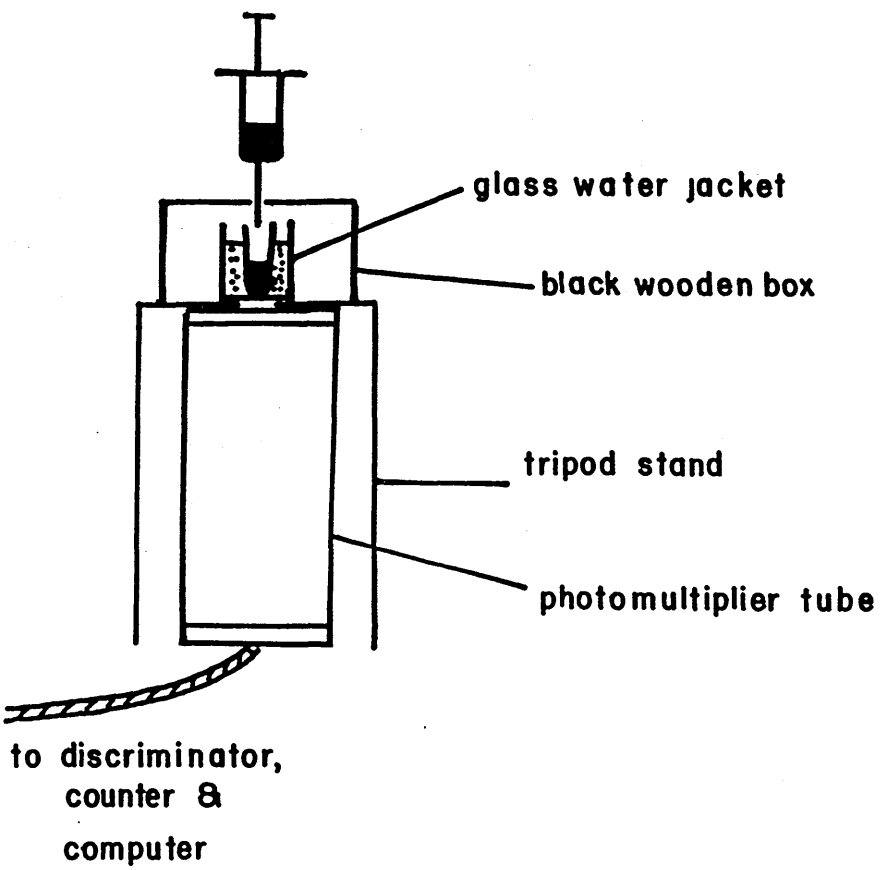


Figure 13 - Luminometer 'C'

Apparatus for measurement of PMN endogenous chemiluminescence.

tritium inulin standards were devised as follows, from which three were selected to document apparatus sensitivity prior to each experiment. The standards were made in 5mm diameter glass fluorimiter tubes and heat sealed. Each tube contained 2-20µl tritiated inulin (TRA 324, 904 mCi/mmol), 300µl of triton toluene (made from 2l toluene, 1l triton x-100, 0.2g POPOP, 8g PPO) and a variable amount of double distilled water and $CHCl_3$ (Table 11). Particle light emission by the tubes was counted in a Packard tricarb scintillation counter and in luminometers 'A' (EHT 1.5Kv, Dv 0.55) and 'B' (EHT 9.5v, Dv 4.0). The trituim samples were placed in the luminometer sample housing in turn in an LP_3 tube in which they were supported on a 3mm cork. It may be seen (Table 11) that luminometer 'A' recorded 2.5 $\pm$ 0.09 (mean $\pm$ SD, n = 9) times more chemiluminescence counts than luminometer 'B'. Over a sample eight month period the sensitivity of the luminometers to routine testing with Samples 1, 4 and 7 remained stable (Table 12). 'Machine background' refers to chemiluminescence counts/10s recorded in the absence of a trituim sample. This value was routinely substracted from measurements obtained in the presence of trituim.

Geometry of light detection

Unless otherwise stated PMN luminol chemiluminescence experiments involved additon of stimulus solution or suspension to cell suspension in equal volumes (500µl) to effect rapid mixing. This large change in volume, however, meant that direct comparison of background (unstimulated or resting) PMN chemiluminescence and the maximum response to stimulation could not be made unless the effect of the volume change on the geometry or efficiency of light detection by the photomultiplier tube was determined. Luminometers 'A' and 'B' were therefore calibrated using varying volumes of a solution which emitted

Table 11 - Calibration of luminometers 'A' and 'B' with tritiated inulin standards at room temperature (22°C)

Tube	H_2O (µl)	[^{3}H] Inulin (µl)	Triton Toluene (µl)	$CHCl_3$ (µl)	Scintillation counts/min	Luminometer 'A' c /10s machine BG subtr. (mean ± SD n = 3)	Luminometer 'B' c /10s machine BG subtr. (mean ± SD n = 3)	Ratio 'A' to 'B'
Machine BG	-	-	-	-	-	273 ± 36	126 ± 24	2.2
1	0	20	300	0	4,653,433	159,249 ± 2,782	64,058 ± 70	2.5
2	0	20	300	30	54,493	9,108 ± 42	3,671 ± 186	2.5
3	10	10	300	30	26,597	5,718 ± 180	2,308 ± 58	2.5
4	25	5	300	30	17,470	2,596 ± 139	1,014 ± 55	2.6
7	10	10	300	30	397,754	21,051 ± 166	8,162 ± 184	2.6
8	10	10	300	30	31,660	5,208 ± 306	2,156 ± 38	2.4
9	10	10	300	10	249,011	70,241 ± 2,458	27,437 ± 281	2.6
10	10	10	300	2	3,338,925	—	—	-
11	10	10	300	1	3,418,042	96,837 ± 1,170	40,690 ± 1,397	2.4
12	-	-	300	0.5	45	172 ± 57	72 ± 38	2.4
13	-	-	300	30	20	5	-0.04	-
14	-	-	300	30	25	2.3	0.03	-

Table 12 - Stability of luminometers tested by three quenched tritiated inulin samples over eight months.

Tritium Sample Number	Luminometer 'A' counts/10s mean ± SD n = 4 (machine BG subtracted)	Luminometer 'B' counts/10s mean ± SD n = 16 (machine BG subtracted
1	153,636 ± 6,507	57,679 ± 4,266
4	3,322 ± 457	968 ± 86
7	24,649 ± 1,207	8,551 ± 792

chemiluminescence at a constant rate. 7ml of 10mM ATP/luciferase solution (2.7) was aliquotted to 6 LP_3 tubes in volumes in the range 0.1 - 2.0 ml.

The tubes were placed in turn into the luminometer housing on three occasions each over a ten min period and chemiluminescence counts/10s measured. The results obtained for luminometer 'B' are presented in Fig. 14. The broken line represents the values expected at each volume point if no change in the geometry of light detection at 1ml had occurred. For both luminometers the conversion factors for volumes of 0.5ml (fo.5) and 1.5ml (f1.5) were thus derived (Table 13).

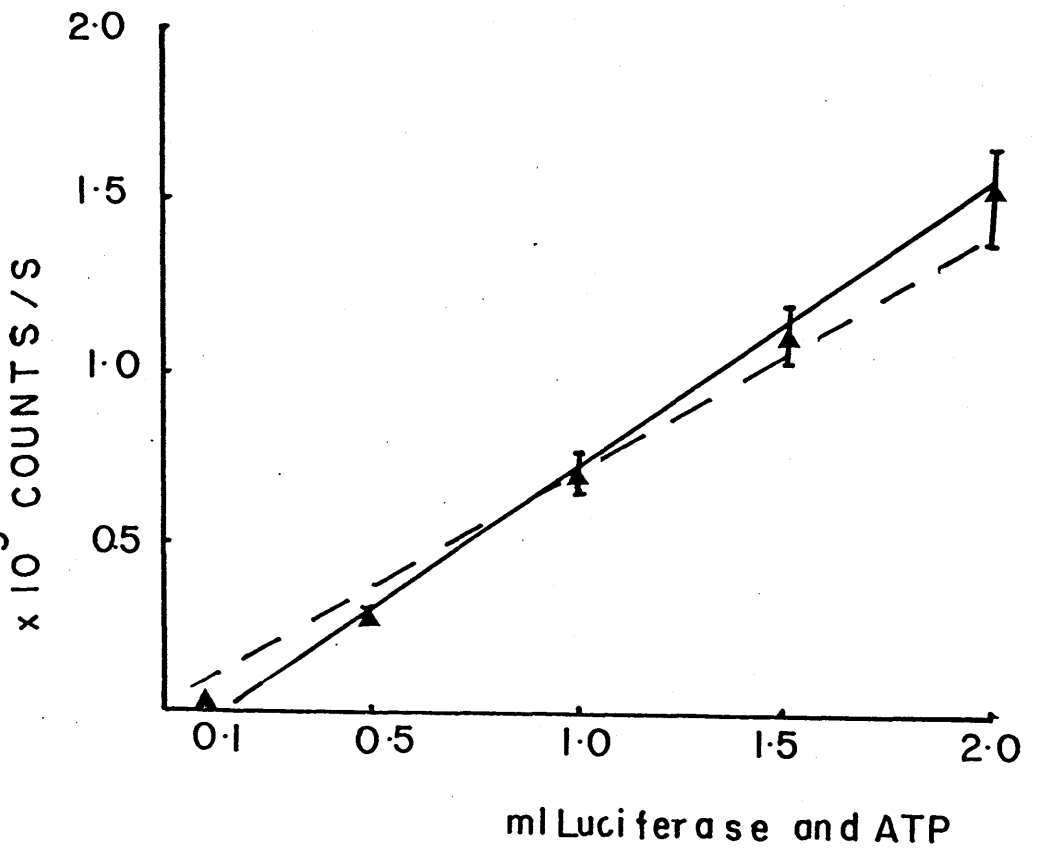


Figure 14 Geometry of light detection by luminometer 'B'

Chemiluminescence counts/s measured by luminometer 'B' in presence of 0.1-2.0ml of ATP/'luciferase solution' (10mM ATP, firefly luciferase, 2mM $MgCl_2$, 20mM sodium arsenate) pH 7.4, room temperature. Points represent mean ± S.D., n = 3. The broken line represents the values expected at each volume point if no change in the geometry of light detection at 1ml had occurred.

	Luminometer 'A'	Luminometer 'B'
f 0.5	1.5	1.2
f 1.5	0.9	0.9

Table 13 - Geometric conversion factors for luminometers 'A' and 'B'

2.9 Isolation of human PMN from whole blood

2.9.1 Method

24 ml whole blood freshly venesected from a normal volunteer (or a patient with rheumatoid arhtritis where stated) was heparinised (25 iu/ml). The majority of erythrocytes were removed by dextran sedimentation based on the method of Skoog and Beck, (1956). The heparinised blood was mixed with 6ml 6% (w/v) dextran (approximate molecular weight 70,000) in 0.9% (w/v) NaCl and allowed to stand in a polythene measuring cylinder for 60min at room temperature (22^{o}C) (Fig. 6 (a)). The straw coloured supernatant was aliquotted into two universal containers and centrifuged at 400g for 10min. The cell pellets were gently resuspended (using a 5mm bore polythene pipette) in Krebs medium pH 7.4 containing 0.1% (w/v) bovine serum albumin, layered on Ficoll-Paque and centrifuged at 400g for 40 min (based on the method of Boyum, (1968)).

The mononuclear cell layer at the interface between Krebs medium and Ficoll-Paque was then removed from each container. The cell pellets comprising erythrocytes and PMN were washed in Krebs medium

(0.1% (w/v) bovine serum albumin) and centrifuged at 400g for 5 min. Using a modification of the method of Fallon et al, (1962), the remaining erythrocytes were lysed by first resuspending the pellets in 2ml 0.9% (w/v) NaCl and then adding 6ml cold double distilled water. The suspensions were shaken gently for 2 min after which 2ml cold 3.5% (w/v) NaCl was added to each and the containers were again centrifuged at 400g for 5 min. Unless otherwise stated this procedure was always performed twice, followed by removal of supernatant containing haemolysed erythocytes and aspiration of any visible red cell membranes from the PMN pellets. The PMN were then pooled and washed twice in Krebs medium (0.1% (w/v) bovine serum albumin) pH 7.4, before final resuspension in 4ml Krebs medium pH 7.4 without added bovine serum albumin. Following the suggestion of Andersen and Amirault, (1979), this suspension was poured through a 0.2mm pore diameter stainless steel filter to remove any macroscopic cell debris. PMN were stored on ice for up to 7 hours.

2.9.2 Criteria of Preparation

Following isolation from whole blood, PMN were characterised on the basis of four criteria:

1. Purity
2. Yield
3. Viability

Yield and Purity of PMN

In order to optimise the number and specificity of experiments which could be carried out on PMN isolated from a 24ml sample of whole blood, PMN were required in high yield (number isolated expressed as percentage of number in original blood sample), and in high purity.

The mean of three cell counts performed with a haemocytometer

(improved Neubauer, Hawksley, Cristalite) and phase contrast microscope established the total number of PMN recovered by each isolation procedure, the mean ± SD of 25 procedures being 2.1 ± 0.5 x 10^7 PMN. Sample preparations stained with haemotoxylin and eosin were >95% pure for neutrophil PMN (Fig 15).

The total number of PMN in randomly selected whole blood samples prior to isolation was detemined using a Coulter counter (Model 'S') and differential white cell counting of a stained whole blood film. The mean PMN yield for ten isolations from normal whole blood carried out during the first eighteen months of this work was 22.9 ± 7.8% (± SD). The yield and purity of PMN at each stage of a single isolation procedure in this period is shown in Table 14. It may be seen that the largest loss of PMN occurred during the red cell hypotonic lysis, giving a final yield of only 19% of PMN in the original whole blood sample. As detailed in 2.9.1 the red cell lysis step involved exposure of cells to unbuffered cold double distilled water on two occasions for two minutes each, the shorter 30 sec lysis period recommended by Fallon et al, 1962, having been found ineffective. Red cell haemoglobin inhibits PMN chemiluminescence (Table 10, 1.4.2) and thus the lower yield of PMN was sacrificed for the higher purity. In the last six months of this work, however, the red cell hypotonic lysis suddenly became and remained more efficient such that cells needed only to be exposed once to cold double distilled water for 60-90 sec. The yield of PMN correspondingly incresed to 37.9 ± 6.9% (mean ± SD, n = 3). The unexpectedly low number of PMN found in the 'post dextran' sample (Table 14) was attributable to a mixing artefact of this high viscosity solution in the Counter counter.

3. PMN Viability

PMN were required for experimental purposes for an average of 4

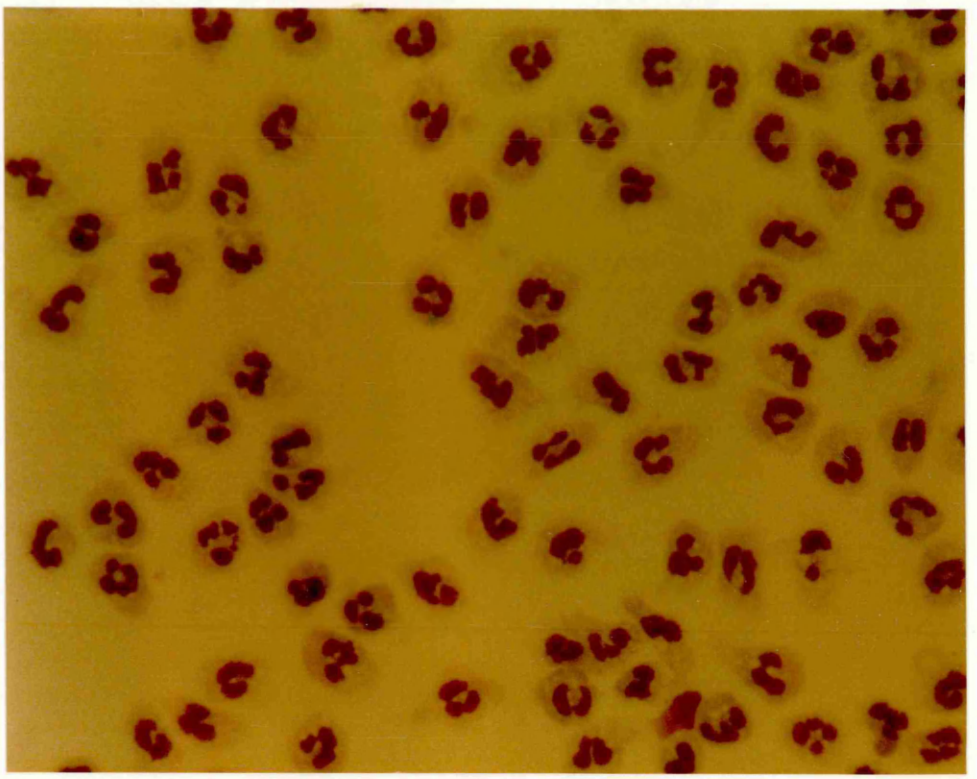


Figure 15 - Sample preparation of PMN isolated from whole peripheral blood and stained with Geimsa.
(magnification x 2,000)

Table 14 - A typical isolation of PMN from whole blood showing yield and purity of PMN in cell preparation at each stage.

		Vol.	Total WCC x 10^7	Total PMN x 10^7	Yield of PMN	Purity of PMN
1	Whole blood	20ml	10.4	6.3	100%	61%
2	Post Dextran	14ml	5.5	2.7	43%	49%
3	Mononuclear cell layer	6ml	3.2	0.25	4%	16%
4	Post Ficoll	20ml	4.4	3.9	62%	88%
5	Post RBC lysis	20ml	4.8	4.6	73%	95%
6	Final Prep	5ml	1.3	1.2	19%	96%

hrs following their isolation from whole blood. It was therefore desirable that the majority of PMN should remain viable throughout this period and that optimum conditions for their storage should be defined. The viability of PMN was established by measurement of the rate of lactate dehydrogenase release (2.6) and rate of loss of PMN ATP content (2.7) while the optimum storage temperature was defined by measurement of the maximum chemiluminescence response to a standard stimulus of PMN which had been stored at $4^{o}C$, $25^{o}C$ or $37^{o}C$ for 0-220 min following their isolation from whole blood (Fig 16). After 3 hrs at $25^{o}C$ or $37^{o}C$ PMN peak height chemiluminescence fell to 25% and 21% of the initial value, while the peak height response of PMN stored on ice at $+4^{o}C$ was still 78% of the initial value. PMN were therefore always stored on ice following isolation from whole blood and were allowed to equilibrate at this temperature for a minimum of 1 hour before the first experiment was performed.

Rate of lactate dehydrogenase release

For PMN stored on ice, there was <5%/hr release of lactate dehydrogenase. The percentage lactate dehydrogenase measured in the supernatant in a typical PMN preparation at 0-150 min following isolation were as shown in Table 15.

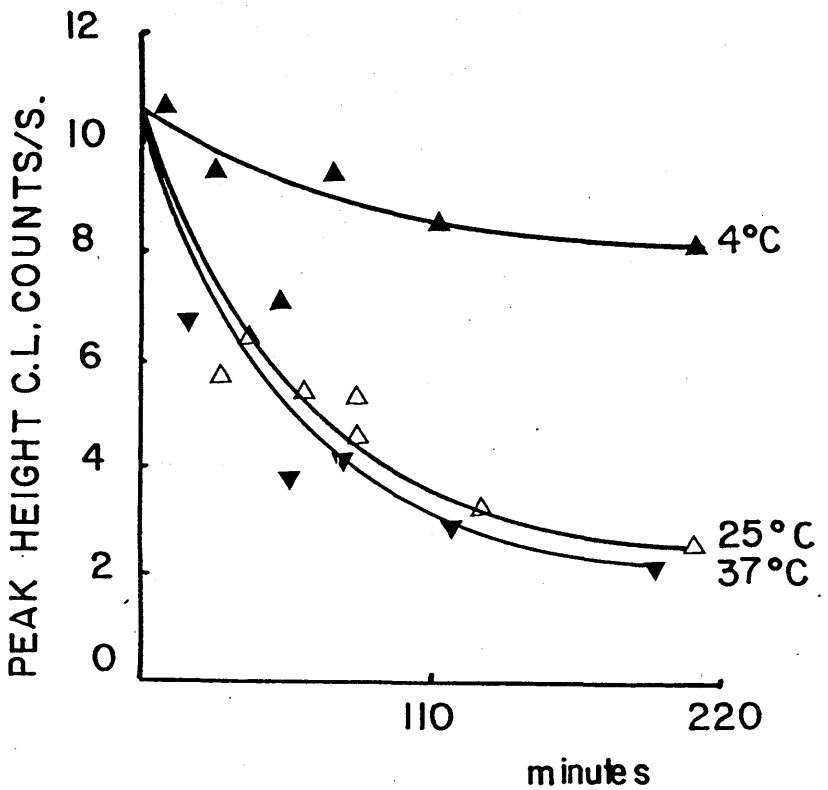


Figure 16 Effect of storage temperature on PMN luminol chemiluminescence

Maximum chemiluminescence, following stimulation by 4 x 10^9/ml latex particles, of 6 x 10^5 PMN/ml which had been previously stored at 4°C (▲), 25°C (△) or 37°C (▼) for 0-220 mins in $A_{2/1}$, at 37°C for 5 minutes immediately prior to stimulation.

Table 15 Rate of lactate dehydrogenase release by PMN following isolation from whole blood.

Time on ice after separation	% Lactate dehydrogenase in supernatant
0 min	18%
80 min	18%
15o min	8%

% lactate dehydrogenase in supernatant of 6.9×10^6 PMN/ml Krebs medium pH 7.4

The high basal value was attributed to the residue of lactate dehydrogenase released by red cells during their lysis.

Rate of loss of PMN ATP content

The mean PMN ATP content measured after one hour on ice following their separation from whole blood was 0.037 ± 0.016 femtomoles/cell (± SEM, n = 5). There was a 9.2 ± 11%/hr mean ± SD, n = 5 decrease in PMN ATP content over the subsequent 3-4 hours.

2.10 Measurement of oxygen consumption by PMN using an oxygen Electrode

PMN oxygen consumption was measured polarigraphically using a Clark type electrode (from Rank Brothers, Bottisham, Cambridge, U.K.) connected to a pen chart recorder. The electrode was held at 0.6V and separated by an oxygen permeable teflon membrane from the cell chamber whose temperature was maintained at 37°C by thermostatically controlled circulating water.

The cell suspension (1.2ml, 10^6PMN/ml Krebs medium pH 7.4) was

introduced to the cell chamber and continuously stirred by a magnetic follower. A tight fitting plunger was placed in position immediately above the fluid so that there was no longer any air fluid interface. A 5 min equilibration time was allowed following which a constant rate of oxygen consumption was detected by the electrode. Cell stimuli (volume <5ul) were introduced to the suspension by injection from a Hamilton syringe (Bonaduz,Switz'land)via a capillairy tube in the chamber plunger and any subsequent change in the rate of oxygen consumption by the PMN was recorded.

The electrode was calibrated by assuming that the fully air saturated solution of Krebs medium contained 210μM oxygen.

2.11 Justification for use of luminol dependent chemiluminescence as a monitor of oxygen radical production by PMN.

The use of luminol dependent chemiluminescence as a monitor of oxygen radical production by PMN was justified by:

1. Comparison with PMN endogenous chemiluminescence
2. Demonstration of its relationship to oxygen consumption by PMN.
3. Demonstration of its dependence on oxygen radical production by PMN.

2.11.1. Comparison with PMN endogenous chemiluminescence

Using luminometer 'C' (2.8.1) the endogenous chemiluminescence of PMN stimulated by latex particles in the absence of luminol was detected (Fig 17). A biphasic time course was observed, the first phase reaching a maximum at 25 seconds after the additon of stimulus and the larger second phase reaching a maximum at around 180 seconds. For 1.5×10^6 PMN, however, the peak height of chemiluminescence was only 2.75×10^2 counts/s. The first phase may simply have represented

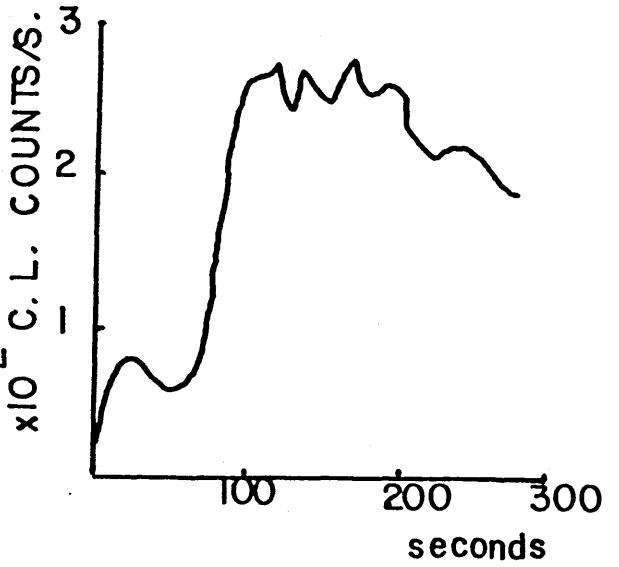


Figure 17 Endogenous PMN chemiluminescence

Chemiluminescence trace produced by 1.5×10^6 PMN/ml in the absence of luminol, following the addition (at time 0 seconds) of 4×10^9/ml latex particles, Krebs medium, pH 7.4, 37°C, Luminometer 'C'.

a cell/stimulus mixing artefact. In the presence of luminol, using luminometer 'A' the peak height of 0.5 - 8.5 x 10^5 PMN stimulated by the same concentration of latex particles produced a peak height of chemiluminescence in the range 0.5 - 15x10^4 counts/s. (Fig 18). PMN luminol dependent chemiluminescence was thus larger in magnitude, easier to measure and required fewer cells than PMN endogenous chemiluminescence.

2.11.2 Demonstration of relationship of PMN luminol chemiluminescence to oxygen consumption by PMN

The concentration of luminol which would be used in this and all further PMN chemiluminescence experiments was determined by measurement of chemiluminescence generated by 5 x 10^5 PMN/ml and a fixed concentration of stimulus in the presence of luminol in the range 100μm - 100nM (Fig 19). 10μM was the concentration of luminol selected as this afforded a large but submaximal PMN chemiluminescence response.

PMN oxygen consumption was measured using the oxygen electrode as detailed in the previous section. In a typical experiment, the oxygen consumption of 9.3 x 10^5/ml PMN which had been equilibrated at 37°C for 5 min was 0.8nmoles/min. Addition of latex particles was followed by an increase in the rate of oxygen consumption which reached a maximum at 35 sec (3.4 nmoles/min) plateaued for 180 sec and gradually decayed (Fig 20). The temporal relationship of the luminol chemiluminescence trace produced by the same concentration of PMN, isolated on the same day, was observed (Fig. 20).

2.11.3. Demonstration of dependence of PMN luminol chemiluminescence on oxygen radical production

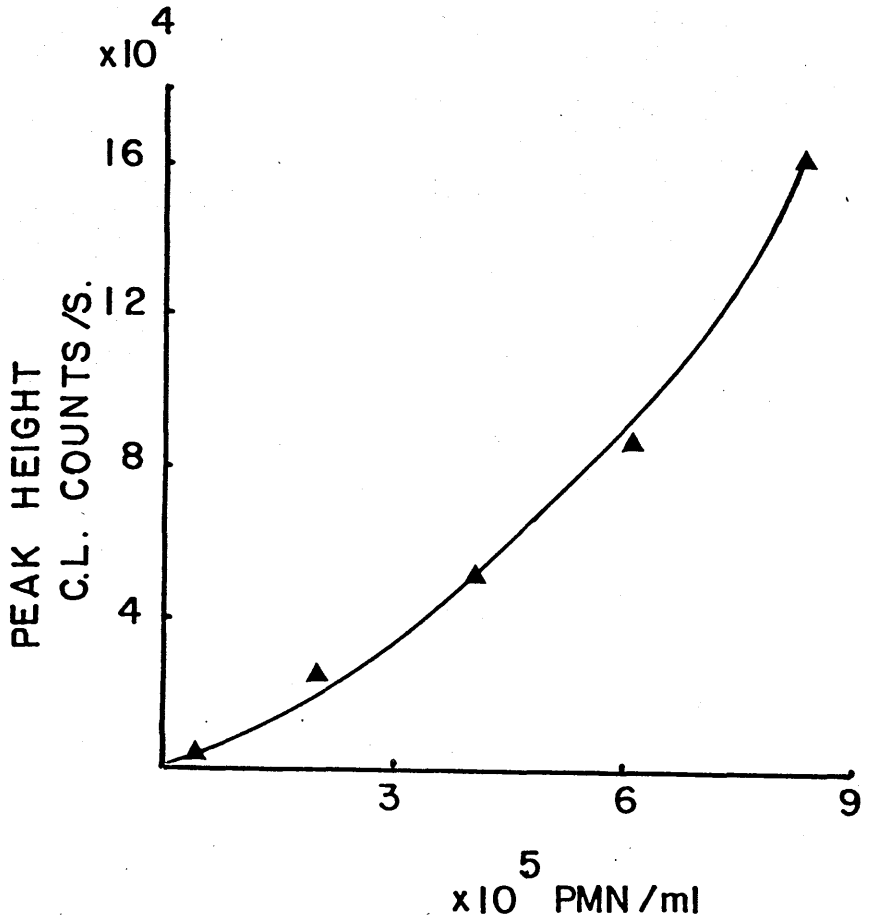


Figure 18 Relationship of PMN luminol chemiluminescence to cell concentration

The graph shows the results from one experiment in which 1 - 8 x 10^5 PMN/ml were stimulated by addition of 4 x 10^9 /ml latex particles in the presence 10μM luminol (0.1% w/v DMSO), Krebs medium, pH 7.4, 37°C.

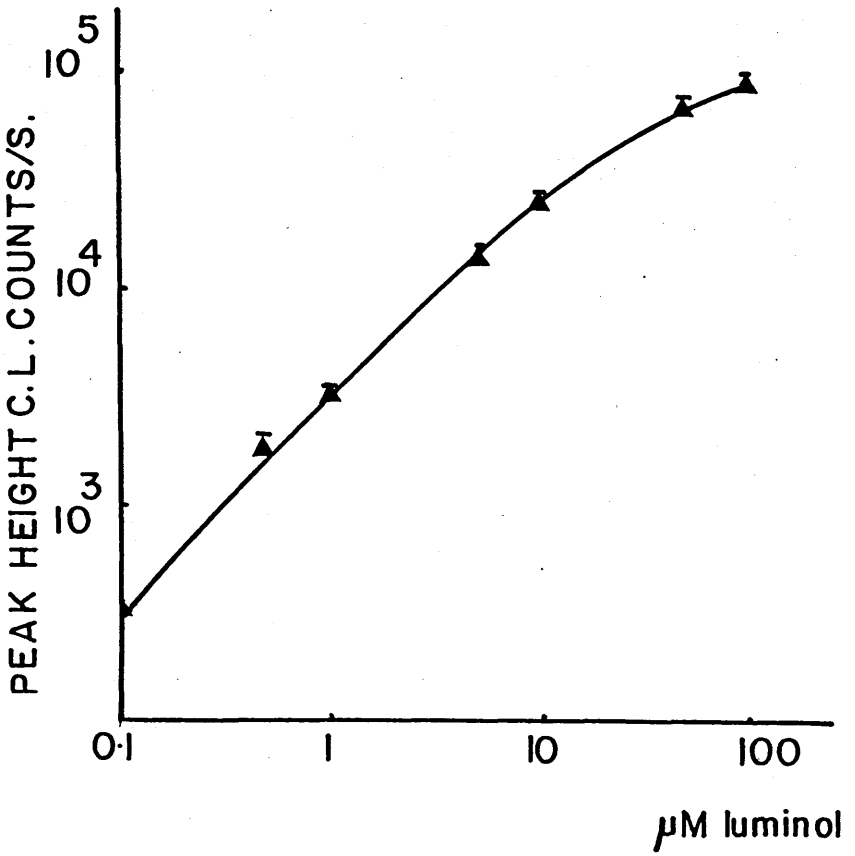


Figure 19 Relationship of PMN chemiluminescence to luminol concentration

The graph shows the results from one experiment in which 5×10^5 PMN/ml were stimulated by addition of 2×10^9/ml latex particles in the presence of 0.1 - 100μM luminol. The concentration of DMSO was maintained at 0.1% (w/v) for each luminol concentration. Points show the mean ± SEM of two or three replicate determinations, Krebs medium, pH 7.4, 37°C.

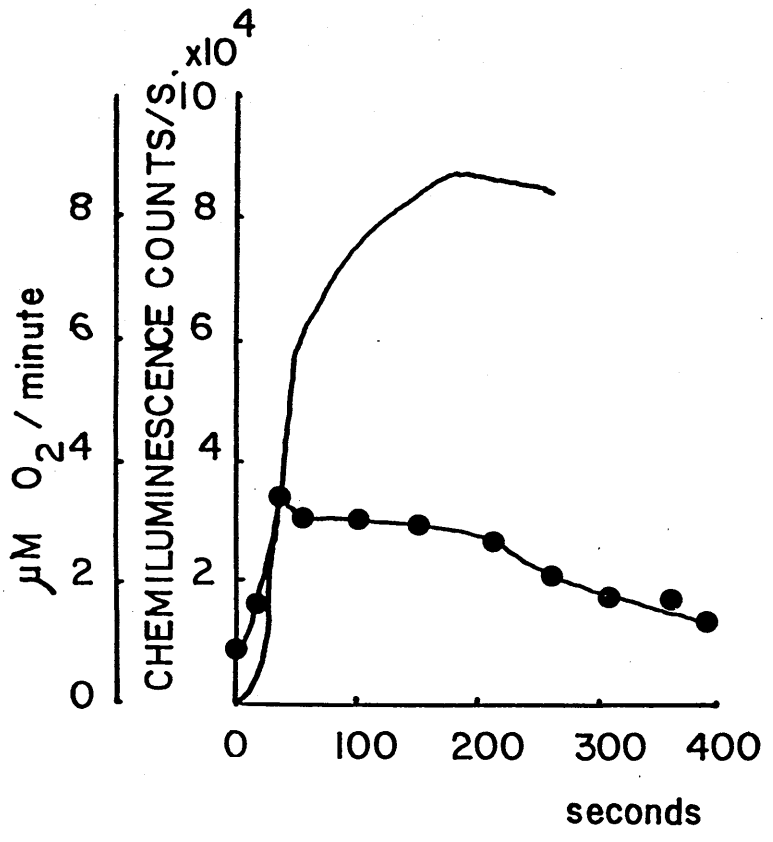


Figure 20 Comparison of stimulated PMN oxygen consumption and luminol chemiluminescence

The luminol chemiluminescence trace (unbroken line) and oxygen consumption (●) of 9.3×10^5 PMN/ml, following addition, at time 0 seconds, of 4×10^9 /ml latex particles, were compared in separate consecutive experiments on the same day. Krebs medium, pH 7.4, 37°C, 10μM luminol (0.1% (w/v) DMSO).

The dependence of PMN luminol chemiluminescence on the generation of oxygen radicals by the cells was confirmed using the known oxygen radical scavengers superoxide dismutase and catalase. When PMN were preincubated with 10-200μg/ml superoxide dismutase for 3 min prior to their stimulation by latex particles in the presence of luminol a 35 - 71% inhibition of peak height chemiluminescence occurred (Fig. 21). The inhibition caused by 10μg/ml superoxide dismutase was abolished when the enzyme was first heated to 100°C for 10 min. Catalase (62.5μg/ml) caused an 88% inhibition which was larger than expected from the published data, (Johnston and Lehmeyer 1977).

2.12 Standardisation of PMN luminol chemiluminescence measurement

In order to compare and contrast the chemiluminescence responses of PMN which had been isolated on different occasions, or which were stimulated by different agents,

a) in the presence and absence of drugs or oxygen radical scavengers and

b) with the results of other researchers

four parameters of chemiluminescence measurement and the conditions of PMN chemiluminescence assay were defined.

2.12.1 Methods of chemiluminescence quantitation

1. Background chemiluminescence - chemiluminescence in counts/s of resting or unstimulated PMN incubated with 20μM luminol in 500μl Krebs medium for 3 or 5 min (as stated) at pH 7.4, 37°C.

Where indicated in the text, background chemiluminescence was 'corrected', using the geometric correction factor (2.8.2) to allow for the effect of volume change incurred by subsequent addition of stimulus on the efficiency of light detection by the luminometer.

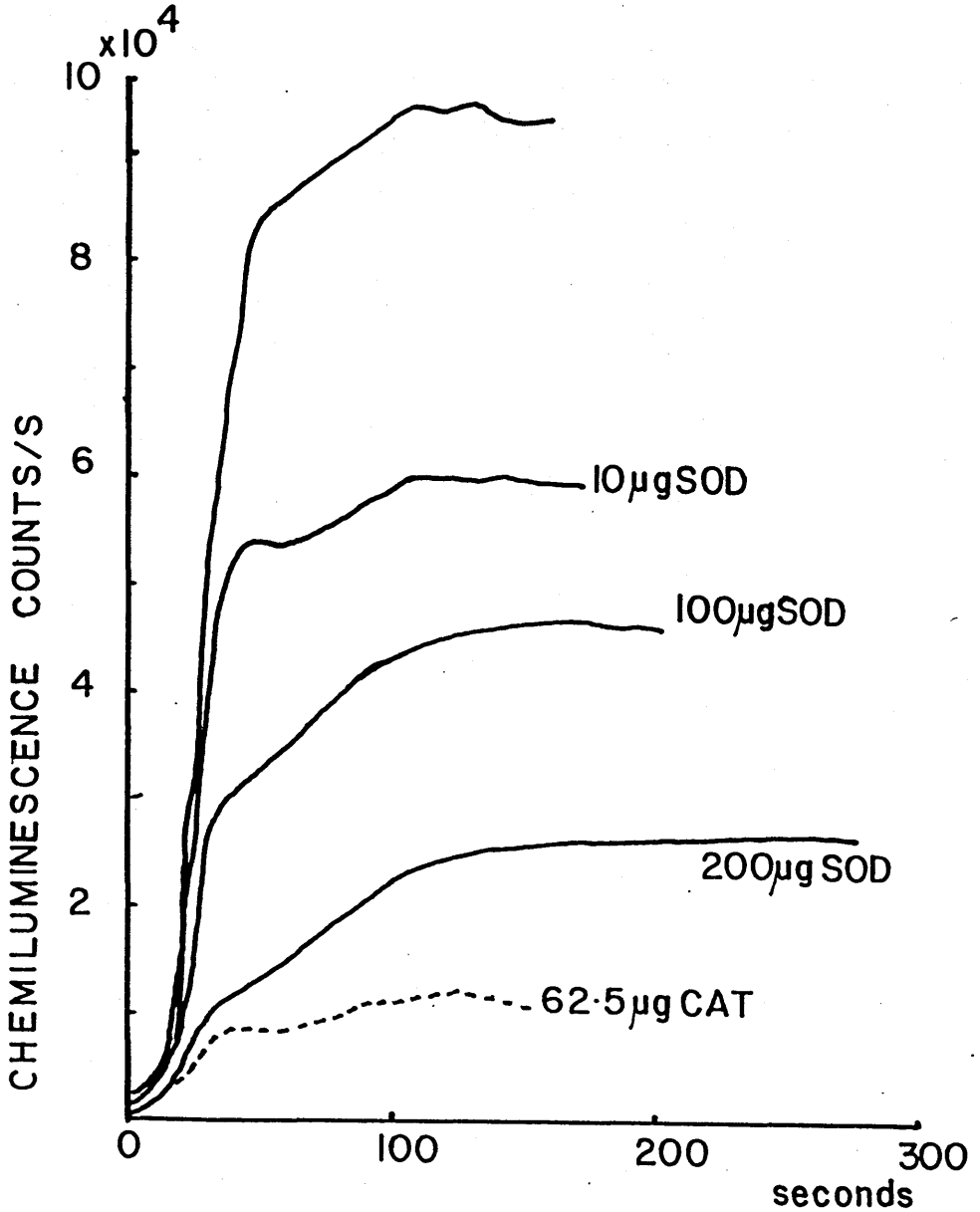


Figure 21 Effect of oxygen radical scavengers on PMN luminol chemiluminescence

Chemiluminescence traces produced by $8.5_9 \times 10^5$ PMN/ml stimulated, at time 0 seconds, by 4×10^9/ml latex particles in the presence and absence of 10-200µg/ml superoxide dismutase (SOD) or 62.5ug/ml catalase (CAT), Krebs medium, pH 7.4, 10µM luminol (0.1% (w/v) DMSO).

2. Peak height chemiluminescence - the maximum chemiluminescence in counts/s achieved following addition of stimulus to PMN which were previously resting, in the presence of luminol in Krebs medium, pH 7.4, 37°C.

3. Stimulation Index - the ratio of peak height chemiluminescence to corrected background chemiluminescence.

4. Background or Peak Height Chemiluminescence as percentage of expected value - Even for PMN stored on ice at 4°C the rate of decrease in peak height and background chemiluminescence was not negligible (18.9 ± 1.8%/hour and 14.6 ± 1.8%/hour (mean ± SD n = 8) respectively). The Stimulation Index however remained comparatively constant (Fig 22). In experiments examining the effects of an additional reagent such as a drug or oxygen radical scavenger, results were documented either as stimulation indices or as background and peak height chemiluminescence expressed as a percentage of the results which would have been achieved at the same time point in the absence of the reagent derived by interpolation of the time course of chemiluminescence decay on the day of experiment (Fig. 22).

2.12.2 Conditions of Assay

PMN luminol chemiluminescence was known to be influenced by a large number of medium variables (Table 10, 1.4.2). Of these variables the effects of changes in pH, and the presence of albumin in low concentration, were particularly examined. In addition the effect of time of preincubation with luminol on background PMN chemiluminescence was studied.

a) Effect of pH

PMN were preincubated for 3 min and the latex particle stimulus

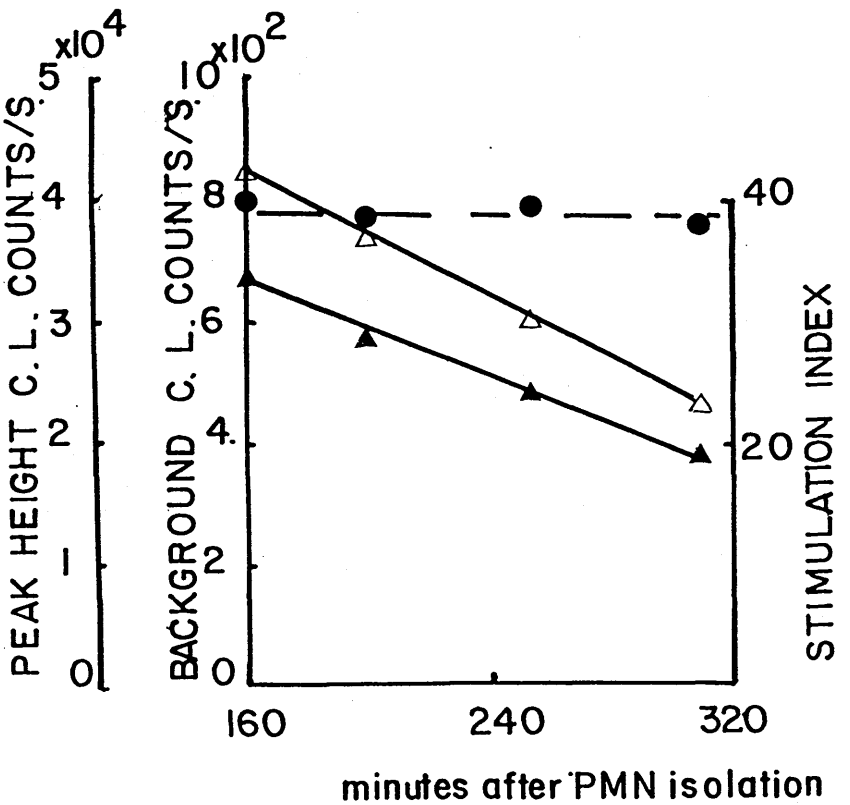


Figure 22 Relationship of three parameters of PMN luminol chemiluminescence to duration of cell isolation from whole blood

The graph shows the results from one experiment in which the background (resting) chemiluminescence (△), peak height (maximum) chemiluminescence (▲) and chemilumipescence stimulation index (see text) (●) of 5 x 10^5 PMN/ml stimulated by 1mg/ml opsonised zymosan were compared at increasing durations of the time for which PMN had been stored at 4 ^{o}C following their isolation from whole blood. PMN were equilibrated in Krebs medium pH 7.4, at 37 ^{o}C for 3 minutes prior to stimulation. 10μM luminol (0.1% (w/v) DMSO).

was added in Krebs medium in which pH was adjusted in the range pH 6.5 - pH 8.4. The peak height of PMN luminol chemiluminescence following stimulation decreased linearly with increasing pH (Fig 23) although the mean background PMN chemiluminescence remained at 676 ± 46 counts/s (± SEM, n = 7). In all future PMN chemiluminescence the pH of Krebs medium was vigorously maintained at 7.4 and always checked and readjusted as necessary following the addition of drugs or oxygen radical scavengers.

b) Effect of albumin in low concentration

Bovine or human serum albumin in low concentrations (up to 0.5% (w/v)) or human or foetal calf serum (1-10% (w/v)) are frequently added to isolated PMN preparations to enhance viability (Hastings et al 1982, Sacks et al 1978, Weening, Wever and Roos, 1975). 0.1% (w/v) bovine serum albumin was found to inhibit stimulated PMN luminol chemiluminescence by 43% (Fig 24) however. Protein was therefore omitted from chemiluminescence assays unless specifically stated.

The concentrations of calcium and magnesium ions and of HEPES buffer were determined by the composition of Krebs medium (2.2). Phenol red, glucose and amino acids were not included in chemiluminescence assays unless stated.

Effect of luminol preincubation time on PMN background chemiluminescence

When the background chemiluminescence of resting PMN which had been preincubated at 37°C, pH 7.4 for 3 mins, was observed immediately following the addition of luminol, a gradual decrease in chemiluminescence counts/s was observed Fig 25 which reached a plateau at approximately 200s following the addition of luminol. To

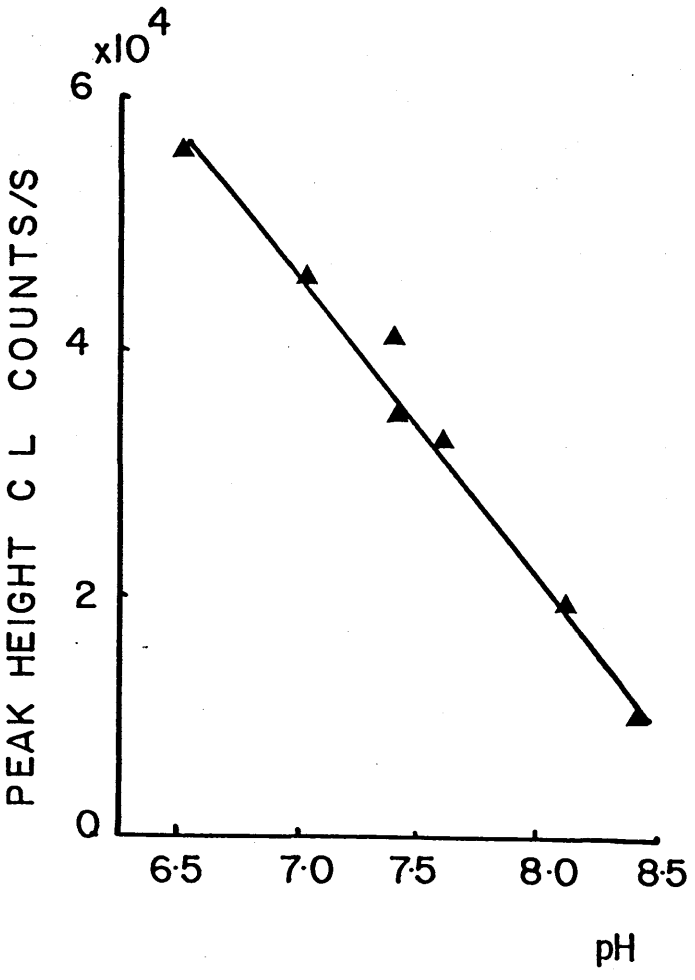


Figure 23 Effect of pH on PMN luminol chemiluminescence

4×10^5 PMN/ml were pre-inc ubated for 3 minutes in Krebs medium with pH re-adjusted in the the range 6.5 - 8.4, prior to stimulation by 4×10^9/ml latex particles. The relationship of the maximum (peak height) chemiluminescence to pH is shown) 37°C, 10μM luminol (0.1% (w/v) DMSO).

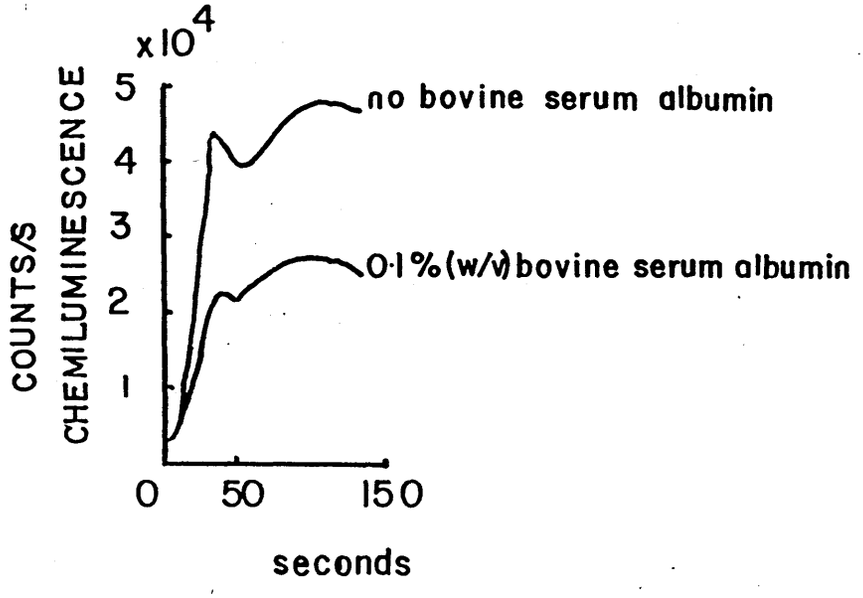


Figure 24 Effect of bovine serum albumin on PMN luminol chemiluminescence

Chemiluminescence traces produced by 6 x 10^5 PMN/ml stimulated at time 0 seconds, by 4 x 10^9/ml latex particles in the presence and absence of 0.1% (w/v) bovine serum albumin. The albumin was included in the 3 minute equilibration of the cells in Krebs medium pH 7.4, 37°C with luminol 10μM (0.1% (w/v) DMSO).

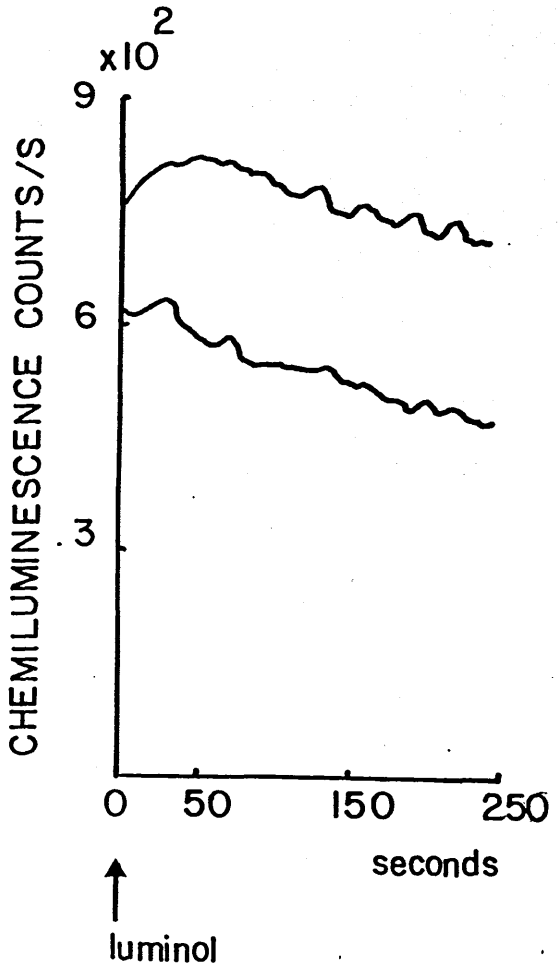


Figure 25 Effect of luminol preincubation time on PMN background chemiluminescence

Chemiluminescence traces produced by 4.6×10^5 PMN/ml which were preincubated, unstimulated, in Krebs medium pH 7.4, 37°C for 3 mins. prior to the addition of luminol (10μM, 0.1% (w/v) DMSO) at time 0 seconds.

standardise chemiluminescence assay conditions, luminol was always added to the cell suspension at the onset of 5 min preincubation at 37°C before background chemiluminescence was recorded.

Chapter 3: Characterisation of luminol-dependent chemiluminescence as an indicator of oxygen radical production by PMN

3.1 Introduction

3.2 Methods
- 3.2.1. General
- 3.2.2. Immune complexes
- 3.2.3. Characterisation of a solid phase chemiluminescence indicator

3.3 Results
- 3.3.1. Chemiluminescence of PMN stimulated by unopsonised latex particles
- 3.3.2. Chemiluminescence of PMN stimulated by opsonised zymosan
- 3.3.3 Chemiluminescence of PMN stimulated by the chemotactic peptide N-formyl-met-leu-phe
- 3.3.4. Chemiluminescence of PMN stimulated by immune complexes
- 3.3.5. Localisation of PMN luminol chemiluminescence.

3.4 Discussion

3.5 Summary

3.1 Introduction

In the work described in the previous chapter, a reproducible method for the isolation of viable PMN from whole blood was developed (2.9), and luminol dependent chemiluminescence was justified and standardised as a monitor of oxygen radical production by these cells (2.11, 2.12). Before examining rheumatoid serum and synovial fluid for the presence of stimuli to the production and release of oxygen radicals by PMN and for the presence of extracellular scavengers of oxygen radicals (Chapter 4), it was first necessary:-

(a) To determine what proportion of PMN luminol dependent chemiluminescencce occurs extracellularly.

(b) To compare and contrast the luminol dependent chemiluminescence of normal PMN exposed to a range of soluble and insoluble phagocytic and chemotactic stimuli, as models of those previously demonstrated at sites of disease activity in rheumatoid arthritis (1.3.1).

and

(c) to define the dose of each stimulus which would cause a PMN chemiluminescence response which was 50% of the maximum and which could therefore be used to identify enhancing or inhibitory effects of additional reagents such as oxygen radical scavengers (or drugs).

Demonstration, hitherto, of the extracellular release of $O_2^{\cdot -}$ and H_2O_2 by stimulated PMN (Babior et al 1973, Root et al 1975) was reliant on fluid phase detectors of oxygen radicals which if phagocytosed or endocytosed by the cells would also measure intracellular oxygen radical production. The chemiluminescence resulting from stimulation of PMN in the presence of 'fluid phase'

luminol might similarly reflect intracellular oxygen radical production (Allred et al 1980). In this chapter, investigation of what proportion of PMN luminol dependent chemiluminescencce occurs extracellularly (3.3.5) included the use of a solid phase non phagocytosable isoluminol chemiluminescence indicator.

Four known activators of the PMN respiratory burst (cf Table 4, 1.2.2) were chosen as models of stimuli previously demonstrated at sites of rheumatoid disease activity, namely:-

(i) latex particles

(ii) opsonised zymosan

(iii) immune complexes

(iv) chemotactic peptide (n formyl met leu phe).

The first three (i) - (iii) represented varieties of phagocytic stimulus (3.3.1 - 3.3.3) of which latex provided an unopsonised particle stimulus and opsonised zymosan provided particles coated predominantly with C3b (Henson 1971(b)). Soluble and insoluble immune complexes provided a stimulus for PMN Fc receptors. One of the n formyl methionyl peptides found in procaryotes (iv) was chosen as a model chemotactic stimulus (3.3.4) (Schiffmann, Corcoran and Wahl, 1975, Hatch et al 1978) although it was recognised that degradative components of the complement pathway are likely to be the chief chemoattractants in rheumatoid arthritis (1.2.2, 1.3.1).

3.2 Method

3.2.1. General

PMN were isolated from normal whole blood as described in 2.9.1 Luminol dependent chemiluminescence was mesured in luminometers 'A' or 'B' (2.8.1). Latex particles, 1μm diameter, were obtained in suspension from Dow diagnostics, such that 10μl suspension further

diluted in 1ml Krebs medium resulted in 4×10^9 latex particles/ml Zymosan from s. cerevisiae yeast was opsonised as described in 2.5 and resuspended in Krebs medium pH 7.4 on the day of experiment at 20mg/ml (9×10^8 particles/ml, particle size 3-4 μm). Chemotactic peptide stock solution (2.2) was diluted in Krebs medium as indicated.

3.2.2. Immune Compexes

Immune complexes were formed in vitro from bovine serum albumin (1mg/ml Krebs medium, pH 7.4) and the purified IgG fraction (in 0.15M phosphate buffered saline pH 7.2) from the serum of a rabbit immunised with bovine serum albumin. The anti bovine serum albumin antibody was kindly donated by Dr. B. D. Williams, Senior Lecturer in Rheumatology, and was 20% specific for bovine serum albumin with equivalence (the ratio of antibody to antigen which gave the maximum concentration of insoluble complexes) occurring at 50μl antibody : 15μg antigen. Human serum depleted of the terminal attack complement component C9 by exposure to polyclonal antibodies to C9 was a kind gift of of Dr. B. P. Morgan, Research Fellow, Department of Medical Biochemistry.

3.2.3. Characterisation of a solid phase chemiluminescence indicator

In order to determine whether PMN luminol chemiluminescence resulted partly or completely from intracellular oxygen radical generation a mechanism for retaining luminol in the extracellular space was required. A 'solid phase' preparation in which luminol was covalently bound to sepharose beads too large to be phagocytosed by PMN would have achieved this. Unfortunately, the molecular configuration of luminol would not permit such bonding. Instead, amino butyl ethyl iso luminol (ABEI), a derivative of isoluminol was

linked to 78 ± 27μm diameter (mean ± SD, n = 20) porous sepharose beads by cyanogen bromide as shown in Fig. 26. This synthesis was kindly performed by Dr. A. K. Campbell, Department of Medical Biochemistry.

The concentration of ABEI in the solid phase preparation and the proportion which was still unbound to sepharose was determined using an acellular chemiluminescence assay for ABEI adapted from the method of Patel (1983).

Microperoxidase/Hydrogen Peroxide assay for ABEI

A stock solution of microperoxidase (sodium salt from horse heart cytochome c) 0.4mg/ml in Tris HCI buffer pH 7.4 was maintained at +4°C for up to six weeks, and was diluted in Krebs medium pH 7.4 to a concentration of 10μg/ml on the day of experiment. Hydrogen peroxide (H_2O_2) (30 volumes) was diluted 0.2% (v/v) in Krebs medium pH 7.4 to a final concentration of 35mM. Fluid phase ABEI was dissolved in 25mM phosphate buffer at a stock concentration of 10mM which was further diluted in 25mM phosphate buffer pH 7.4 on the day of experiment to 10mM. To assay ABEI, the background chemiluminescence of 0.5μg/ml microperoxidase and 0.1 - 1 p moles ABEI in 500ul Krebs medium pH 7.4 was measured before the initiation of reaction by addition of 35mM hydrogen peroxide in 500μl Krebs medium. A rapid increase in chemiluminescence count/s resulted, reaching a maximum 10s following the additon of hydrogen peroxide and thereafter quickly decaying. The linear relationship of peak height to concentration of ABEI in the range 0.1 - 1 p moles was established (Fig 27).

Measurement of solid phase ABEI

The concentration of sepharose beads in the neat solid phase ABEI preparation was 7.5 x 10^6 beads/ml 25mM phosphate buffer.

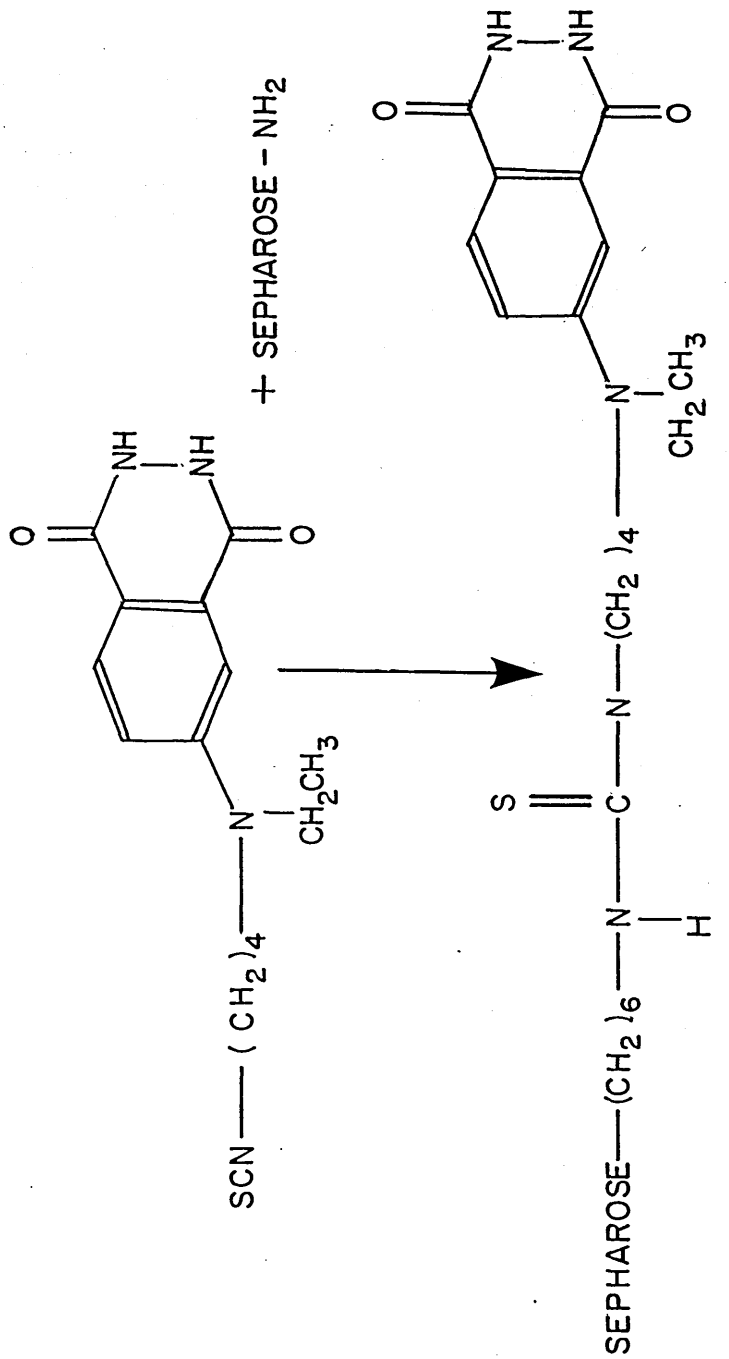


Figure 26 - Solid phase ABEI

Amino butyl ethyl iso luminol (ABEI) was linked to the porous sepharose beads by cyanogen bromide as shown.

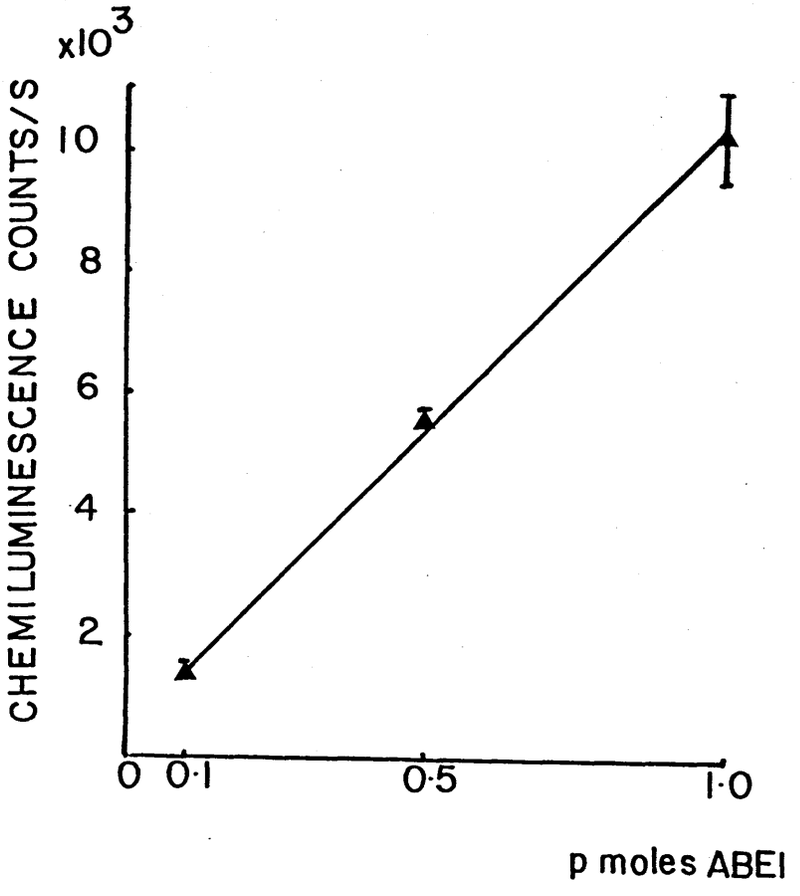


Figure 27 Microperoxidase/hydrogen peroxide assay for ABEI

The graph shows the results from one experiment in which maximum chemiluminescence was measured 10s following the addition of 35mM H_2O_2 in 500µl Krebs medium pH 7.4 to 0.5µg/ml microperoxidase and 0.1 - 1pmoles amino butyl ethyl iso luminol also in 500ul Krebs medium pH 7.4. Room temperature. Points represent means ± SD of 3 observations.

Chemiluminescence of solid phase ABEI in the presence of microperoxidase was initiated by addition of H_2O_2 as above. Light emission was so great that the solid phase preparation had to be diluted to prevent damage to the luminometer photomultiplier tube. In this way it was established that 10µl of 1:100 solid phase ABEI produced the same amount of chemiluminescence as 4 picomoles fluid phase ABEI. By extrapolation 50µl neat solid phase ABEI would give equivalent chemiluminescence to 2 nanomoles fluid phase ABEI. Supernatant from the solid phase preparation, obtained by centrifugation at 10,000g for 2 min yielded only 2.7% of the chemiluminescence of the whole solid phase preparation thus indicating that the majority of the ABEI was bound to the sepharose beads.

3.3 Results

The aims of the experiments described in the following four subsections were firstly to compare the different stimuli of PMN luminol chemiluminescence in terms of:

(a) the time of onset of the chemiluminescence response

(b) the duration of the response

(c) the maximum chemiluminescence achieved

and secondly to define the dose of each stimulus which caused a PMN chemiluminescence response which was 50% of maximum and which could therefore be used to identify enhancing or inhibiting effects of additional agents such as drugs or oxygen radical scavengers.

3.3.1 Chemiluminescence of PMN stimulated by unopsonized latex particles

Stimulation of PMN by latex particles in the presence of luminol resulted in a rapid increase in light emission, as shown in Fig. 28, a

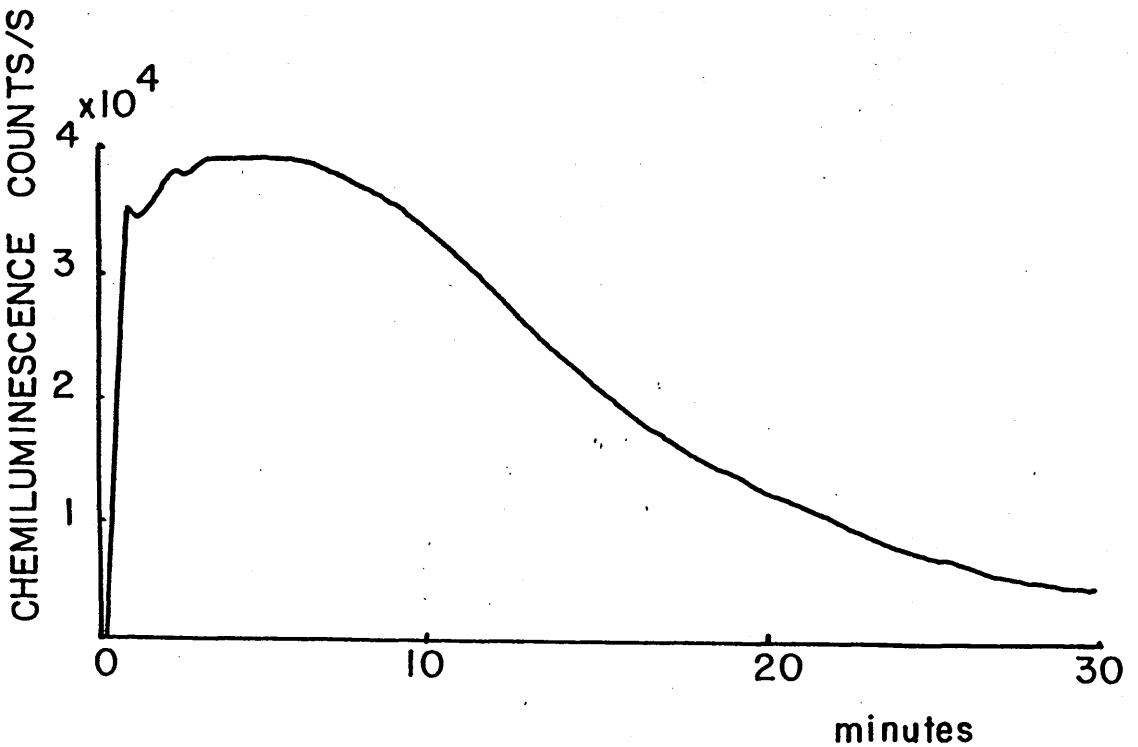


Figure 28 Unopsonised latex particle stimulated PMN luminol chemiluminescence

Chemiluminescence trace produced by 5×10^5 PMN/ml stimulated by addition at time 0 minutes of 4×10^9/ml latex particles. Krebs medium, pH 7.4, 37°C, 10μM luminol.

typical trace produced by luminometer 'B'.

Following the addition of stimulus at 0 min there was a 20s lag before chemiluminescence rapidly increased above the background rate of 3.8×10^2 counts/s to a first peak or shoulder at 60s from there rising to a large second phase with a maximum of 3.9×10^4 counts/s, 290s following the addition of stimulus. Thereafter the chemiluminescence decayed in an exponential fashion but at 30 min was still 4.4×10^3 counts/s and thus raised above the original background level. In order that the maximum number of experiments could be accomplished latex particle induced PMN luminol chemiluminescence was generally observed for only 300s following addition of stimulus, until the peak height of the second phase had been recorded.

Effect of latex particle concentration

Using 10μM luminol, the relationship between PMN chemiluminescence stimulation index (see 2.10) and the concentration of stimulus was established, (Fig 29). A half maximal response occurred at a concentration of 2×10^9 latex particles/ml. Unless otherwise stated this concentration was used in all subsequent latex paticle stimulated PMN chemiluminescence experiments as it enabled either an enhancement or an inhibition of chemiluminescence by any additional substance to be detected.

3.3.2 Chemiluminescence of PMN stimulated by opsonized zymosan

Stimulation of PMN by 3mg/ml opsonized zymosan in the presence of luminol resulted in a steady increase in chemiluminescence above background following a lag of 30s (Fig 30, trace (d)). Light intensity reached a single peak within 360s and then decayed. Stimulation of PMN with unopsonised zymosan (Fig 30, trace (a)) caused only a two fold rise in chemiluminescence above background

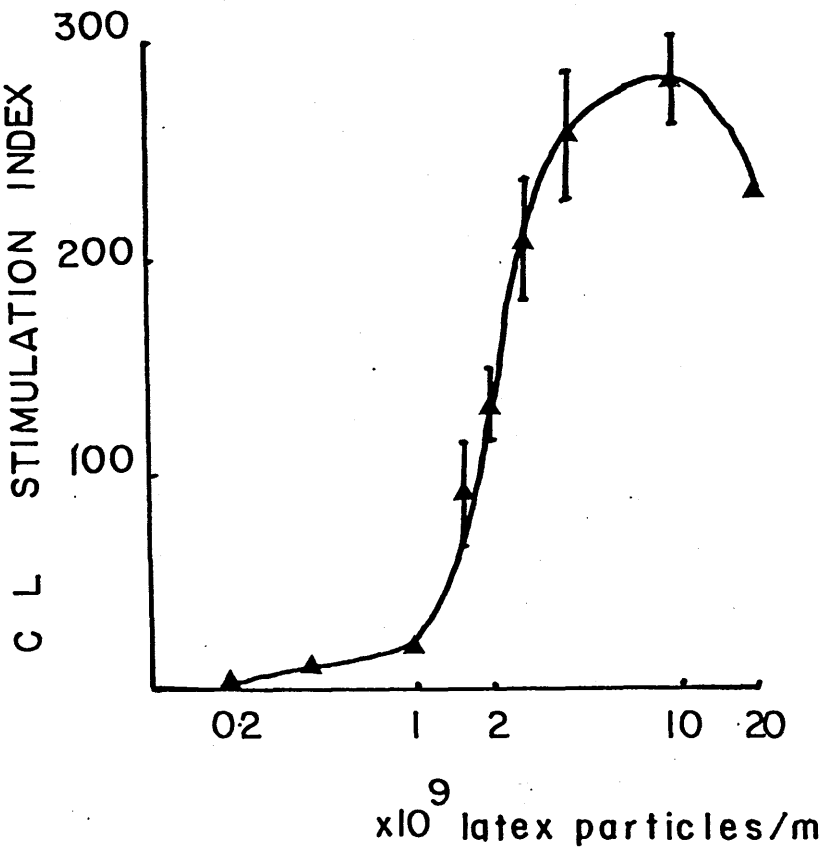


Figure 29 Relationship between PMN chemiluminescence stimulation index and latex particle concentration

The graph shows the results of one experiment in which 5×10^5 PMN/ml were stimulated by the additon of $0.2\text{-}20 \times 10^9$/ml latex particles. Points show mean ± SEM for up to three replicate determinations. Krebs medium pH 7.4, 37°C, 10μM luminol.

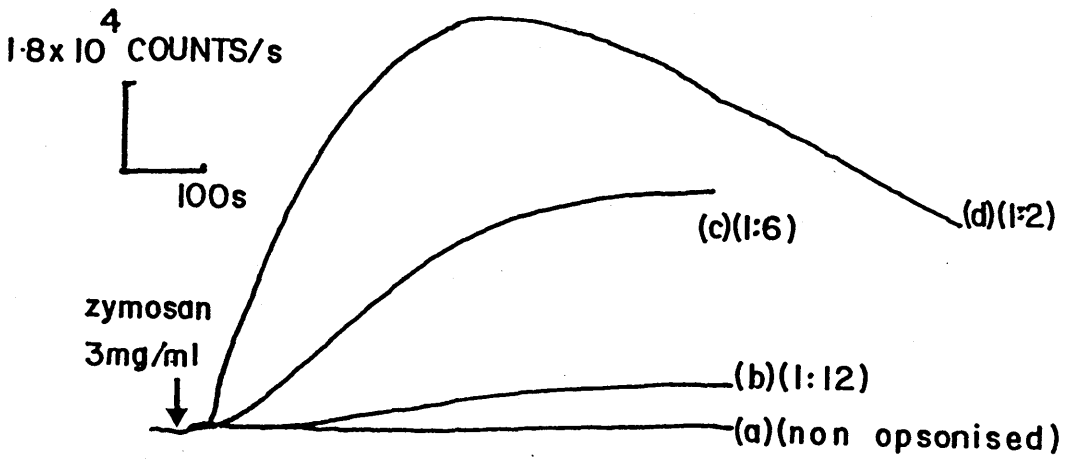


Figure 30 Chemiluminescence of PMN stimulated by opsonised zymosan - effect of serum dilution during opsonisation.

The graph shows chemiluminescence traces produced by four preparations of 5×10^5 PMN/ml stimulated by addition (at arrow) of 3mg/ml zymosan which was (a) not opsonised or (d)-(b) opsonised by prior exposure at 37°C for 30 minutes to normal human serum diluted (1:2) - (1:12) in phosphate buffered saline pH 7.4. In all cases the zymosan was washed three times in phosphate buffered saline before resuspension in Krebs medium pH 7.4. PMN chemiluminescence was observed in Krebs medium, pH 7.4, 37°C, 10μM luminol.

demonstrating that at least 98% of chemiluminescence observed in the first trace was attributable to receptor mediated stimulation of the PMN by the opsonised zymosan (Henson 1971(b)).

Increased dilution of serum in phosphate buffered saline pH 7.4 during the opsonisation of zymosan (see 2.5) over the range 1:2 to 1:12 was likewise accompanied by a decrease in the magnitude of chemiluminescence produced by PMN subsequently stimulated by the zymosan as shown in sample traces (d) to (b) (Fig. 30), in agreement with the results of Easmon et al, 1980. In all cases the zymosan was washed three times in phosphate buffered saline following opsonisation.

Zymosan was subsequently always opsonised in the presence of 50% (w/v) serum in phosphate buffered saline. As with latex particles (3.3.1), using 10μM luminol, the relationship between PMN chemiluminescence Stimulation Index and the concentration of stimulus was established for opsonised zymosan (Fig. 31). 1mg/ml opsonized zymosan gave the half maximal response and was used in all subsequent experiments unless otherwise indicated.

3.3.3 Chemiluminescence of PMN stimulated by chemotactic peptide (N-formyl-met-leu-phe)

Stimulation of PMN by the synthetic chemotactic peptide N-formyl-met-leu-phe in the presence of 10μM luminol resulted in a rapid increase of chemiluminescence, following a lag of 20s, which rose to a first peak or shoulder at 60s (Fig 32) after which there was a further increase to a second large peak at 180 - 240s following addition of stimulus. The chemiluminescence then decayed in exponential fashion.

When the time course of chemotactic peptide induced luminol chemiluminescence and oxygen consumption were compared (Fig. 33) chemotactic peptide produced a large, rapid increase in the rate of

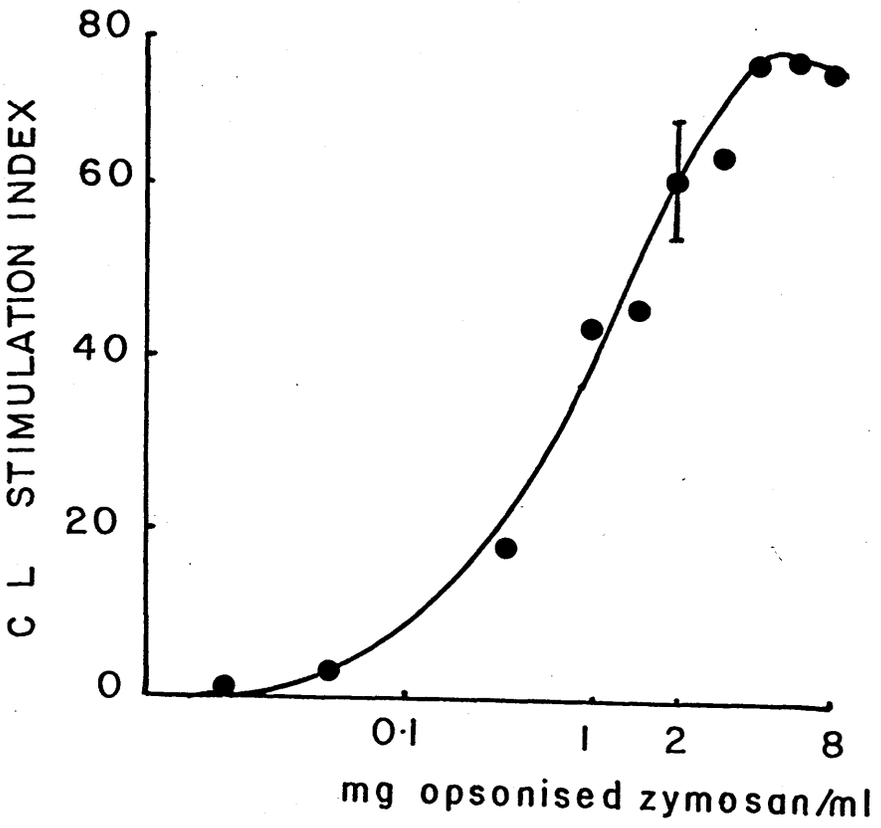


Figure 31 Relationship between PMN chemiluminescence stimulation index and opsonised zymosan concentration

The graph shows the results of one experiment in which 5 x 10^5 PMN/ml were stimulated by the addition of 0.02-8mg/ml opsonised zymosan (opsonising serum diluted 1:2). The points represent single observations with the exception of that for 2mg/ml opsonised zymosan which was the mean $\pm$ SEM of three observations. Krebs medium pH 7.4, 37°C, 10μM luminol.

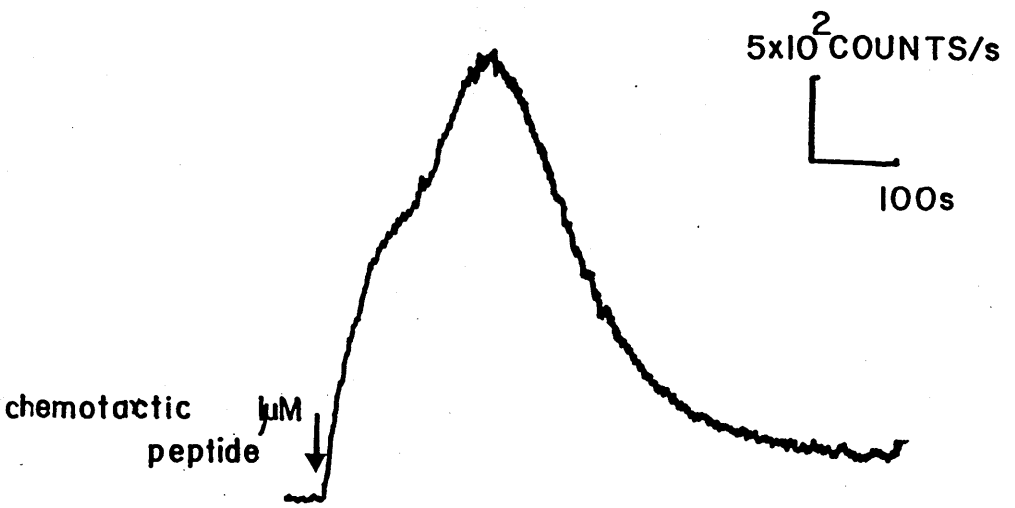


Figure 32 Chemotactic peptide stimulated PMN luminol chemiluminescence

Chemiluminescence trace produced by $5x10^5$ PMN/ml stimulated by addition, at arrow, of 1uM chemotactic peptide (n formyl met leu phe). Krebs medium, pH 7.4, 37°C, 10μM luminol.

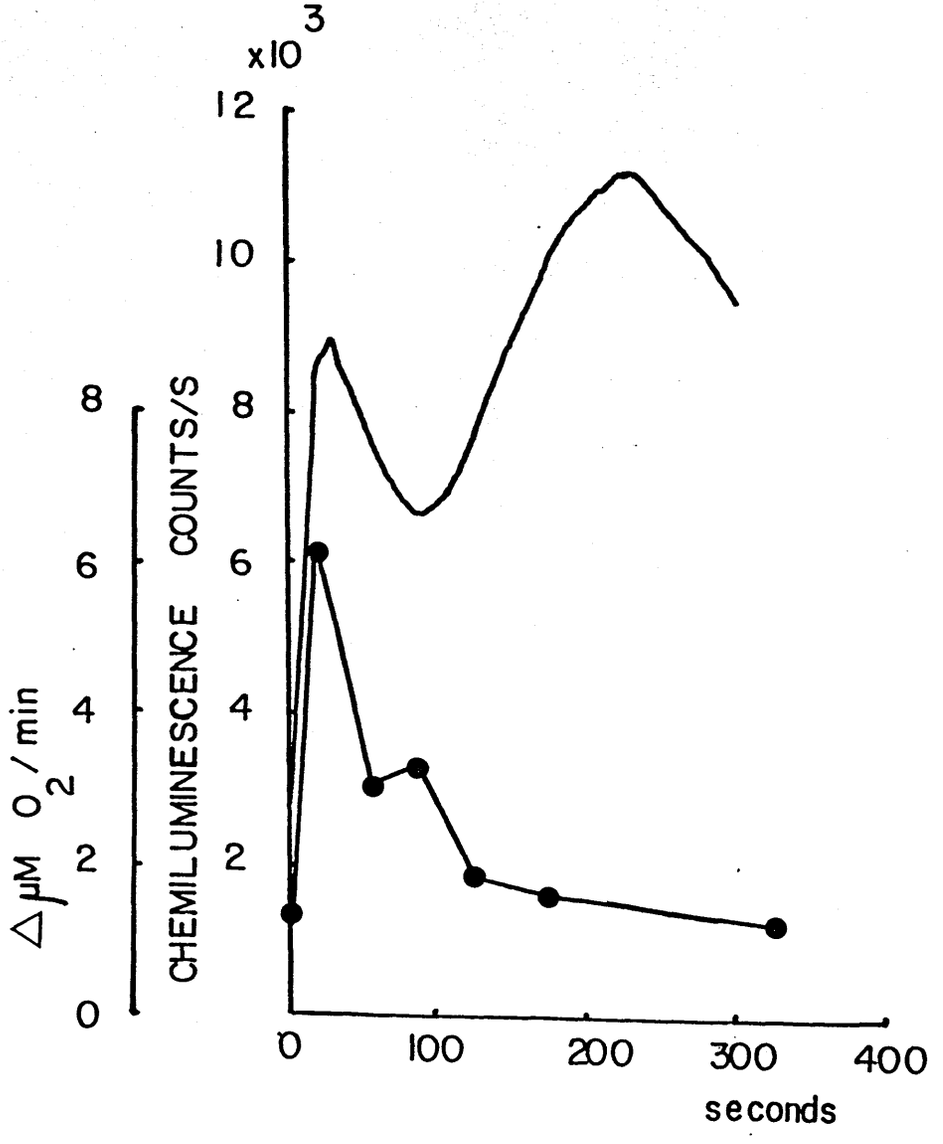


Figure 33 Comparison of oxygen consumption and luminol chemiluminescence of chemotactic peptide stimulated PMN

The graph shows the oxygen consumption (●) and chemiluminescence (unbroken line) of 4×10^6 PMN/ml stimulated by addition at time 0 seconds of 1µM chemotactic peptide. Krebs medium, pH 7.4, 37°C, 10µM luminol.

oxygen consumption (cf latex particles 2.9) reaching a maximum at 20s which corresponded with the first phase of chemotactic peptide induced chemiluminescence. The rate of oxygen consumption thereafter decayed although chemiluminescence increased to a second maximum in this particularly biphasic trace. This dissociation of chemiluminescence from oxygen consumption in the second phase suggested that chemiluminescence in this phase resulted entirely from interaction of luminol with radicals derived from those generated in the first phase.

As with latex particles (3.3.1) and opsonized zymosan (3.3.2), the relationship between PMN chemiluminescence Stimulation Index and the concentration of stimulus was established for chemotactic peptide (data not shown). 0.1μM chemotactic peptide gave the half maximal response and was used in all subsequent experiments unless otherwise indicated.

The relationship between magnitude and time course of the chemiluminescence responses to the three stimuli for PMN taken from one preparation is shown in Fig. 34. The peak height of the response to latex beads was 15 fold larger than the peak height of the response to chemotactic peptide. The response to opsonised zymosan was of intermediate size.

3.3.4 Chemiluminescence of PMN stimulated by immune complexes

Following the addition of insoluble rabbit anti-bovine serum albumin - bovine serum albumin immune complexes formed at equivalence (50μl IgG : 15μg bovine serum albumin), PMN luminol chemiluminescence increased rapidly above background following a lag of 20s, reaching a first peak or shoulder at 60 - 100s following addition (Fig. 35(a)) and then either plateaued or rose to a second larger peak at approximately 200s, after which chemiluminescence gradually decayed.

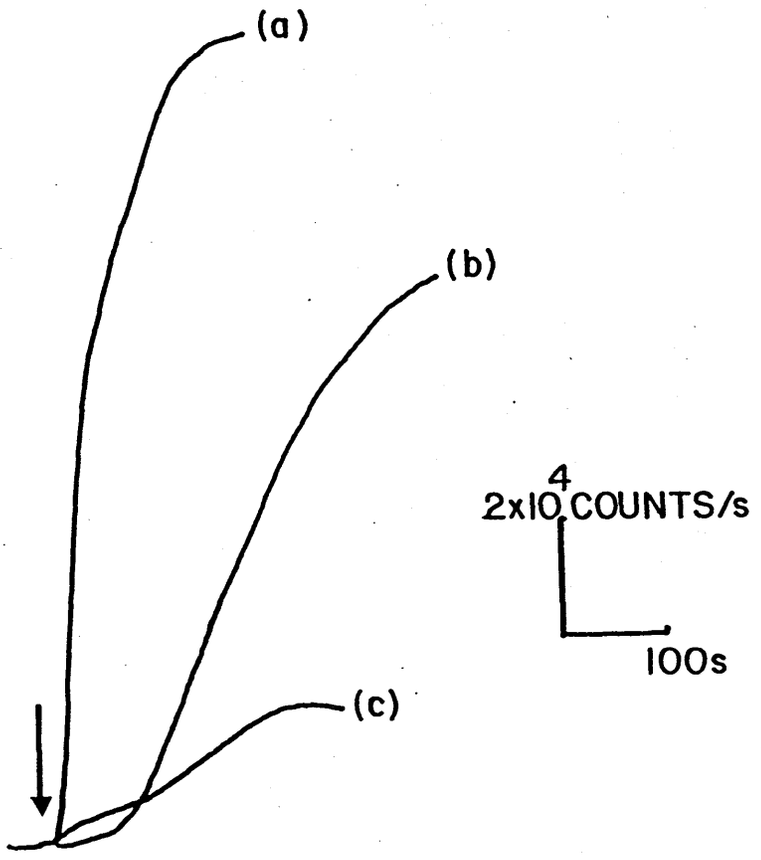


Figure 34 Comparison of PMN chemiluminescence responses to three different stimuli

Chemiluminescence traces produced by 5.7×10^5 PMN/ml following addition (at arrow) of (a) 4×10^9/ml latex particles, (b) 2mg/ml opsonised zymosan (c) 1μM chemotactic peptide. Krebs medium pH 7.4, 37°C, 10μM luminol.

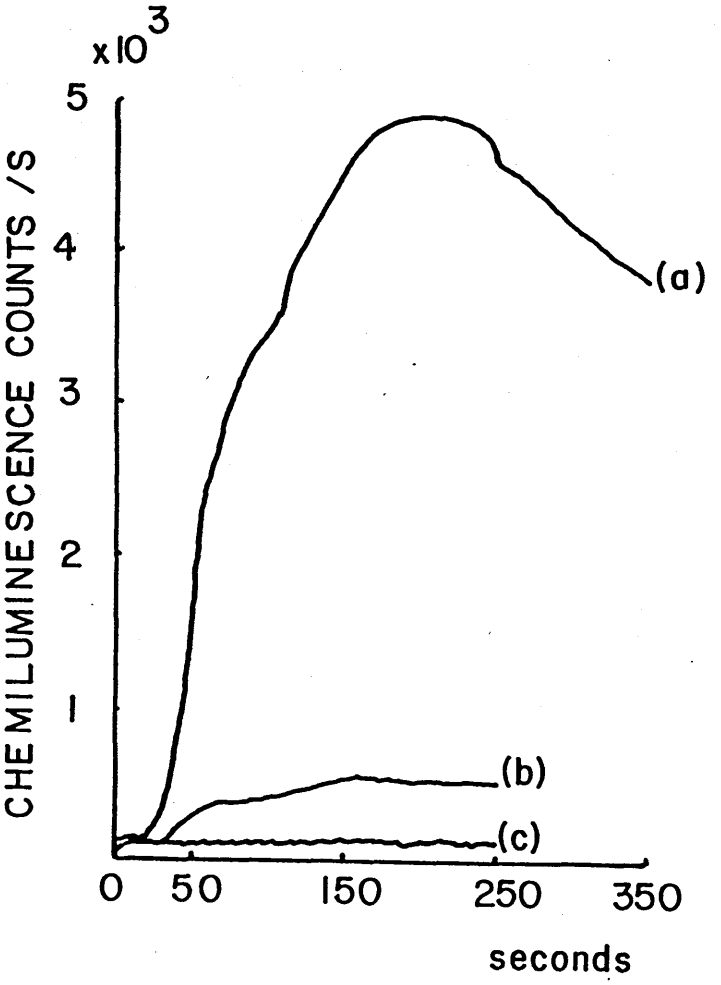


Figure 35 Chemiluminescence of PMN stimulated by immune complexes formed at equivalence

Chemiluminescence traces produced by $5x10^5$ PMN/ml stimulated by addition, at time 0 seconds of (a) 50µl rabbit (1gG) anti bovine serum albumin: 15µg bovine serum albumin, (b) 50ul rabbit (1gG) anti bovine serum albumin alone and (c) 15µg bovine serum albumin. Krebs medium pH 7.4, 37°C, 10µM luminol.

Addition of antigen alone (15µg bovine serum albumin) caused no change in the background luminol chemiluminescence produced by resting PMN (Fig. 35(c)) but addition of antibody alone (50µl 1gG) produced a small, biphasic increase in PMN chemiluminescence reaching a maximum at 170s (Fig. 35(b)) which was 11% of the peak achieved when PMN were stimulated by immune complexes. This low level increase of luminol PMN chemiluminescence produced by addition of 1gG alone suggested that PMN were stimulated either by monomeric 1gG, or by spontaneously formed 1gG aggregates, or by a low concentration of anti-bovine serum albumin - bovine serum albumin immune complexes resulting from contamination of the stimulus additive syringe with bovine serum albumin.

When PMN were stimulated by immune complexes formed from the combination of antigen and antibody in differing ratios, a variation in the luminol chemiluminescence response was observed. The chemiluminescence stimulation index (2.10) was maximal when immune complexes were formed at equivalence (50µl 1gG : 15-20ug bovine serum albumin) (Fig. 36) and decreased with increasing antigen or antibody concentrations. The maximum chemiluminescence was therefore produced when PMN were stimulated by the highest concentration of insoluble immune complexes.

The PMN luminol chemiluminescence response to stimulation by immune complexes formed at equivalence increased with increasing concentrations of immmune complexes as shown in Fig. 37.

When PMN were preincubated with 2% normal human serum (v/v in Krebs Medium pH 7.4) at 37°C for 5 min prior to the addition of immune complexes formed at equivalence (50µl 1gG : 15µg bovine serum albumin 1:1) the PMN chemiluminescence response was 3 times greater than that observed when immune complexes were added in the absence of serum

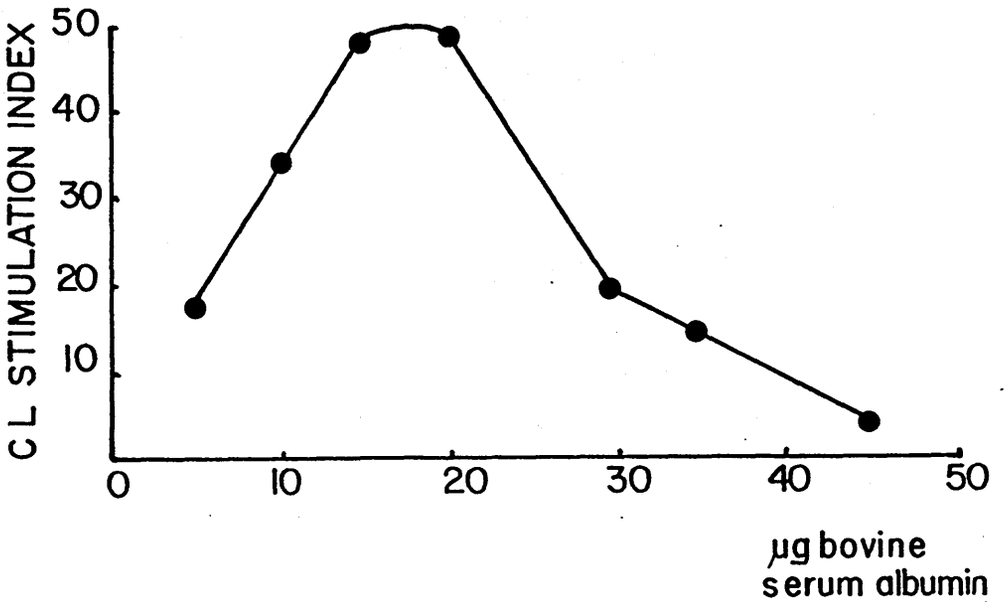


Figure 36 Chemiluminescence of PMN stimulated by immune complexes formed in the presence of varying quantities of antigen

The graph represents the results of one experiment in which 5 x 10^5 PMN/ml were stimulated in the presence of immune complexes formed by combination of 50µl rabbit (1gG) anti bovine serum albumin (antibody) with varying quantities bovine serum albumin (antigen). Krebs medium pH 7.4, 37°C, 10µM luminol.

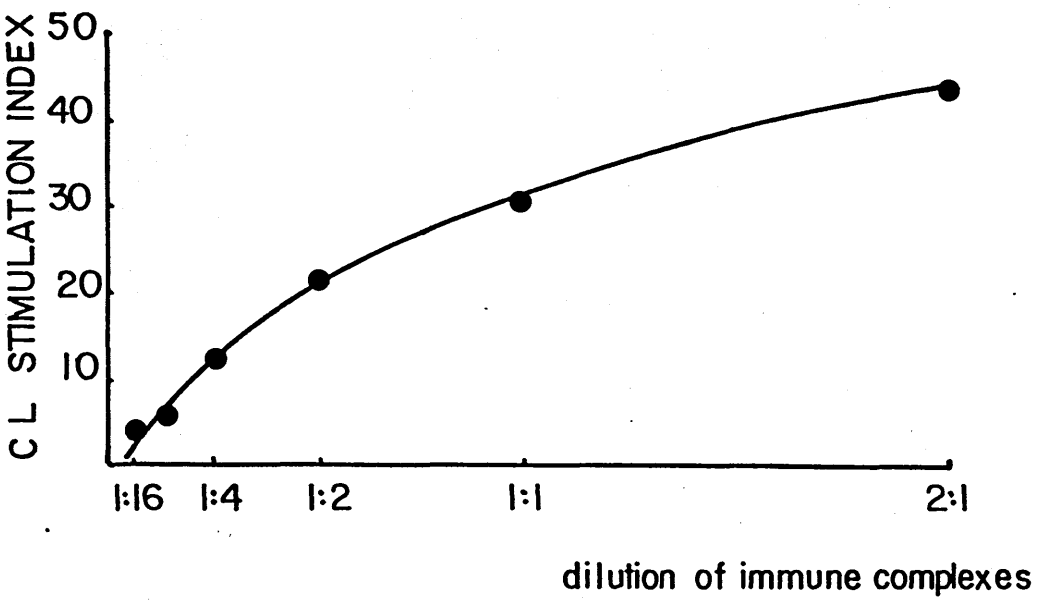


Figure 37 Relationship of PMN chemiluminescnece stimulation index and concentration of immune complexes (insoluble).

Chemiluminescence of $5x10^5$ PMN/ml stimulated by immune complexes formed at equivalence (50ul rabbit (1gG) anti bovine serum albumin: 15µl bovine serum albumin) and diluted in Krebs medium pH 7.4, 2:1 - 1:16, 37°C, 10µM luminol.

(Fig. 38 (b) and (a)). The serum was obtained from freshly venesected whole blood which had been allowed to clot at room temperature for 45 min, and was not heat treated. A 2.6 fold enhancement of PMN chemiluminescence also occurred when PMN were stimulated by immmune complexes, (formed at equivalence) in the presence of serum which had been depleted of the terminal attack complement component C9 (Fig. 38 (c)).

3.3.5 Localisation of PMN luminol chemiluminescence

In order to determine what proportion of luminol dependent chemiluminescence, and thus what proportion of oxygen radicals produced by stimulated PMN occurred extracellularly, the following experiments were carried out:-

1. Stimulation of PMN with latex paticles in the presence of a solid phase chemiluminescence indicator which was non phagocytosable and thus would not detect intracellular oxygen radicals.
2. Stimulation of PMN in the presence of the microfiliament inhibitor cytochalasin B which was known not only to inhibit phagocytosis but also to enhance extracellular secretion of myeloperoxidase (Zurier, Hoffstein and Weissmann 1973(b)).
3. Investigation of the effect of time of addition of the impermeant superoxide dismutase on PMN luminol dependent chemiluminescence to determine whether its previously demonstrated inhibitory effect (2.11.3) occurred predominantly intra- or extracellularly.

1) PMN chemiluminescence in the presence of a solid phase chemiluminescence indicator

Before attempting to measure specifically PMN extracellular chemiluminescence using the solid phase sepharose bead linked ABEI (amino butyl ethyl iso luminol) chemiluminescence indicator (3.2.3) it

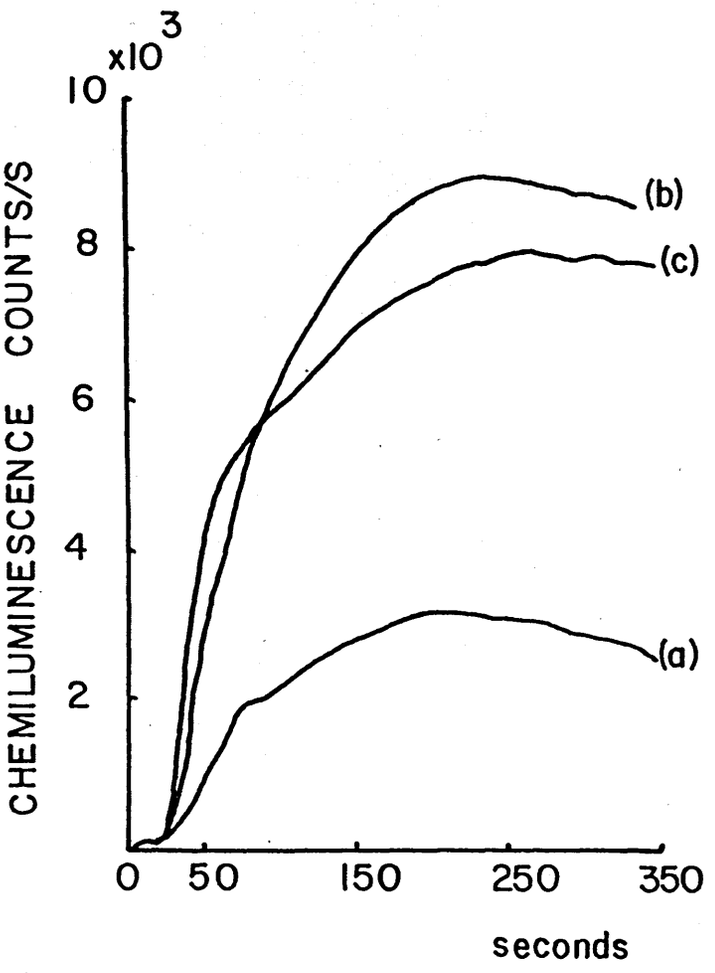


Figure 38 Effect of serum (normal and C9 depleted) on the Chemiluminescence of PMN stimulated by insoluble immune complexes.

Chemiluminescence traces produced by 5 x 10^5 PMN/ml which were pre incubated with (b) 2% (v/v) normal human serum, (c) 2% (v/v) human serum depleted of complement component C9 or (a) no serum, before stimulation by addition (at 0 seconds) of immune complexes formed at equivalence (50ul rabbit (1gG) anti bovine serum albumin: 15µg bovine serum albumin). Krebs medium pH 7.4, 37°C, 10µM luminol.

was necessary

(i) To determine the time course and characteristics of the chemiluminescence which would result from stimulation of PMN (by latex particles) in the presence of fluid phase ABEI

(ii) to determine whether the presence of control sepharose beads would enhance or inhibit latex particle stimulated PMN 'fluid phase' ABEI chemiluminescence

and (iii) to determine what chemiluminescence would result from stimulation of PMN in the presence of the supernatant from the solid phase ABEI preparation alone. The supernatant had only been responsible for 2.7% of the chemiluminescence resulting from reaciton of solid phase ABEI in the microperoxidase/H_2O_2 assay (3.2.3)

(i) Characterisation of PMN ABEI chemiluminescence - comparison with luminol

The chemiluminescence of PMN stimulated by latex particles in the presence of 10nmoles (10μM) fluid phase ABEI was monitored and compared with that of PMN stimulated in the presence of 10nmoles (10μM) luminol (Fig 39). IN the presence of ABEI there was a rapid increase in chemiluminescence counts/s above background following a lag of 10s, and reaching a peak at 30s which corresponded to the first phase of luminol chemiluminescence althogh its magnitude was only 6% of that of the luminol first phase. Thereafter the ABEI chemiluminescence rapidly decayed, in general plateauing at a level intermediate between background and peak height (Fig 40(a)) although as in Fig. 39, a secondary gradual rise was sometimes observed. In no ABEI trace however was a large rise to a secondary peak observed as with PMN luminol chemiluminescence.

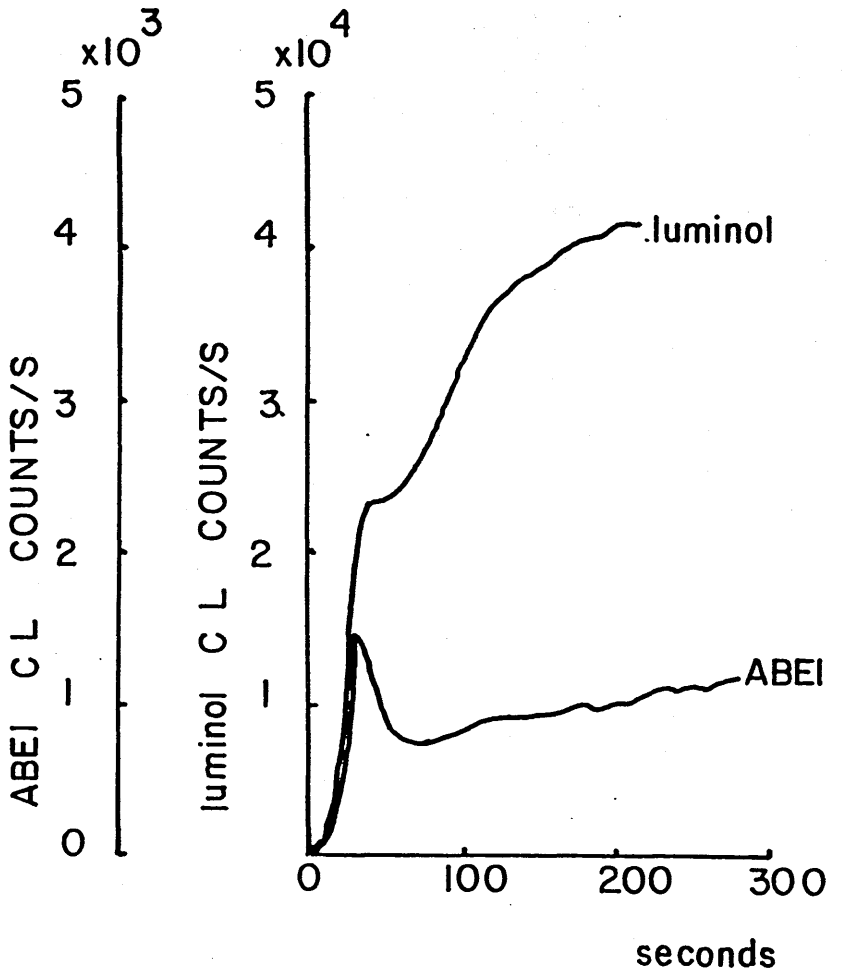


Figure 39 Comparison of luminol and ABEI PMN Chemiluminescence

Chemiluminescence traces produced by 1 x 10^6 PMN/ml stimulated by addition (at time 0 seconds) of 4×10^9 latex particles/ml in the presence of 10μM luminol or 10uM ABEI (amino butyl ethyl iso luminol). Krebs medium pH 7.4, 37°C

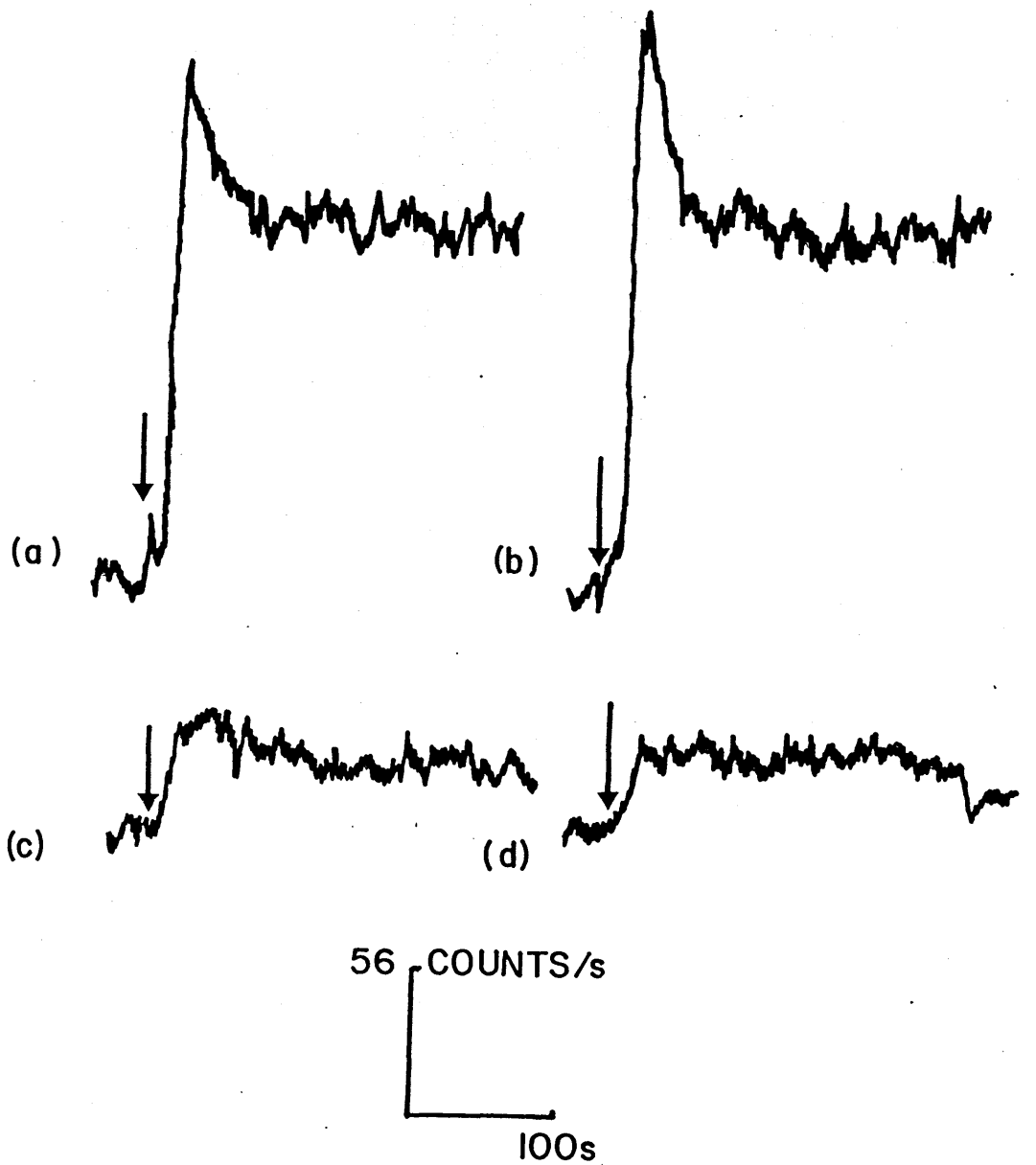


Figure 40 Measurement of extracellular PMN chemiluminescence using 'solid phase' sepharose linked ABEI

Chemiluminescence traces produced by $1x10^6$ PMN/ml stimulated at arrows by addition of $4x10^9$/ml latex particles in the presence of
(a) 2μM ABEI (= 2nmoles ABEI)
(b) 2μM ABEI + 50ul 'control' sepharose beads
(c) 50μl sepharose linked ABEI
(d) 50μl supernatant from sepharose linked ABEI

Krebs medium, pH 7.4, 37°C.
In an acellular microperoxidase/H_2O_2 assay for ABEI (3.2.3) the chemiluminescence of 50μl sepharose linked ABEI was equivalent to 2nmoles fluid phase ABEI.

(ii) Effect of control sepharose beads on PMN ABEI chemiluminescence

Using the microperoxidase/H_2O_2 assay it had been established that 50µl of the solid phase ABEI preparation would give chemiluminescence equivalent to 2nmoles (2µM) of the 'fluid phase' ABEI (3.2.3). To determine whether the sepharose beads would have any enhancing or inhibitory effect on PMN chemiluminescence, the cells were stimulated by latex particles in the presence of 2µM ABEI, and in the presence and absence of 50µl control (non ABEI linked) sepharose beads (Fig 40, (a) and (b)). The magnitude and character of the chemiluminescence was not affected by the control beads.

(iii) Chemiluminescence of PMN stimulated in the presence of supernatant from 'solid phase' ABEI

Stimulation of PMN by latex particles in the presence of 50µl supernatant from 'solid phase' ABEI yielded a low level of chemiluminescence (Fig 40(d)) in which a biphasic pattern could not be distinguished but the peak height was only 10% of that attained in the presence of 2nmoles (2µM) ABEI (Fig 40 (a) and (b)).

(iv) Chemiluminescence of PMN stimulated in the presence of 'solid phase' ABEI

Stimulation of PMN by latex particles in the presence of 50µl 'solid phase' ABEI also yield a low level of chemiluminescence (Fig 40 (c)) in which a first phase was more discernible, but the peak height was again only 17% of that attained when cells were stimulated in the presence of 2nmoles (2µM) ABEI, the concentration of ABEI which gave an equivalent chemiluminescence in the microperoxidase/H_2O_2 assay (3.2.3). When the chemiluminescence counts over the first 210 seconds following addition of stimulus were integrated for traces (c) and (d) Fig 40 (solid phase ABEI and solid phase ABEI supernatant

respectively) the chemiluminescence obtained with the supernatant was 75% of that obtained with the whole preparation. Thus a minimum of 25% of the solid phase ABEI chemiluminescence had occurred extracellularly. This fraction, however, only represented 5% of the integrated chemiluminescence counts when cells were stimulated in the presence of 2μM ABEI (Fig 40 (a)).

The discrepancy in the sizes of fluid phase and solid phase ABEI chemiluminescence may have been attributable to inadequate mixing of PMN with the latter. Further, in the case of the acellular chemiluminescence generated by the microperoxidase/H_2O_2 assay, the reacting substances would have had more ready access to ABEI bonded to internal surfaces of the sepharose beads.

2) Effect of Cytochalasin B

Cytochalasin B, a metabolite of the mold Helminthosporium dematiodeum (Carter, 1967) was known to reversibly inhibit PMN locomotion, phagocytosis and glycolysis via an action on the peripheral cytoplasmic filamentous network (Zigmond and Hirsch 1972). Decreased PMN oxygen consumption (Roos et al 1976) increased releasse of $O_2^{\cdot -}$ and H_2O_2 (Goldstein et al 1975; Curnette and Babior 1975; Root and Metcalf 1977) and increased release of lysosomal enzymes (Zurier et al 1973(b)) had also been shown to ensue from its presence during the stimulation of PMN by phagocytic stimuli, and Cytochalasin B had thus increasingly been used as a model for 'frustrated phagocytosis' (Weissmann 1982) (1.2.1). By its prevention of formation of the phagolysosome, cytochalasin B potentially offered a further means of dissecting PMN luminol chemiluminescence to determine what proportions occurred intra- or extra-cellularly.

Following incubation of PMN with cytochalasin B (5μg/ml) and luminol at 37°C for 5 min, PMN background chemiluminescence was

decreased (18.6 ± 6%, mean ± S.D., n = 3) compared with PMN incubated without cytochalasin B. Following addition of chemotactic peptide chemiluminescence counts/s immediately (i.e. no lag) increased. The resultant trace was not only 2-3 fold larger than chemotactic peptide induced chemiluminescence in the absence of cytochalasin B (Fig 41), but was also markedly biphasic. The first phase reaced a maximum at 40-50s and the second, larger phase reached a maximum of 230s.

By contrast, when PMN preincubated with cytochalasin B and luminol were stimulated by opsonised zymosan (Fig 42) only a small, slow increase in chemiluminescence counts/s resulted, reaching a maximum which was only 12% of that achieved in the absence of cytochalasin B.

3) Effect of time of addition of superoxide dismutase on its inhibition of PMN luminol chemiluminescence

Stimulation of PMN in the presence of added extracellular superoxide dismutase is likely to be accompanied by passage of this enzyme intracellularly either during the formation of the phagolysosome or during endocytosis but not by simple diffusion. Thus the inhibition of PMN luminol chemiluminescence which occurred when PMN were pre-incubated with superoxide dismutase prior to their stimulation by latex particles (2.11, Fig 21) may have resulted from scavenging of intracellular $O_2^{\cdot -}$. If the action of superoxide dismutase on PMN chemiluminescence occurred predominantly extracellularly however, addition of the enzyme after the addition of stimulus would be expected to provide the same degree of inhibition as occurs when it is added prior to the stimulation.

To test this hypothesis superoxide dismutase was added to the PMN suspension 20s and 90s following addition of stimulus, in separate

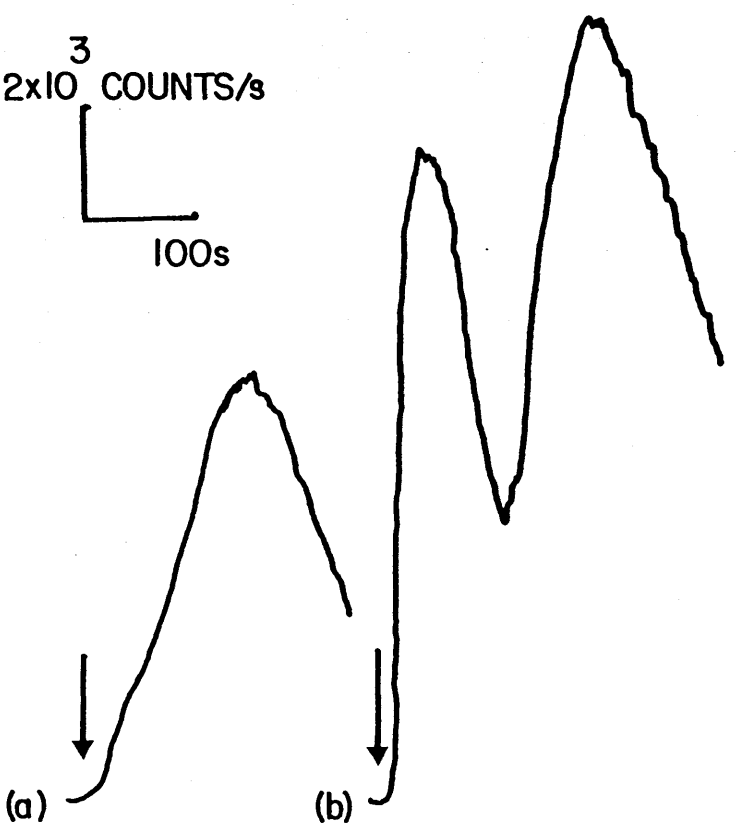


Figure 41 Effect of cytochalasin B on chemotactic peptide induced PMN chemiluminescence

Chemiluminescence traces produced by $5x10^5$ PMN/ml stimulated at arrows by addition of 1μM chemotactic peptide. The usual PMN chemiluminescence response to this stimulus is shown in (a). The response in the presence of 5μg/ml cytochalasin B with which the cells were pre-incubated for 5 minutes prior to the addition of stimulus is shown in (b). Krebs medium, pH 7.4, 37°C, 10μM luminol.

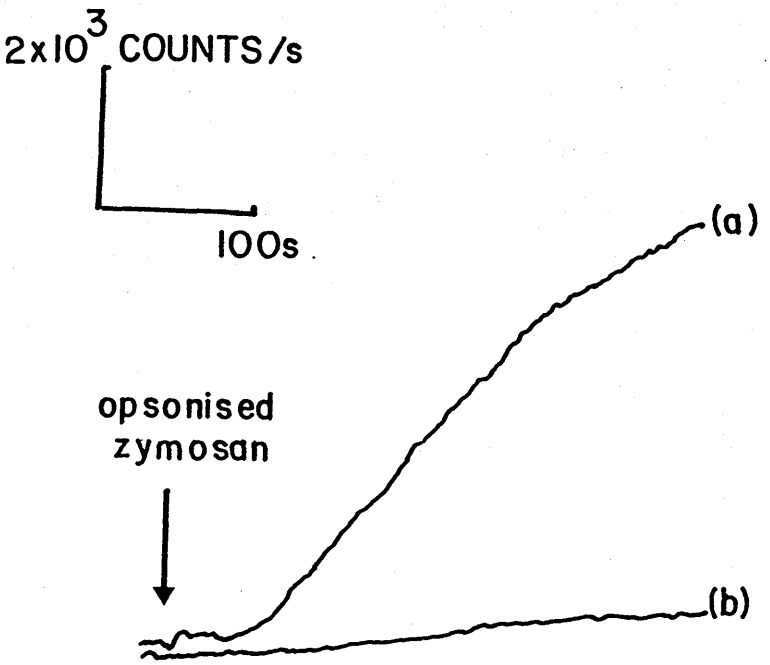


Figure 42 Effect of cytochalasin B on opsonised zymosan induced PMN chemiluminescence

Chemiluminescence traces produced by $5x10^5$ PMN/ml stimulated at arrow by addition of 1mg/ml opsonised zymosan. The usual PMN chemiluminescence response to this stimulus is shown in (a). The response in the presence of 5μg/ml cytochalasin B with which the cells were pre-incubated for 5 minutes prior to the additon of stimulus is shown in (b). Krebs medium, pH 7.4, 37°C, 10μM luminol.

experiments (Fig 43 (b) and (c)). Preincubation of the cells with superoxide dismutase (50μg/ml) prior to their stimulation with latex particles resulted in a 75% inhibition (Fig 43 (a)), whereas addition of the enzyme at 20s resulted in only a 46% inhibition and addition at 90s resulted in no inhibition, rather a slight enhancement was observed (Fig 43 (c)).

3.4 Discussion

The experiments described in this chapter characterised the luminol dependent chemiluminescence of PMN exposed to four 'model stimuli' and established the amount of each stimulus which was required to give a half maximal response. Three stimuli (latex particles, insoluble immune complexes and chemotactic peptide) evoked biphasic PMN chemiluminescence responses. The biphasic chemiluminescence provoked by chemotactic peptide was enhanced and exaggerated by the presence of the microfilament inhibitor cytochalasin B, and its 'second phase' was unaccompanied by any secondary rise in the rate of oxygen consumption. Although difficulties were encountered in the use of a 'solid phase' isoluminol chemiluminescence indicator, part of PMN chemiluminescence was found to be extracellular thus supporting release of oxygen radicals by these cells.

For each of the model stimuli, PMN luminol dependent chemiluminescence traces were characterised by the time of onset of the response following additon of stimulus, the duration of the response and the maximum peak height achieved. Unless specifically indicated all experiments were carried out in the absence of extracellular protein and under the conditions of temperature, pH, luminol concentration and cation concentration previously defined (2.12.2). Some similarities but several differences were noted on

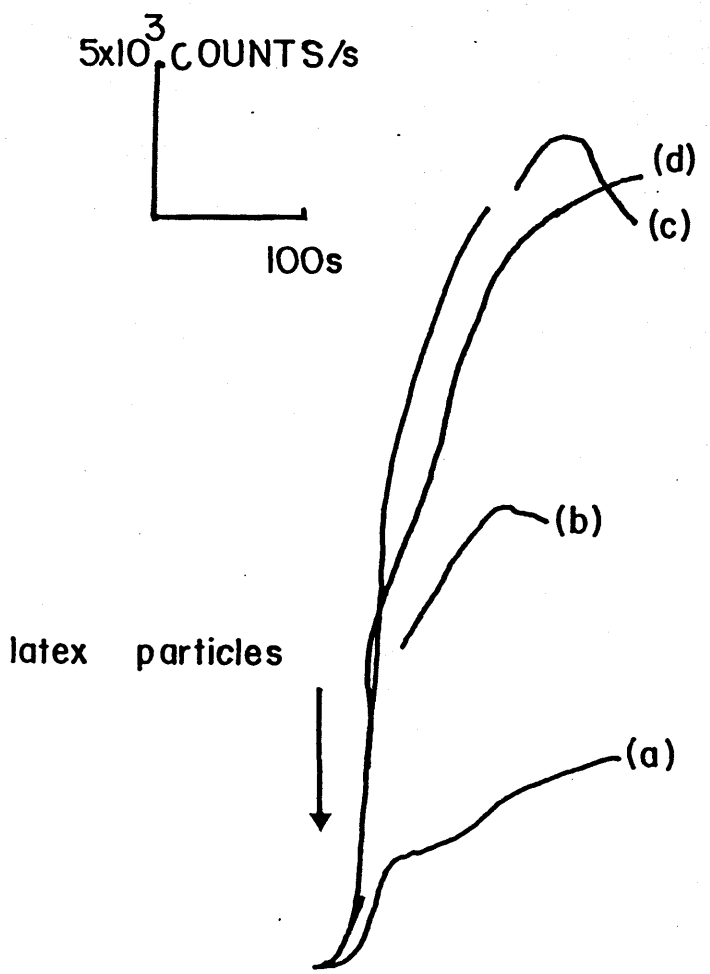


Figure 43 Effect of time of addition of superoxide dismutase on its inhibition of PMN luminol chemiluminescence

Chemiluminescence traces produced by 5.5×10^5 PMN/ml stimulated by addition at arrow of 4×10^9/ml latex particles in (a) cells were pre-incubated with 50μg/ml superoxide dismutase for 5 minutes.

(b) 50μg/ml superoxide dimutase was added 20s after stimulus.

(c) 50μg/ml superoxide dismutase was added 90s after stimulus.

(d) no superoxide dismutase was present.

Krebs medium, pH 7.4, 37°C, 10μM luminol.

comparison of these traces with those published by other groups prior to or concurrently with the work of this thesis. While this may in part be attributable to the increased sensitivity of the 'home built' luminometers used in our laboratory, more important factors are likely to have been other variations used in the generation and detection of PMN luminol dependent chemiluminescence (Table 10, 1.4.2), leading to differences not only in the rate of PMN stimulation and oxidation, but also in the content of the available oxygen radical pool. Examples of the different luminol concentrations, temperatures and protein concentrations used by other groups are provided in Table 16.

The concentrations of latex particles, opsonised zymosan and chemotactic peptide which gave half maximal chemiluminescence responses were found to be $2x10^9$ latex particles/ml, 1mg opsonised zymosan/ml and 0.1μM chemotactic peptide respectively. Rather surprisingly, unopsonised latex particles gave the largest PMN luminol chemiluminescence response (Fig 34). Several groups of workers had provided evidence for ingested particles themselves being substrates for PMN chemiluminescence (Cheson et al 1976; Rosen and Klebanoff 1976; Nelson et al 1977). The differences in magnitude and time course of latex particle induced PMN chemiluminescence in the absence of luminol (Fig 17, 2.11.1), and in the presence of luminol or ABEI (Fig 39) would indicate that latex was not itself a substrate for chemiluminescence.

The 'background' chemiluminescence of 'resting' PMN observed prior to their stimulation has been recorded by other workers in the presence (Hatch et al 1978; De Sole et al 1982) and absence (Johnston an Lehmeyer 1976) of luminol, and is likely to correspond to the basal rate of cytochrome c reduction which has likewise been measured in unstimulated PMN (Weening, Wever and Roos, 1975). Evidence has been

Table 16

Measurement of PMN luminol dependent chemiluminescence - comparison of conditions of luminol concentration, temperature and protein concentration used in this thesis with those of other workers 1976-1983

Reference	luminol concentration	temperature	protein concentration
1. This thesis (1981-1983)	10μm	37oC	nil
2. Hatch et al (1978)	100μm	31oC	0.5%(w/v) bovine serum albumin
3. Easmon et al (1980)	20μm	37oC	0.1%(v/v) foetal calf serum
4. Hallett et al (1981)	10μm	37oC	0.1%(w/v) bovine serum albumin
5. Williams and Cole (1981)	10μm	37oC	nil
6. Dahlgren & Stendhal (1982)	10nm	22oC	nil
7. De Sole et al (1982)	1μm	22oC	nil
8. Bender & Van Epps (1983)	0.1μm	ambient	nil
9. James et al (1983)	90μm	37oC	1%(v/v) foetal calf serum

provided however for the stimulation of phagocytes in vitro by non phagocytosable polystyrene surfaces per se (Johnston and Lehmeyer 1976; Williams and Cole 1981 (b)).

The biphasic luminol chemiluminescence traces evoked by stimulation of PMN by latex particles, immune complexes or chemotactic peptide were characterised by the appearance of a first phase or 'shoulder' at 20-100s on the larger second phase. By contrast stimulation of PMN by latex particles in the presence of ABEI resulted in a uniphasic chemiluminescence whose maximum corresponded temporally with the first phase of PMN luminol chemiluminescence (Fig 39). The biphasic nature of the chemotactic peptide stimulated trace was enhanced and exaggerated by the presence of cytochalasin B (Fig 41). Hatch et al (1978) obtained only a single phase trace, reaching a maximum within two minutes of addition of n-f-met-phe. Dahlgren and Stendhal (1982) and Bender and Van Epps (1983) obtained biphasic traces with n-f-met-leu-phe. Bender and Van Epps (1983) found that the relative heights of their two phases could be altered by varying the cell or luminol concentrations.

With regard to immune complexes, Starkebaum et al (1981) demonstrated only a uniphasic trace reaching a maximum at around 12 min on stimulating PMN with immune complexes in the presence of 0.1μM luminol. Gale et al (1984) however did demonstrate a biphasic chemiluminescence time course similar to that of this thesis when they stimulated PMN with neat aggregated gamma globulin. Using preformed complexes of albumin and rabbit anti albumin, both groups confirmed that maximum chemiluminescence was obtained with insoluble immune complexes formed at equivalence (cf Fig 36). Starkebaum et al (1981) were likewise able to stimulate a low level of PMN luminol chemiluminescence using only monomeric IgG and also found that normal

human serum enhanced the chemiluminescence provoked by aggregated IgG (cf 3.3.4). This enhancement was partially decreased if the serum was heat inactivated to remove the effects of complement (Starkebaum et al 1981; Smith and Wood 1969).

The basis for the biphasic nature of certain PMN luminol dependent chemiluminescnece responses is not fully understood. Evidence to support two, not mutually exclusive, hypotheses has been provided. Firstly, study of PMN isolated from patients with known myeloperoxidase deficiency, and studies of the effects of superoxide dismutase and azide on luminol dependent chemiluminescence have led to the conclusion that the chemiluminescencce is dependent on at least two radical generating processes, involving $O_2^{\cdot -}$ and myeloperoxidase respectively (Rosen and Klebanoff 1976; Dahlgren and Stendhal 1983). Secondly studies involving cytochalasin B have allowed the conclusion that one of the phases is intracellular while the other is extracellular (Dahlgren and Stendhal (1982), Bender and Van Epps 1983).

In the experiments reported in this chapter, support for the first hypothesis was provided by the observation that the second phase of chemotactic peptide stimulated PMN chemiluminescence was not accompanied by any secondary rise in the rate of oxygen consumption (Fig. 33), suggesting that second phase chemiluminescence arose from a different species of radical whose presence was not directly dependent on that of environmental oxygen. Support for the second hypothesis was provided by the inhibition of zymosan induced PMN chemiluminescence, and enhancement of chemotactic peptide induced chemiluminescence effected by the presence of cytochalasin B. Potential support for both hypotheses was provided by the decreased effectiveness of superoxide dismutase as an inhibitor of PMN chemiluminescence when added to the cell suspension subsequent to the

addition of the latex particle stimulus. The absence of a large second phase when PMN were stimulated by latex particles in the presence of ABEI may indicate an inability for ABEI to react with the oxygen radicals of the second phase with which luminol reacts - whether intra or extracellularly.

Inhibition of opsonised zymosan stimulated PMN luminol chemiluminescence by the presence of cytochalasin B (3.3.5) was concurrently demonstrated by Williams and Cole (1981(a)) who confirmed previous observations that while PMN oxygen consumption under these circumstances is decreased, production of $O_2^{\cdot -}$ is increased (Goldstein et al 1975). Hallett and Campbell (1983) working with elicited rat PMN in our laboratory subsequently provided evidence for the stimulation of PMN by unopsonised latex particles and by chemotactic peptide via two separate pathways, the former independent of and the latter dependent on a rise in intracellular calcium. Particle stimulated chemiluminescence involved endocytosis and was inhibited by cytochalasin B whereas chemotactic peptide stimulated chemiluminescence was dependent on the exocytosis of myeloperoxidase and was enhanced by cytochaslasin B. It was thus concluded that a major part of the former occurred intracellularly, while a major part of the latter occurred extracellularly.

Thus the finding that the chemiluminescence of PMN stimulated by latex particles in the presence of 'solid phase' ABEI only accounted for 5% of the chemiluminescence achieved by cells stimulated in the presence of an equivalent concentration of 'fluid phase' ABEI (3.3.5) may be explained if either the majority of PMN chemiluminescence with this stimulus is intracellular (endocytosis dependent) or if ABEI in particular is unable to react with extracellular radicals.

At the time of writing no other attempts to measure PMN oxygen

radical production using a 'solid phase' detection mechanism have been reported. Johnston and Lehmeyer 1976) used a 'solid phase' stimulus (i.e. non phagocytosable surface bound immune complexes) to promote cytochrome c reduction, scopoletin oxidation and chemiluminescence, but in all cases the oxygen radical detection mechanism remained in the 'fluid phase'. Further investigation of the site of exocytosis dependent PMN chemiluminescence (e.g. chemotactic peptide stimulated) is required using a solid phase indicator once the problems of mixing and indicator availability have been overcome (3.3.5).

3.5 Summary

The luminol dependent chemiluminescence of normal human peripheral blood PMN stimulated by unopsonised latex particles opsonised zymosan, the synthetic chemotactic peptide n-formyl-met-leu-phe and immune complexes was characterised, and the intracellular/extracellular location of the chemiluminescence was investigated:-

(a) The magnitude and time courses of the chemiluminescence response for each stimulus was compared and the amount of stimulus required for a half maximal chemiluminescence response was determined.

(b) The PMN luminol chemiluminescence evoked by insoluble immune complexes (formed at equivalence) was greater than that evoked by soluble complexes, and was in turn enhanced by the presence of normal serum.

(c) The chemotactic peptide n-formyl-met-leu-phe elicited a biphasic PMN chemiluminescence response which was enhanced and exaggerated by the presence of the microfiliament inhibitor cytochalasin B. The second phase of the chemotactic peptide induced PMN

chemiluminescence was found to be dissociated from the rate of PMN oxygen consumption measured polarographically. Opsonised zymosan stimulated PMN chemiluminescence was inhibited by the presence of cytochalasin B.

(d) A solid phase isoluminol chemiluminescence probe was synthesised, characterised and used to investigate what proportion of PMN chemiluminescence occurs extracellularly. It was established that a minimum of 25% of the solid phase isoluminol chemiluminescence resulting from stimulation of PMN by unopsonised latex particles occurred extracellularly.

(e) The inhibitory effect of the $O_2^{\bullet -}$ scavenger superoxide dismutase on latex particle stimulated PMN luminol chemiluminescencce was found to decrease as the time interval between addition of stimulus and addition of scavenger increased. Maximal inhibition was obtained if cells were preincubated with superoxide dismutase prior to stimulation.

Chapter 4

Investigation of rheumatoid serum and synovial fluid for the presence of stimulatory or inhibitory factors of PMN luminol-dependent chemiluminescence

4.1 Introduction

The proposal that oxygen radicals may play an important role in the pathogenesis of rheumatoid arthritis (Del Maestro 1980, Greenwald 1981) was dependent not only on the production of these highly reactive agents by PMN in response to stimultation by immune complexes or chemotactic complement components, but also on their release into the extracellular milieu where concentrations of the oxygen radical scavengers superoxide dismutase and catalase were known to be several orders of magnitude lower than those of the cytoplasm (McCord 1974) (1.2.3, 1.3.2.).

The presence of immune complexes (Winchester, Kunkel and Agnello 1971, Hay et al 1979) and chemotactic complement components (Ward and Zvaifler, 1971) in rheumatoid synovial fluid is well established. Chemotactic complement components (chiefly derived from C3) have also been demonstrated in synovial fluid from seronegative inflammatory arthropathies (Ward and Zvaifler 1971). Prior to commencement of the work of this thesis, the in vivo stimulation of PMN in both groups of arthropathy had been further supported by the demonstration of PMN intracellular complement and immunoglobulin in each, although only rheumatoid PMN contained inclusions of rheumatoid factor (Zvaifler 1974). Increased circulatory immune complexes had also been demonstrated in the peripheral blood of 60% of the rheumatoid patients studied by Hay et al (1979), but except in the presence of systemic disease (e.g. vasculitis) there was little evidence of serum complement activation in rheumatoid arthritis (Winchester et al 1971) and immune complex inclusions had not been detected in peripheral blood PMN (Cats, Lafeber and Klein, 1975).

In Chapter 3 of this thesis luminol dependent chemiluminescence was established as a sensitive continuous indicator of oxygen radical

production by PMN exposed in vitro to phagocytic and chemotactic stimuli. Evidence was also provided for part of this chemiluminescence occurring extracellularly, thus confirming release of oxygen radicals, and their potential availability for tissue damage. In order to use luminol dependent chemiluminescence to investigate the production of oxygen radicals by PMN in vivo in patients with rheumatoid arthritis, and to examine the extracellular fate of such radicals it was necessary to:-

(a) determine whether any abnormalities existed in the generation of oxygen radicals by rheumatoid PMN following their exposure in vitro to a model stimulus.

(b) determine whether rheumatoid synovial fluid and serum would stimulate PMN luminol chemiluminescence

and

(c) determine whether rheumatoid synovial fluid and serum would contain factors capable of inhibiting PMN luminol chemiluminescnece via oxygen radical scavenging.

Abnormalities of rheumatoid PMN chemotaxis and phagocytosis had previously been demonstrated in vitro and in vivo (Mowat and Baum, 1971, Hanlon, Panayi and Laurent, 1980, Jessop et al 1973). Decreased phagocytosis in vitro by rheumatoid synovial fluid and peripheral blood PMN (Bodel and Hollingsworth, 1966, Numo, Lapadula and Covelli, 1979) had been atrributed to the prior ingestion of immune complexes by PMN in vivo (Turner, Schumacher and Myers 1973).

Two pre-requisites to the work of this chapter were firstly, definition of the range of any quantitative variation in the chemiluminescence responses of normal PMN, and secondly, a method for depleting viscous synovial fluid of all live cells while retreiving a large proportion of the cells in viable form. Persistence of any live

or effete cells within rheumatoid synovial fluid would be a potential source of artefactual PMN chemiluminescence stimulation.

4.2 Methods

4.2.1 General Methods

Whole blood samples were obtained from five normal subjects (2.4). Whole blood and/or synovial fluid samples were also obtained from 13 patients with rheumatoid arthritis (one with Felty's syndrome), 3 patients with seronegative inflammatory arthopathies and one patient with chondrocalcinosis. Clinical details of these patients, identified throughout the chapter by the letters (A) to (R), are given in Tables 17 and 18.

PMN were isolated from whole blood samples as described in 2.9.1. Whole blood, allowed to clot at room temperature for 45 min and then centrifuged at 1000g for 5min, was also used for the provision of serum where indicated.

Luminol and chemotactic peptide (n-formyl-met-leu-phe) were diluted in Krebs medium to the desired concentrations from stock solutions in DMSO (2.2) Zymosan was opsonised according to the method detailed in 2.5.

In experiments examining the effect of synovial fluid or serum on PMN resting chemiluminescence, unless otherwise stated, the cells were pre-incubated in 500µl Krebs medium, pH 7.4 at 37°C with luminol for 5 minutes prior to the addition of 200µl serum or synovial fluid diluted in Krebs medium to a total of 500µl. The final concentration of serum or synovial fluid was thus 20% (v/v).

4.2.2 Separation of synovial fluid PMN

In order to compare the chemiluminescence responses to rheumatoid

Table 17

Clinical details Patients (A) to (J) Chapter 4

Identification	Sex (M or F)	Sample Type	Age	Diagnosis	Rheumatoid Factor	Duration of arthropathy	Drug Therapy
(A)	F	SF	61yrs	Rheumatoid Arthritis	+ve	18 yrs	Penicillamine
(B)	M	SF	49yrs	Rheumatoid Arthritis	+ve	7 yrs	Naproxen
(C)	M	SF	57yrs	Rheumatoid Arthritis	+ve	8 yrs	Indomethacin Hydroxychloroquine
(D)	F	SF	57yrs	Rheumatoid Arthritis	+ve	19yrs	Prednisolone Piroxicam Penicillamine
(E)	F	SF	32yrs	Monoarthropathy	-ve	4 yrs	Methrazone
(F)	F	SF	17yrs	Polyarthropathy	-ve	2 yrs	Indomethacin
(G)	F	SF	62yrs	Rheumatoid Arthritis	-ve	4 yrs	Gold
(H)	F	SF	63yrs	Rheumatoid Arthritis	+ve	23 yrs	Benorylate Gold
(J)	M	SF	62yrs	Rheumatoid Arthritis	-ve	7 yrs	Ibuprofen Hydroxychloroquine

Table 18

Clinical Details Patients (K) - (R) Chapter 4

Identification	Sex (M or F)	Sample Type	Age	Diagnosis	Rheumatoid Factor	Duration of arthropathy	Drug Therapy
(K)	M	SF	62yrs	Chondrocalcinosis	-ve	9 yrs	nil
(L)	F	SF	51yrs	Rheumatoid Arthritis	-ve	39 yrs	Penicillamine
(M)	M	blood	45yrs	Rheumatoid Arthritis	+ve	7 yrs	Indomethacin Prednisolone
(N)	F	blood	58yrs	Rheumatoid Arthritis (Felty's syndrome)	+ve	12 yrs 6 months	Indomethacin
(O)	F	SF and blood	57yrs	Rheumatoid Arthritis	+ve	9 yrs	Indomethacin
(P)	F	SF	41yrs	Psoriatic Arthritis	-ve	6 months	Indomethacin
(Q)	F	SF	27yrs	Rheumatoid Arthritis	+ve	4 yrs	Gold
(R)	M	blood	46yrs	Rheumatoid Arthritis	+ve	2 1/2yrs	Fenbufen

synovial fluid PMN with those of control synovial fluid, or whole blood PMN it was necessary to establish a method for isolating viable PMN from synovial fluid. Similarly in order to look for a stimulating effect of rheumatoid synovial fluid on normal PMN it was necessary to denude synovial fluid of all live cells. While the viscosity of rheumatoid synovial fluid is less than that of normal or non inflammatory synovial fluids (Schumacher 1981), the viscosity of each is greater than that of plasma. After centrifugation of a freshly aspirated, non heparinised sample of synovial fluid from rheumatoid patient (D) (Table 17) at 400g for 10min (c.f. isolation of PMN from whole blood, 2.9.1), the pellet, resuspended in Krebs medium contained only 40% of cells in the whole sample. The remaining 60% of cells were visible on phase contrast microscopy of the 400g supernatant. When this supernatant was added to normal PMN at 37°C in the presence of luminol (Fig 44(a)) a rapid increase in chemiluminescence counts/s was observed. An increase of similar magnitude also occurred however when the synovial fluid was added to Krebs medium and luminol alone (Fig 44(b)) and could be explained by interaction of oxygen radicals produced by the synovial fluid cells with luminol. Persistence of live cells in synovial fluid was hence a potential source of 'artefactual' normal PMN chemiluminescence stimulation by rheumatoid synovial fluid.

Method for removal of all live cells from synovial fluid

This chemiluminescence of cells, retained in synovial fluid following centrifugation, was in turn used as an assay for the presence of live cells in the determination of the minimum speed required to eliminate all live cells from synovial fluid. Thus a sample of synovial fluid, freshly aspirated from rheumatoid patient (G) (Table 17) was centrifuged at increasing speeds in the range 400-100,000g (Fig 45).

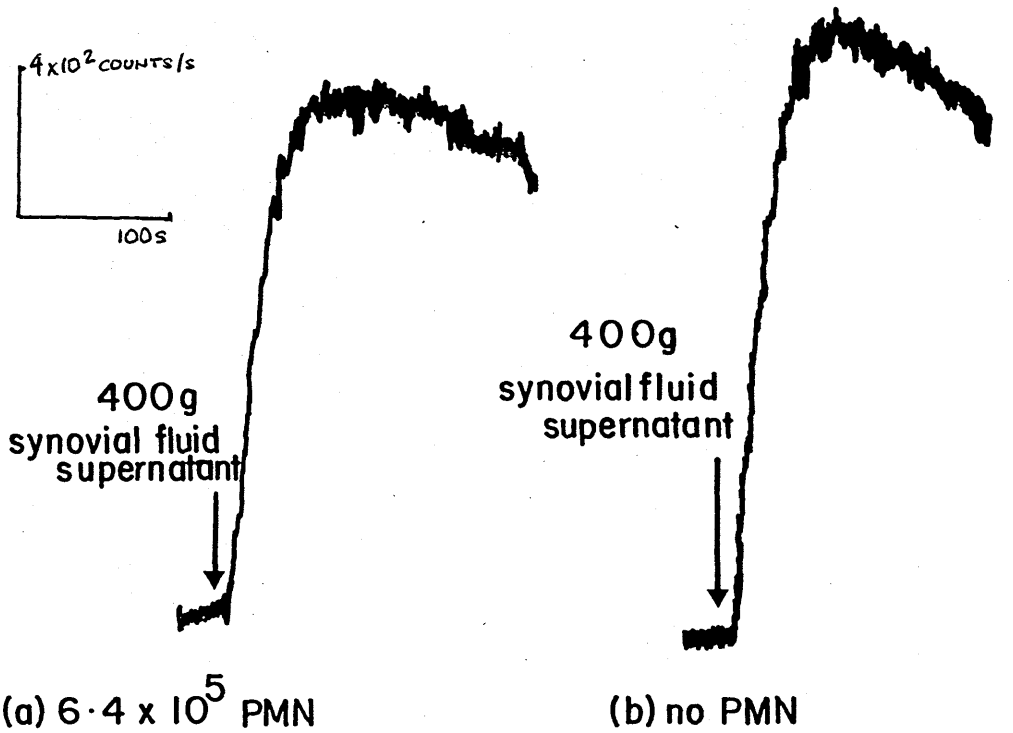


Figure 44 Luminol chemiluminescence of rheumatoid synovial fluid following centrifugation at 400g for 10 minutes

Chemiluminescence produced by addition at arrows of 20% (v/v) freshly aspirated rheumatoid synovial fluid which had been centrifuged at 400g for 10 minutes (to remove cells) to (a) 6.4×10^5 PMN/ml, 10μM luminol and (b) no PMN, 10uM luminol. The increase in chemiluminescence counts/s in each case was accounted for by persistence of live cells in the synovial fluid samples, despite centrifugation, and their reaction with luminol. Krebs medium, pH 7.4, 37°C.

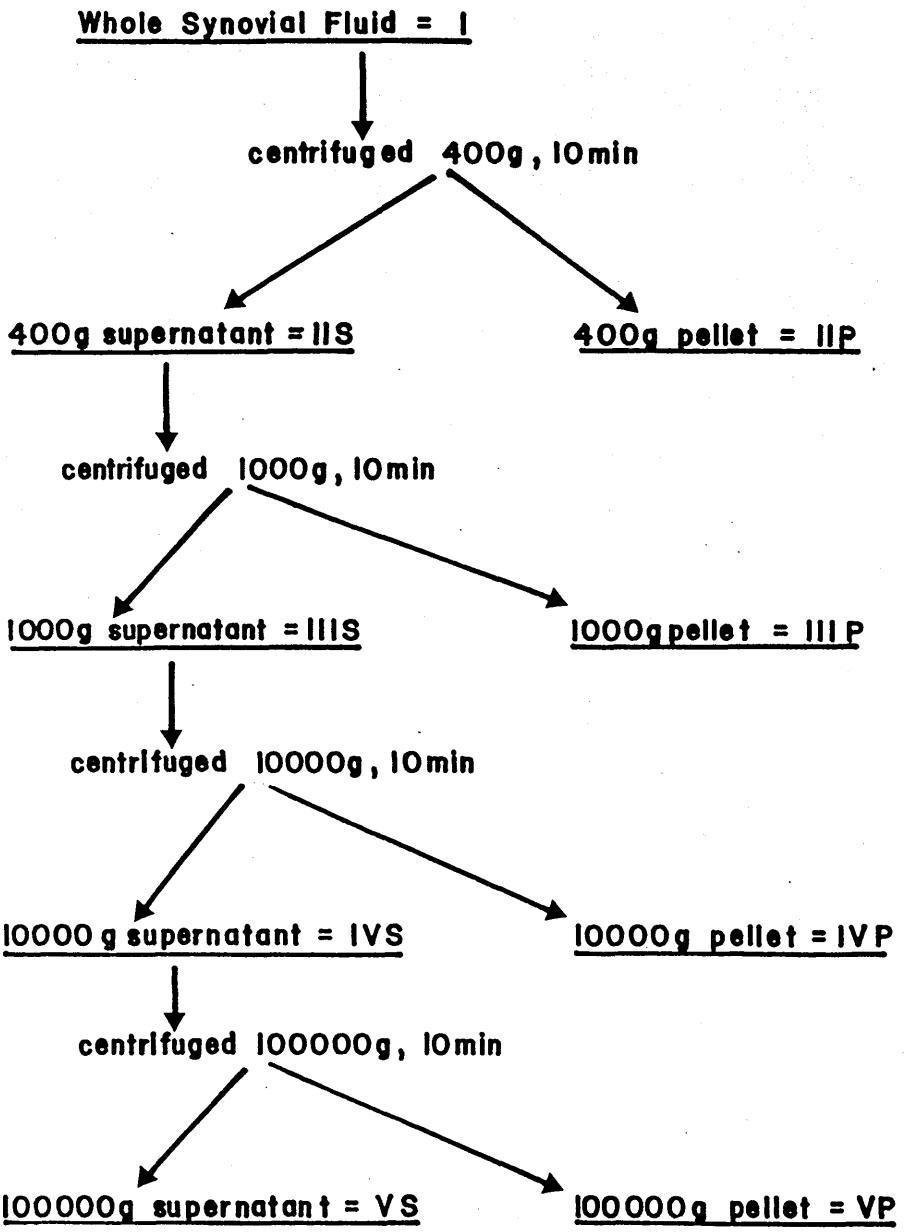


Figure 45 - Flow chart for the separation of PMN from synovial fluid by differential centrifugation

Following each centrifugation, any cell pellet remaining was resuspended in Krebs medium (volume equal to that of original synovial fluid/supernatant sample) and a portion of the supernatant was retained, the remainder going forward for centrifugation at the next highest speed as indicated (Fig 45). Total and differential white cell counting, ATP assay (2.7) and chemiluminescence studies were then performed on the resuspended cell pellets and retained supernatants.

For chemiluminescence studies 200µl of supernatant or resuspended pellet was diluted in Krebs medium pH 7.4, to a total volume of 500µl and equilib rated at 37°C for 3 min before addition to an equal volume of Krebs medium pH 7.4, 37°C containing 20µM luminol (but no PMN). Chemiluminescence was measured in luminometer 'B' before and 120s following addition of sample. To confirm that any resultant increase in chemiluminescence counts/s was attributable to the presence of live cells a further 500µl of Krebs medium pH 7.4, 37°C containing 2µM chemotactic peptide was then injected (final volume 1.5ml) and chemiluminescence observed for an additional 300s.

The mean background chemiluminescence of 20µM luminol in Krebs medium was 3.1 $\pm$ 7 counts/s. (n = 22 $\pm$ SD). Addition of synovial fluid sample supernatants or pellets was followed by a rapid increase in chemiluminescence counts/s reaching a maximum at 50 sec and then decaying (Fig 46). Addition of chemotactic peptide was followed by a biphasic increase in chemiluminescence, the peak of the second larger phase occurring at 150-300s following addition of stimulus (Fig 37). Relative to the concentration of PMN, chemotactic peptide produced a larger response in the pellet samples (PMN suspended in Krebs medium) than in the supernatant samples (Fig 46 and Table 19) which raised the possibility that synovial fluid was inhibiting PMN luminol chemiluminescence in the supernatant samples. No chemiluminescence

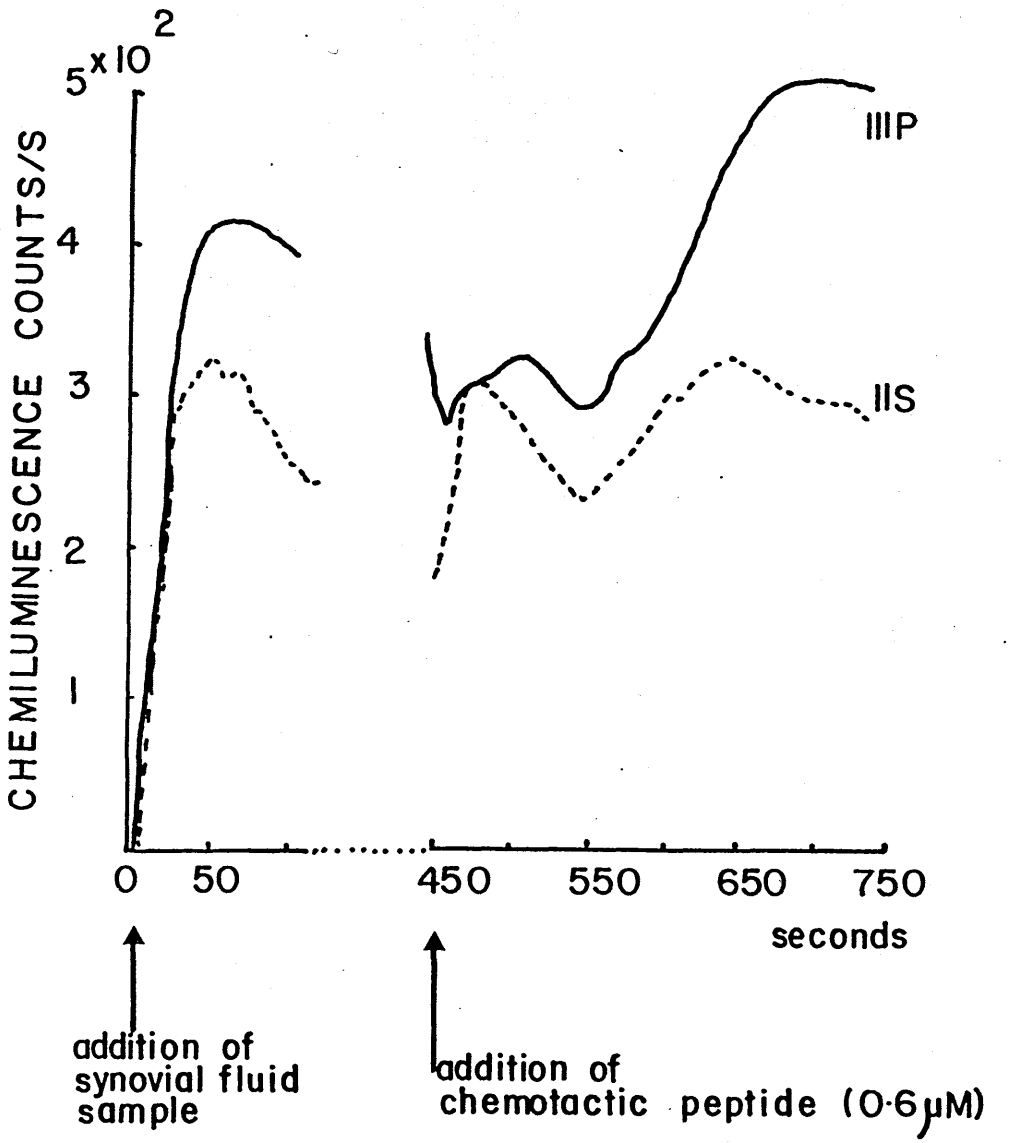


Figure 46 Chemiluminescence of rheumatoid synovial fluid following differential centrifugation

Chemiluminescence traces produced by addition to 10uM luminol at time 0 seconds of 200μl supernatant from synovial fluid centrifuged at 400g for 10 minutes (dotted line - 11S), and the cell pellet obtained by centrifugation of an equivalent volume of 11S at 1000g for 10 minutes, resuspended in 200μl Krebs medium (unbroken line - 111P). The increased chemiluminescence which resulted from the addition of 0.6μM chemotactic peptide to eahc at 450s is also shown. Krebs medium, pH 7.4, 37°C, (PMN concentrations determined by phase contrast microscopy were:- 11S = 9.9×10^9 PMN3ml, 111P = 5.6×10^9 PMN/ml).

Table 19

Chemiluminescence of synovial fluid samples following differential centrifugation. Effect of addition of chemotactic peptide.

Synovial fluid sample	Peak chemiluminescence ± sd cps (n=2 or 3)	% of CL of whole synovial fluid	Peak Chemiluminescence after addition of chemotactic peptide 0.6um (n = 2 or 3)	% of CL of whole synovial fluid
I	354 ± 69	100%	384 ± 58	100%
II S	334 ± 13	94%	324 ± 36	85%
II P	110 ± 21	31%	252 ± 23	65%
III S	147 ± 17	41%	121 ± 12	31%
III P	486 ± 196	137%	578 ± 208	150%
IV S	3.7 ± 1.0	0%	6.6 ± 3	0%
IV P	127 ± 14	35%	309 ± 76	80%
V S	5.9 (n = 1)	0%	5.4 (n = 1)	0%
V P	no pellet visible	-	-	-
No synovial fluid	3.1 ± 7 (n = 22)	-	-	-

above background luminol chemiluminescence was detectable in the 10,000g (IVs) or the 100,000g (Vs) supernatants, Table 19, indicating that all viable PMN were removed by centrifugation at 10,000g for 10min.

Examination of PMN concentrations(Table 20) showed that 89% of PMN were removed following centrifugation at 1000g for 10min (IIIs). The unexpectedly large number of PMN found in the 10,000g supernatant was therefore likely to represent artefactual counting of effete cells and debris by the Coulter counter, particularly as no visible cell pellet was found after further centrifugation at 100,000g. The lack of correlation between PMN concentration and ATP (Table 20) in the supernatant samples suggested interference of synovial fluid in the ATP assay.

On the basis of the synovial fluid differential centrifugation chemiluminescence results (Table 19), in all experiments in which elimination of cells from synovial fluid was required, the synovial fluid was centrifuged at a minimum of 10,000g for 10min and pellet discarded before use.

Method for isolation of viable PMN from synovial fluid

For isolation of viable PMN from synovial fluid the centrifugation speed was not increased above 400g for 10 min. Although higher centrifugation speeds were associated with an increased yield of PMN (Table 20), they were also associated with increased PMN aggregation. The method for PMN isolation was hence as follows:

10-20ml synovial fluid, freshly aspirated from the knee joint effusion of a patient with rheumatoid or other inflammatory arthritis was centrifuged in a universal container at 400g for 10min. The

Table 20

PMN and ATP concentration in synovial fluid samples following differential centrifugation

Synovial Fluid Sample	Total White cells	Total PMN	PMN as % of PMN in whole SF	ATP (p moles)
I	$12.1 \times 10^9/_1$	$7.8 \times 10^9/_1$	100%	< 1 pmole
II S	$9.9 \times 10^9/_1$	$5.45 \times 10^9/_1$	68%	< 1 pmole
II P	$2.7 \times 10^9/_1$	$2.5 \times 10^9/_1$	31%	3.5 pmole
III S	$1.9 \times 10^9/_1$	$0.87 \times 10^9/_1$	11%	1.1 pmole
III P	$5.6 \times 10^9/_1$	$4.53 \times 10^9/_1$	57%	20 pmole
IV S	$1.4 \times 10^9/_1$	$1.4 \times 10^9/_1$	17.5%	< 1 pmole
IV P	$2.8 \times 10^9/_1$	$1.57 \times 10^9/_1$	19.6%	4.5 pmole
V S	not assayed	-	-	-
V P	no pellet visible	-	-	-

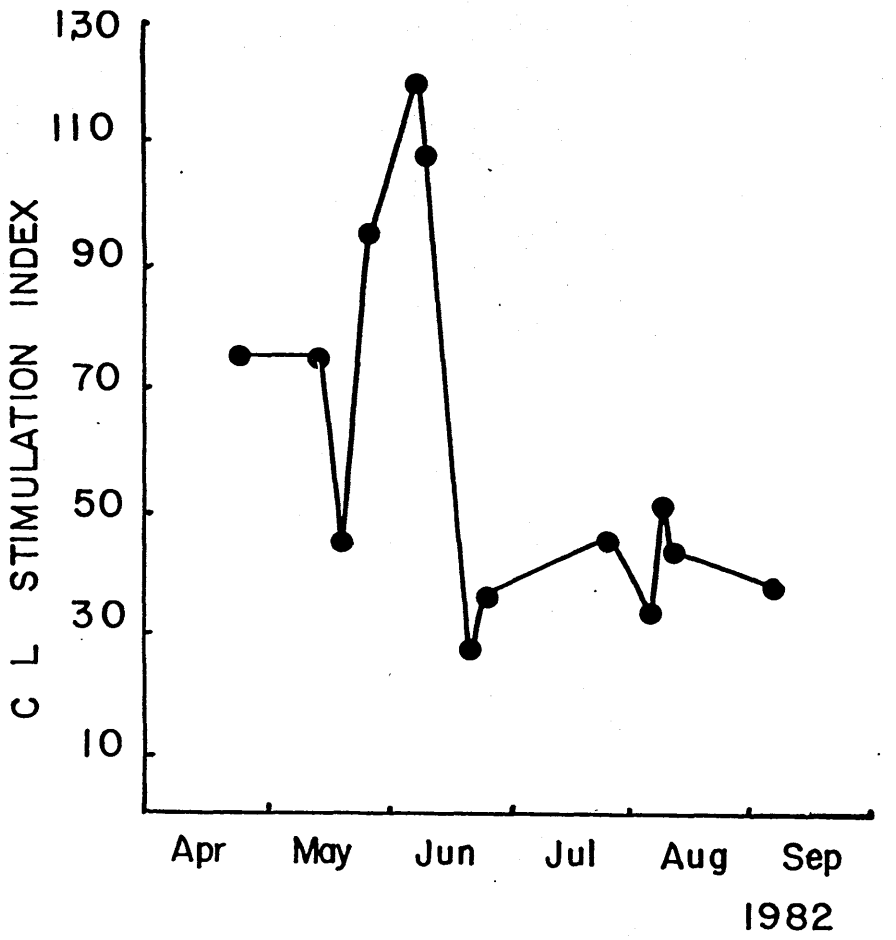


Figure 47 Variation in the chemiluminescence stimulation index of peripheral blood PMN isolated from one normal subject during a six month period

Points represent the chemiluminescence stimulation index achieved by 5×10^5 PMN/ml stimulated by 4×10^9 ml latex particles. The cells were isolated at the same time of day from the peripheral blood of one healthy 30 year old female on 13 occasions over a six month period. Krebs medium, pH 7.4, 37°C, 10µM luminol.

supernatant was removed and the pellet was gently resuspened in Krebs medium pH 7.4 (without bovine serum albumin), layered on Ficoll and centrifuged at 400g for 40 min. The mononuclear cell layer was removed and the PMN pellet was resuspened in Krebs medium, washed and centrifuged twice at 400g for 5 min before final resuspension in Krebs medium pH 7.4 and storage on ice. Unless the synovial fluid aspiration had produced traumatic blood staining, there was insufficient red cell contamination to warrant a hypotonic lysis step (c.f. 2.9.1). Stray red cell membranes were pipetted from the top of the PMN pellet between washings.

4.3 Results

4.3.1 Luminol chemiluminescence of rheumatoid PMN - comparison with normal PMN

In order to use luminol dependent chemiluminescence to demonstrate either prior stimulation of rheumatoid PMN in vivo or to investigate any abnormalities in their response to stimulation in vitro, it was first necessary to examine the extent to which the chemiluminescence of normal PMN varied either when resting or when exposed to a fixed concentration of standard stimulus in vitro. Unexpected difficulty was encountered in establishing such a 'normal range' for luminol dependent PMN chemiluminescence.

Using luminometer 'A' over a six month period a considerable variation in the chemiluminescence responses of PMN isolated from the peripheral blood of one healthy 30 year old female, to stimulation by $4x10^9$ latex particles/ml was observed (Fig. 47). The mean stimulation index (2.12.1) in this period was thus 65·0 ± 9·4(± S.E.M., n = 13) and the range was 27.6 - 121. When comparison was made with the

stimulation indices and background and peak height chemiluminescence of PMN isolated from the whole blood of four other normal subjects aged 24 - 32 years (Table 21), considerable inter-subject variation in all three parameters was observed although the character and time course of the chemiluminescence traces for each were qualitatively the same.

Despite the considerable intra (Fig 47) and inter (Table 21) subject variation for normal PMN resting and stimulated luminol chemiluminescence, early comparison was made with PMN isolated from the blood or synovial fluid of 5 patietns with rheumatoid arthritis and 2 patients with seronegative inflammatory arthropathies (Table 22). The patients and normals were not age or sex matched. Again, within the rheumatoid patient group considerable intersubject variation in PMN chemiluminescence occurred, preventing, in this small population, the demonstration of any significant differences from the responses of normal subjects (Fig 48). The lower stimulation indices of the rheumatoid synovial fluid PMN (Fig 48(c)) reflected their higher background chemiluminescence (Fig 48(a)) and provided tentative evidence not only for the prior stimulation of these cells in vivo but for their desensitisation to further stimulation.

The absence of a satisfactory 'normal range' of PMN luminol chemiluminescence prevented further quantitative comparison of rheumatoid PMN chemiluminescence. The lack of any qualitative differences between the normal or rheumatoid PMN chemiluminescence traces (Fig 49) formed the basis, however, for the remaining work of this thesis.

Table 21

Peripheral blood PMN chemiluminescence

Quantitative variations - normal subjects

Sex	Age yrs	Number of observations	Background (corrected) mean (± range)	Peak Height mean (± range)	Stimulation Index mean (± range)
F	30	13	18,805 (5,844 - 37,707)	1,024,266 (335,896 - 1,667,105)	65.0 (27.6 - 121.1)
M	32	2	13,058 (12,914 - 13,202)	841,662 (840,971 - 842,353)	64.4 (63.8 - 65.0)
M	29	4	51,950 (16,077 - 126,698)	933,870 (612,994 - 1,612,043)	27.8 (12.7 - 48.7)
F	24	2	16,062 (10,614 - 21,510)	564,752 (486,822) - 642,682)	37.9 (29.9 - 45.9)
M	26	1	13,646	857,006	62.8

Table 22

Peripheral blood and synovial fluid PMN chemiluminescence. Quantitative variations - patients with inflammatory arthropathies

Identification	Sex	Age yrs	Diagnosis	Source of Sample	Background (corrected)	Peak Height	Stimulation Index
(R)	M	45	Rheumatoid Arthritis	Blood	19,749	508,371	25.7
(P)	F	42	Psoriatic Arthritis	Blood	21,872	500,448	22.9
(O)	F	67	Rheumatoid Arthritis	Blood	6,524	349,539	53.6
(O)	F	67	Rheumatoid Arthritis	SF	32,654	1,056,977	32.4
(Q)	F	27	Rheumatoid Arthritis	SF	80,103	1,474,787	18.4
(C)	M	57	Rheumatoid Arthritis	SF	16,101	387,974	24.1
(A)	F	61	Rheumatoid Arthritis	SF	26,156	303,065	11.6
(E)	F	32	Seronagative monoarthopathy	SF	28,148	1,759,190	62.5

5×10^5 PMN/ml isolated from blood or synovial fluid (as indicated) were stimulated by 4×10^9/ml latex particles. 10μM luminol, pH 7.4, 37°C

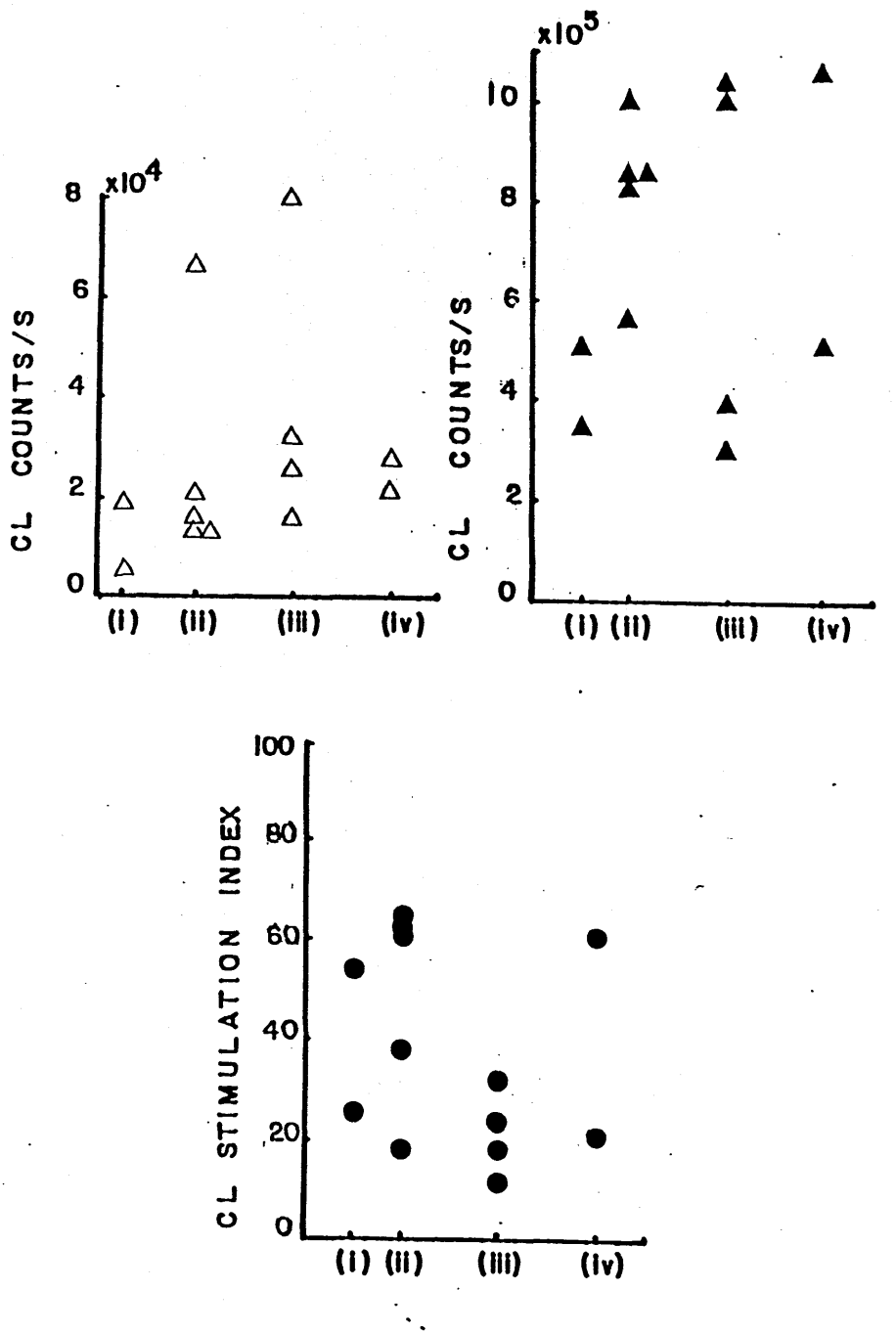


Figure 48 - Quantitative Comparisons of the luminol chemiluminescence of PMN isolated from the serum/synovial fluid of normal subjects and patients with inflammatory arthropathies

Comparison of background (△) and peak height chemiluminescence (▲) and chemiluminescence stimulation indicies of 5×10^5 PMN/ml isolated from (i) rheumatoid blood (●)
(ii) normal blood
(iii) rheumatoid synovial fluid
(iv) synovial fluid of patients with non rheumatoid inflammatory arthropathies

The stimulus for each was 4×10^9/ml latex particles. Krebs medium, pH 7.4, 37°C, 10μM luminol.

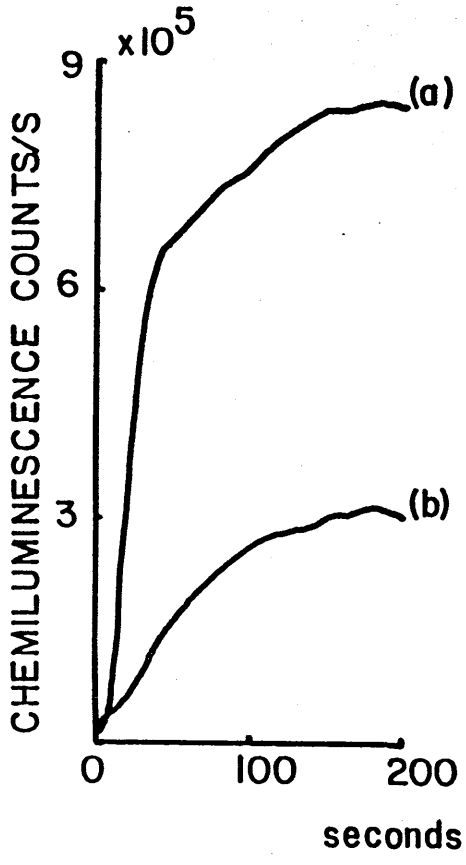


Figure 49 - Qualitative comparison of the latex particle induced luminol chemiluminescence of normal peripheral blood PMN and rheumatoid synovial fluid PMN

Chemiluminescence traces produced when 4×10^9/ml latex particles were added at time 0 seconds, to 5×10^5 PMN/ml. In (a) the PMN were isolated from the peripheral blood of a normal subject. In (b) PMN were isolated from the synovial fluid of a patient with rheumatoid arthritis (Patient(A), Table 17). Krebs medium, pH 7.4, 37°C, 10μM luminol.

4.3.2Investigation of rheumatoid synovial fluid and serum for stimuli of PMN luminol chemiluminescence

The aim of the experiments described in this section was to determine whether rheumatoid synovial fluid, previously shown to contain immune complexes (Winchester et al 1970, Hay et al 1979) and chemotactic complement components (Ward and Zvaifler 1971), would stimulate PMN luminol dependent chemiluminescence and thus provide evidence for the production of oxygen radicals by these cells in patients with rheumatoid arthritis. The capacity for rheumatoid serum to stimulate PMN luminol chem,iluminescence was also examined. Hay et al (1979) had demonstrated increased circulating immune complexes in 60% of their patients, but except in the presence of systemic disease (e.g. vasculitis) there had been little evidence of serum complement activation (Winchester et al 1971).

Synovial Fluid

Freshly aspirated synovial fluid from which all cells had been removed by centrifugaton at 10,000g for 10 min (4.2.2) was diluted in Krebs medium pH 7.4 and added to normal whole blood PMN. Synovial fluid samples similarly prepared from patients with seronegative inflammatory polyarthropathies were used as controls. Of four rheumatoid and two seronegative fluids tested (patients (A) to (F), Table 17), only one, from a patient with rheumatoid arthritis caused an increase in chemiluminescence, after an initial decrease, reaching a maximum at 180s after addition (Fig 50 (i)). This stimulation was slightly decreased by heating the centrifuged synovial fluid at 56^{o}C for 30 minutes to inactivate complement (Smith and Wood, 1969), but was markedly decreased following storage at -20^{o}C for 5 days (Fig 50 (iii)).

Addition of the remaining rheumatoid (Fig 51) and seronegative

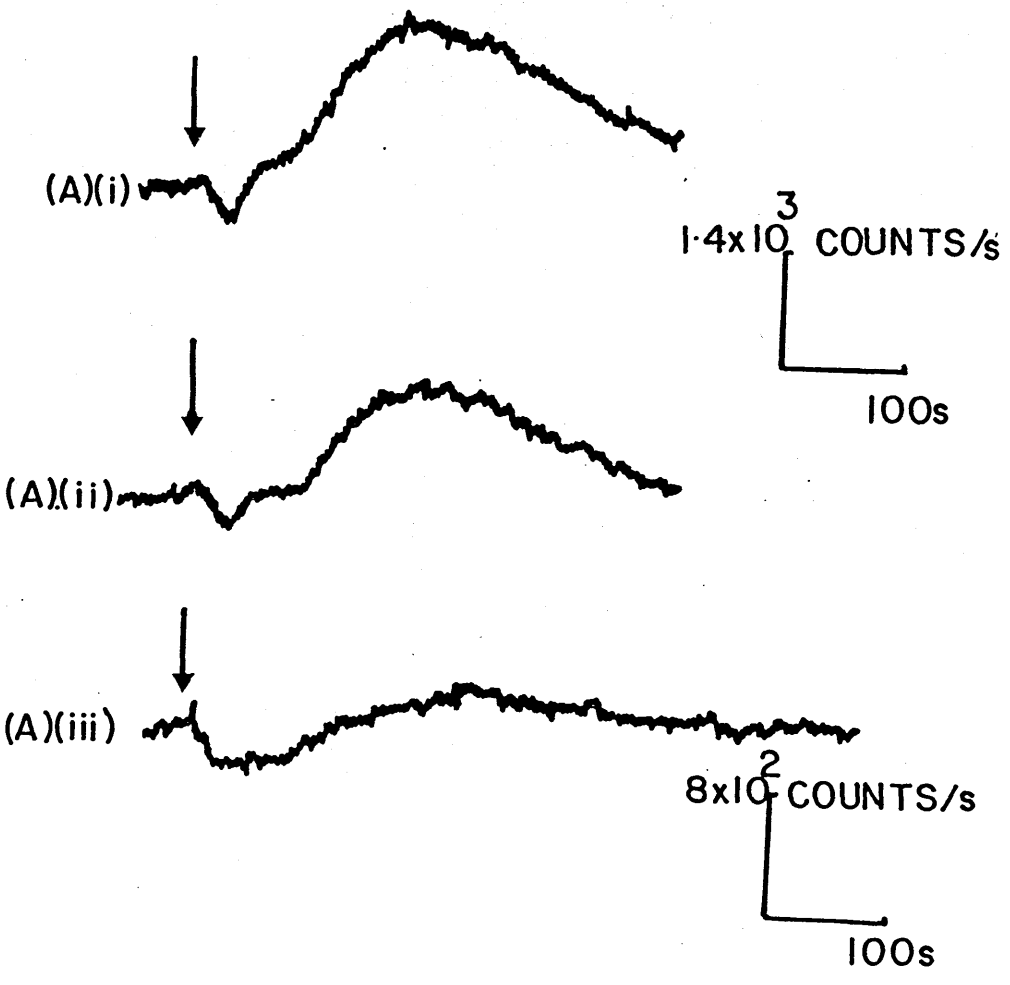


Figure 50 Luminol chemiluminescence of normal peripheral blood PMN exposed to rheumatoid synovial fluid (I)

Chemiluminescence traces produced by 1.2×10^6 PMN/ml stimulated by addition at arrows of 20% (v/v) rheumatoid synovial fluid from patient (A) (Table 17) from which cells had been removed by centrifugation at 10,000g for 10 minutes. In (i) the freshly aspirated, centrifuged fluid had not been further treated. In (ii) it had been heated at 56°C for 30 minutes. In (iii) it had been stored at -20°C for 5 days. Krebs medium, pH 7.4, 37°C, 10μM luminol.

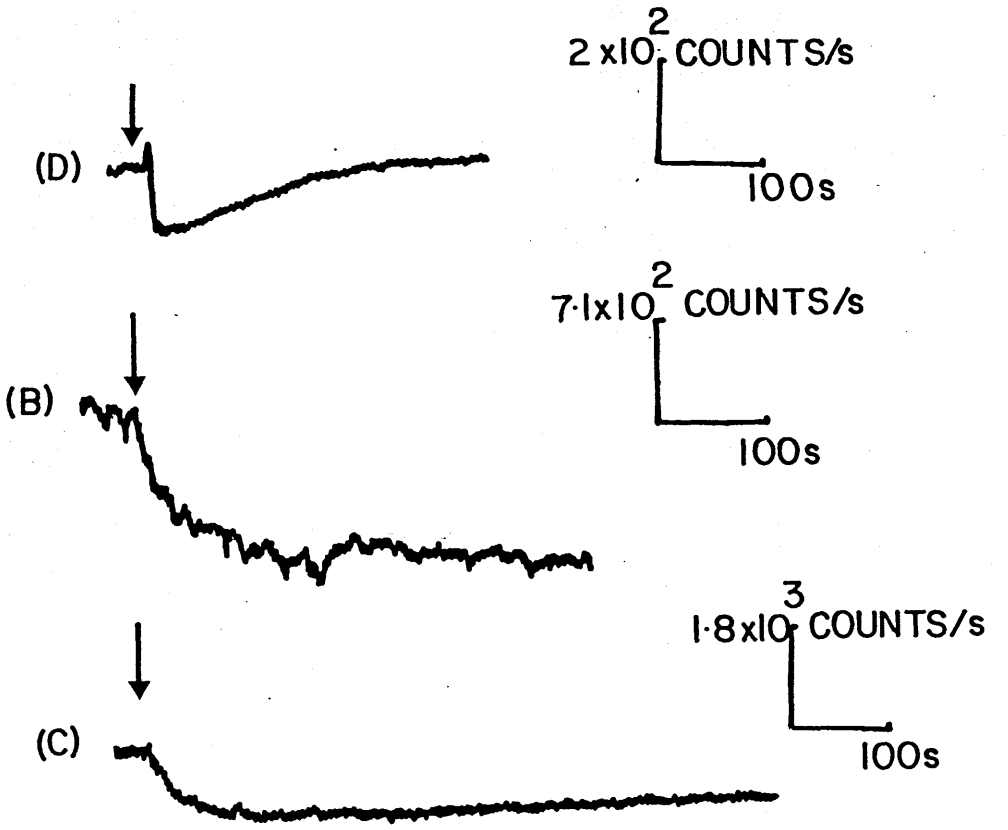


Figure 51 Luminol chemiluminescence of normal peripheral blood PMN exposed to rheumatoid synovial fluid (II)

Chemiluminescence traces which resulted when 20% (v/v) freshly aspirated synovial fluid from rheumatoid patients (D), (B) and (C) (Table 17) were added, at arrows, to 6.4×10^5 PMN/ml, 7.4×10^5 PMN/ml and 1.1×10^6 PMN/ml, respectively, normal peripheral blood PMN. The synovial fluid had been centrifuged at 10,000g for 10 mins to remove all cells. Krebs medium, pH 7.4, 37°C, 10μM luminol.

(Fig 52) synovial fluids to normal whole blood PMN was followed by a decrease in chemiluminescence count/s to values which were 58-70%, for the three rheumatoid samples, and 56-58%, for the two seronegative samples, below the level of resting PMN chemiluminescence. Thereafter, chemiluminescence either reached a plateau at this lower level or increased gradually to a maximum which did not surpass the value of resting PMN chemiluminescence.

Addition of Krebs medium alone (i.e. without synovial fluid) to normal whole blood PMN similarly pre incubated with luminol also resulted in a decrease in chemiluminescence count/s reaching a plateau which was 75% of the original resting PMN chemiluminescence (Fig 53). This decrease may have been accounted for by the dilution of luminol from 20μM to 10μM which occurred as the volume of Krebs medium was increased from 500μl to 1ml. The effect on the geometry of light detection by the luminometer (2.9.2) of this volume change would however have resulted in an increase in chemiluminescence measured.

Thus only one patient (patient (A), Table 17) of four rheumatoid synovial fluid samples tested was able to stimulate normal whole blood PMN chemiluminescence. The remainder, and synovial fluid from two patients with seronegative arthropathies causing an inhibition of PMN chemiluminescence which was greater than that caused by addition of an equal volume of Krebs medium only.

When synovial fluid from rheumatoid patient (A) was added back to autologous synovial fluid PMN separated from the same sample by density gradient centrifugation as described in 4.2.2 no increase in synovial fluid PMN chemiluminescence occurred (Fig 54(i)), rather there was an immediate decrease in PMN chemiluminescence followed by a gradual rise to a value which did not surpass that of the background PMN chemiluminescence. Addition of synovial fluid from rheumatoid

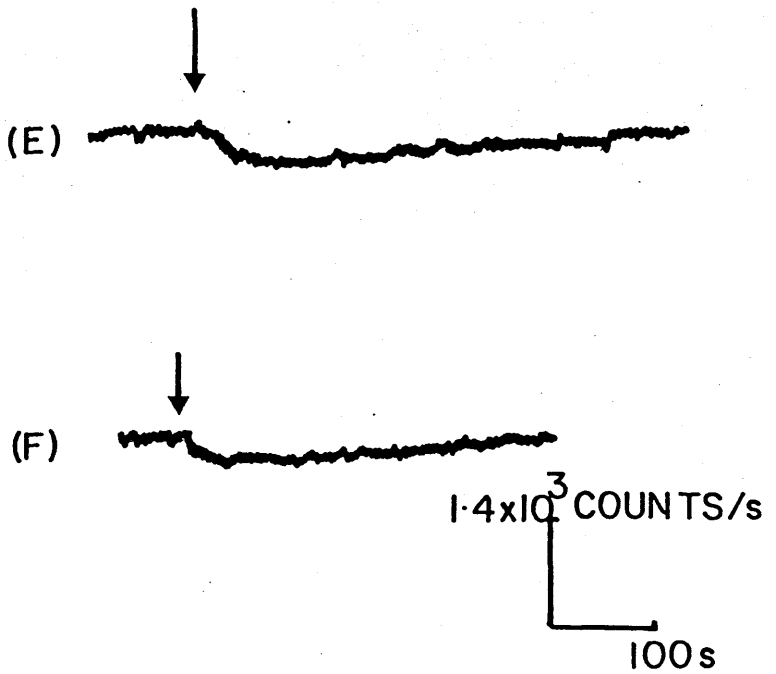


Figure 52 Luminol chemiluminescence of normal peripheral blood PMN exposed to seronegative inflammatory synovial fluid.

Chemiluminescence traces which resulted when 20% (v/v) freshly aspirated synovial fluid from patients (E) and (F) (Table 17) with seronegative inflammatory arthropathies, were added to 1.2×10^6 PMN/ml normal peripheral blood PMN. The synovial fluid had been centrifuged at 10,000g for 10 mins to remove all cells. Krebs medium, pH 7.4, 37°C, 10μM luminol.

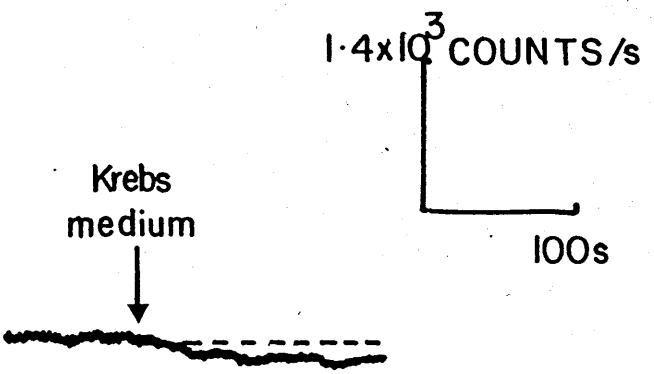


Figure 53 Effect of addition of Krebs medium only on the luminol chemiluminescence of normal peripheral blood PMN

Chemiluminescnece of 4.6×10^5 PMN/ml normal peripheral blood PMN before and after the addition at arrow of 500ul Krebs medium only (no synovial fluid or stimulus). The volume increase equalled that which had occurred by addition of diluted synovial fluid (200µl synovial fludi + 300µl Krebs medium) in Figures 50-52. Krebs medium, pH 7.4, 37°C, 10µM luminol.

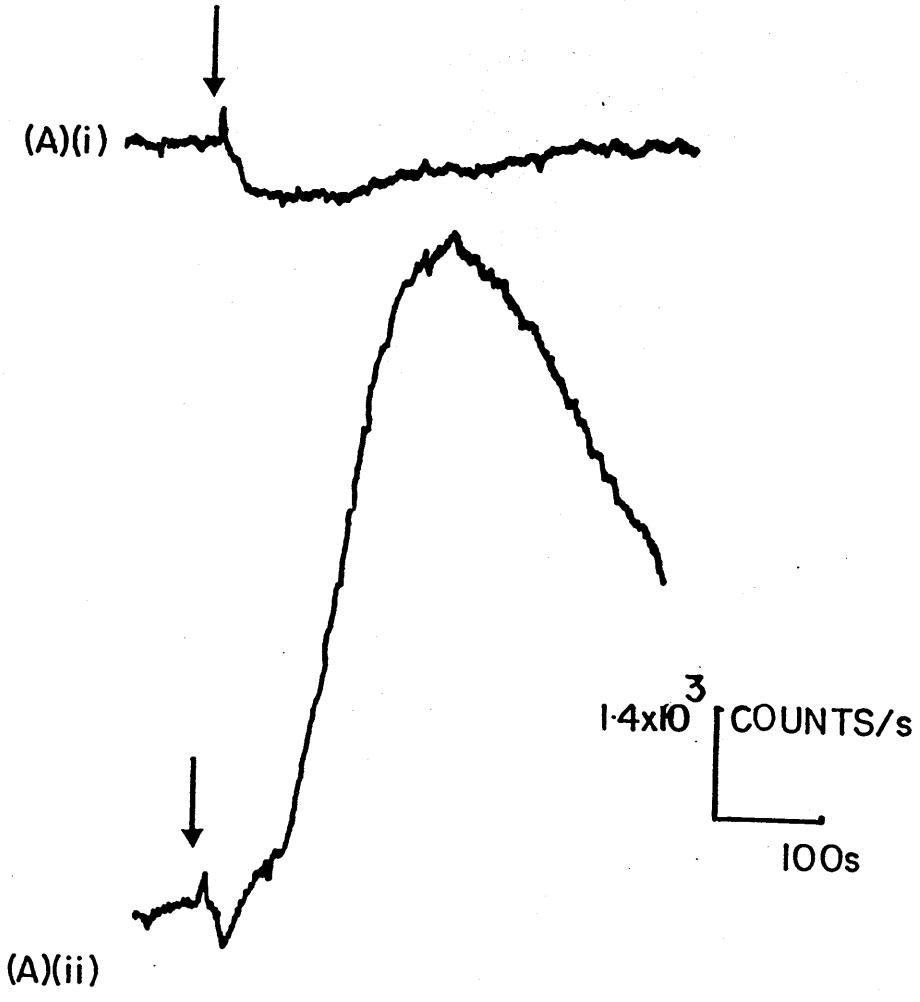


Figure 54 Effect of rheumatoid synovial fluid from patient (A) on the luminol chemiluminescence of autologous rheumatoid and seronegative inflammatory synovial fluid PMN

Chemiluminescence which resulted when 20% (v/v) synovial fluid from rheumatoid patient (A) (Table 17) was added, at arrows, to (i) 1.3×10^6 PMN/ml autologous synovial fluid PMN and (ii) 1.3×10^6 PMN/ml PMN from the synovial fluid of patient (E) with a seronegative monoarthropathy (Table 17). The rheumatoid synovial fluid had been centrifuged at 10,000g for 10 minutes to remove all cells. Krebs medium pH 7.4, 37°C, 10μM luminol.

patient(A) to PMN isolated from the synovial fluid of seronegative monoarthropathy patient (E) resulted in a massive increase in PMN chemiluminescence, after a decrease, reaching a maximum 220s following the addition of fluid (Fig 54 (ii)). As with the stimulation of normal blood PMN the seronegative synovial fluid PMN wre also stimulated by rheumatoid patient (A)'s synovial fluid which had been heated at 56°C for 30 minutes (data not shown) (cf Fig 50 (ii)).

Serum

Serum was prepared from whole blood freshly venesected from three patients with rheumatoid arthritis ((M), (N) and (R), Table 18) one of whom (N), had Felty's syndrome with splenomegaly and a leukopaenia of $1.8 \times 10^9/l$ (28%) neutrophils) according to the method described in 4.2.1. Following the addition of serum, (diluted in Krebs medium) to normal PMN preincubated at 37°C with luminol there was an immediate fall in chemiluminescence count/s to values which were 50%, 44% and 47% of resting PMN chemiluminescence for (M) (N) and (R) respectively at 50-60 seconds (Fig 55). Thereafter there was a gradual rise in PMN luminol chemiluminescence which reached a plateau at 51-69% of the value of resting PMN chemiluminescence. Addition of only 5% (v/v) serum (diluted in Krebs medium to 500µl) from patients (N) and (R) resulted in inhibition of PMN chemiluminescence to levels which were 95% and 90% of the resting value (data not shown).

Rheumatoid serum had thus failed to stimulate normal blood PMN chemiluminescence; instead, an inhibition of resting chemiluminescence had occurred. Rheumatoid serum similarly inhibited the resting chemiluminescence of autologous rheumatoid blood PMN (data not shown). Addition of normal serum similarly prepared from freshly venesected whole blood also caused a rapid fall in the luminol chemiluminescence

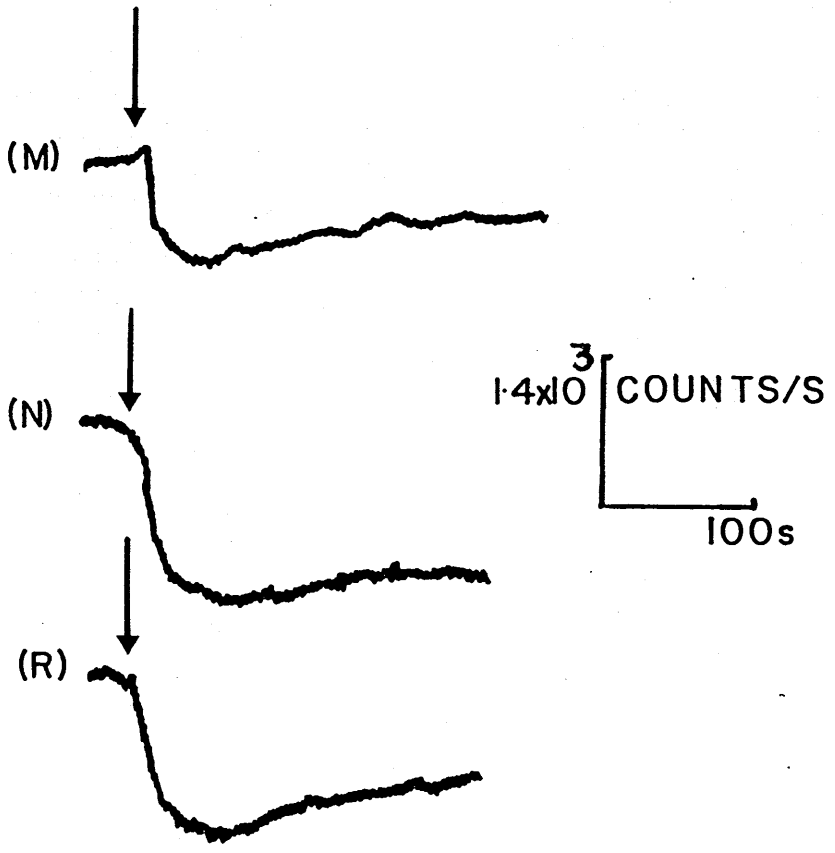


Figure 55 Luminol chemiluminescence of normal peripheral blood PMN exposed to rheumatoid serum

Chemiluminescence traces which resulted when 20% (v/v) serum from rheumatoid patients (M), (N) and (R) (Table 18) was added at arrows to 1.1×10^6 PMN/ml, 5.7×10^5 PMN/ml and 5.7×10^5 PMN/ml normal peripheral blood PMN respectively. The sera were obtained from freshly aspirated whole blood and had not been heat treated. Krebs medium, pH 7.4, 37°C, 10μM luminol.

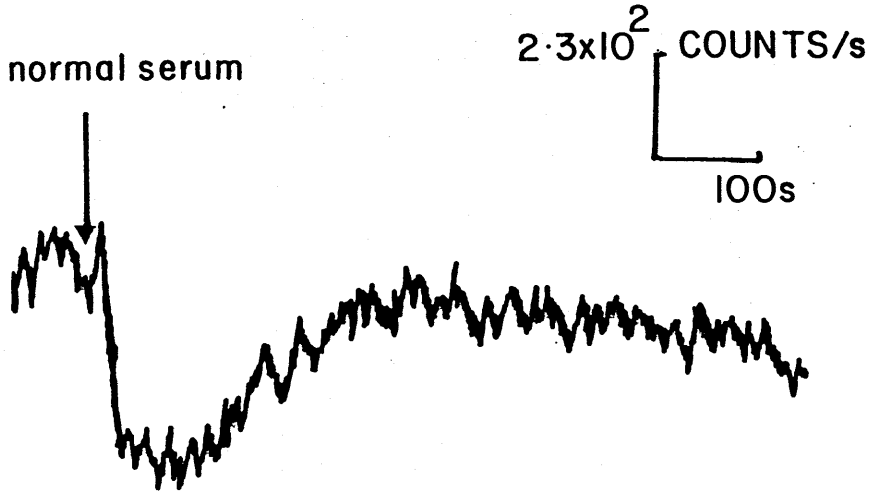


Figure 56 Effect of normal serum on the luminol chemiluminescence of normal peripheral blood PMN

Chemiluminescnece trace produced by 6.5×10^5 PMN/ml normal peripheral blood PMN to which 200µl of normal serum (diluted to 500ul in Krebs medium) was added at arrow. Krebs medium pH 7.4, 37°C 10µM luminol.

of normal PMN (Fig 56) to a value which was 52% of resting chemiluminescence 50 seconds after addition. Thereafter there was a gradual rise in chemiluminescence count/s to a plateau at 90% of the original resting chemiluminescence.

4.3.3 Investigation of synovial fluid and serum for inhibitors of PMN luminol chemiluminescence

Although the number of samples examined was small, the predominantly inhibitory effect of rheumatoid synovial fluid and serum on the resting chemiluminescence of normal PMN in the experiments of the previous section suggested either

(i) the samples did not contain stimuli of PMN chemiluminescence.

or

(ii) the samples contained inhibitors of PMN chemiluminescence instead of/or in addition to stimuli.

Seronegative synovial fluid and normal serum had similarly also inhibited the chemiluminescence of resting PMN. It was now important to establish whether similar inhibitions would occur in the presence of 'model stimuli' of PMN chemiluminescence (cf Chapter 3).

Synovial Fluid

The effect of synovial fluid from four patients (H) - (L), Tables 17 and 18, (three with rheumatoid arthritis and one with chondrocalcinosis) on the luminol chemiluminescence of normal PMN stimulated by the chemotactic peptide n formyl met leu phe was examined (Fig 57). In all cases, immediately following aspiration, the synovial fluid had been centrifuged at 10,000g for 10 minutes to remove cells (4.2.2) and stored at -70°C for six months. Prior to use the samples were heated at 56°C for 30 minutes to inactivate

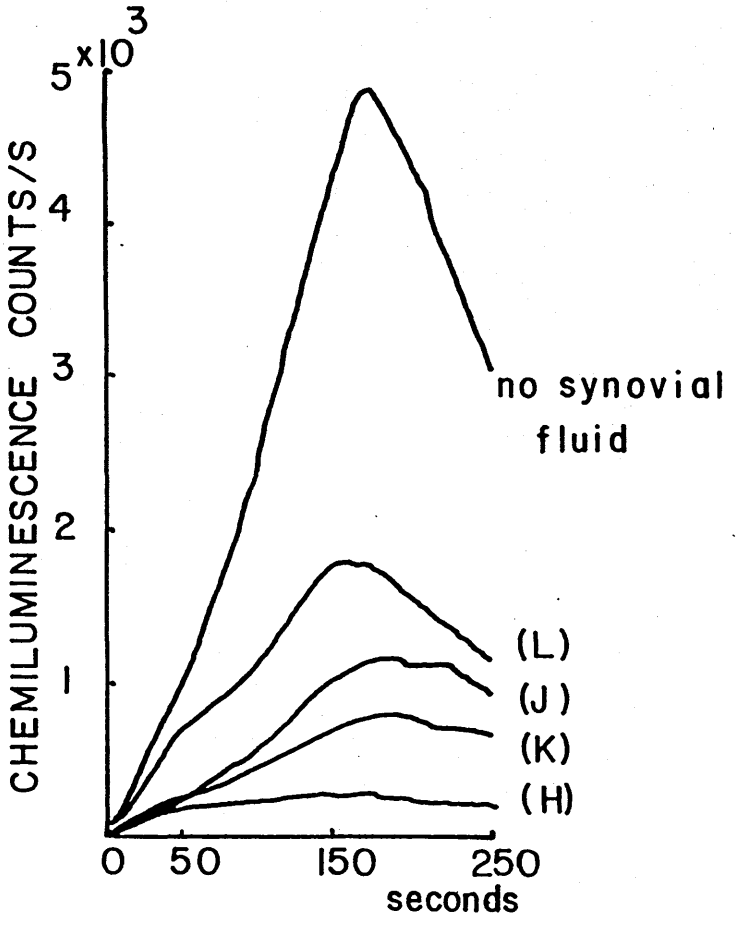


Figure 57 Inhibition of chemotactic peptide stimulated PMN chemiluminescence by synovial fluid

Chemiluminescence traces produced when $5x10^5$ PMN/ml (normal peripheral blood PMN) were stimulated by addition at 0 seconds of 1uM chemotactic peptide. The top trace shows the usual chemiluminescence response to this stimulus. In the remaining traces cells were pre-incubated with 40% (v/v) synovial fluid from rheumatoid patients (L), (J), (H) and patient (K) who had chondrocalcinosis (Tables 17 and 18), for 5 minutes at $37^{\circ}C$ prior to addition of stimulus. Krebs medium pH 7.4, $37^{\circ}C$, 10μM luminol.

complement. Normal whole blood PMN were then pre-incubated with synovial fluid and luminol at 37°C for 5 minutes prior to the addition of chemotactic peptide. All four synovial fluids inhibited the resultant PMN chemiluminescence (Fi g 57), the maximum peak heights being between 5% (H) and 36% (L) of that achieved in the absence of synovial fluid. This concurred with observations made during the differential centrifugation of synovial fluid, (4.2.2, Fig 46 and Table 19), that relative to the concentration of PMN, chemotactic peptide stimulated a larger chemiluminescence response in PMN isolated from rheumatoid synovial fluid and resuspended in Krebs medium than in PMN which had not been so isolated.

Serum

When normal PMN were preincubated with normal human serum and luminol in Krebs medium pH 7.4, 37°C for 3-5 minutes prior to stimulation by chemotactic or phagocytic stimuli the resultant resting and stimulated PMN luminol chemiluminescence was markedly reduced when compared with PMN in the absence of serum (Fig. 58), 40% (v/v) serum in Krebs medium caused an 85% inhibition in peak height of chemotactic peptide induced PMN chemiluminescence while only 1% (v/v) serum in Krebs medium caused a 67% inhibition of latex particle induced chemiluminescence. Normal human serum in the range 10-80% (v/v) in Krebs medium also caused inhibition of resting and stimulated luminol PMN chemiluminescence which was dose related (Fig 59). 80% (v/v) serum caused 84% inhibition of opsonised zymosan stimulated PMN luminol chemiluminescence. Two thirds of this inhibition persisted following dialysis of normal serum against 11 Krebs medium pH 7.4 for 24 hrs. (Table 23) indicating that the inhibitory factor(s) was of high molecular weight.

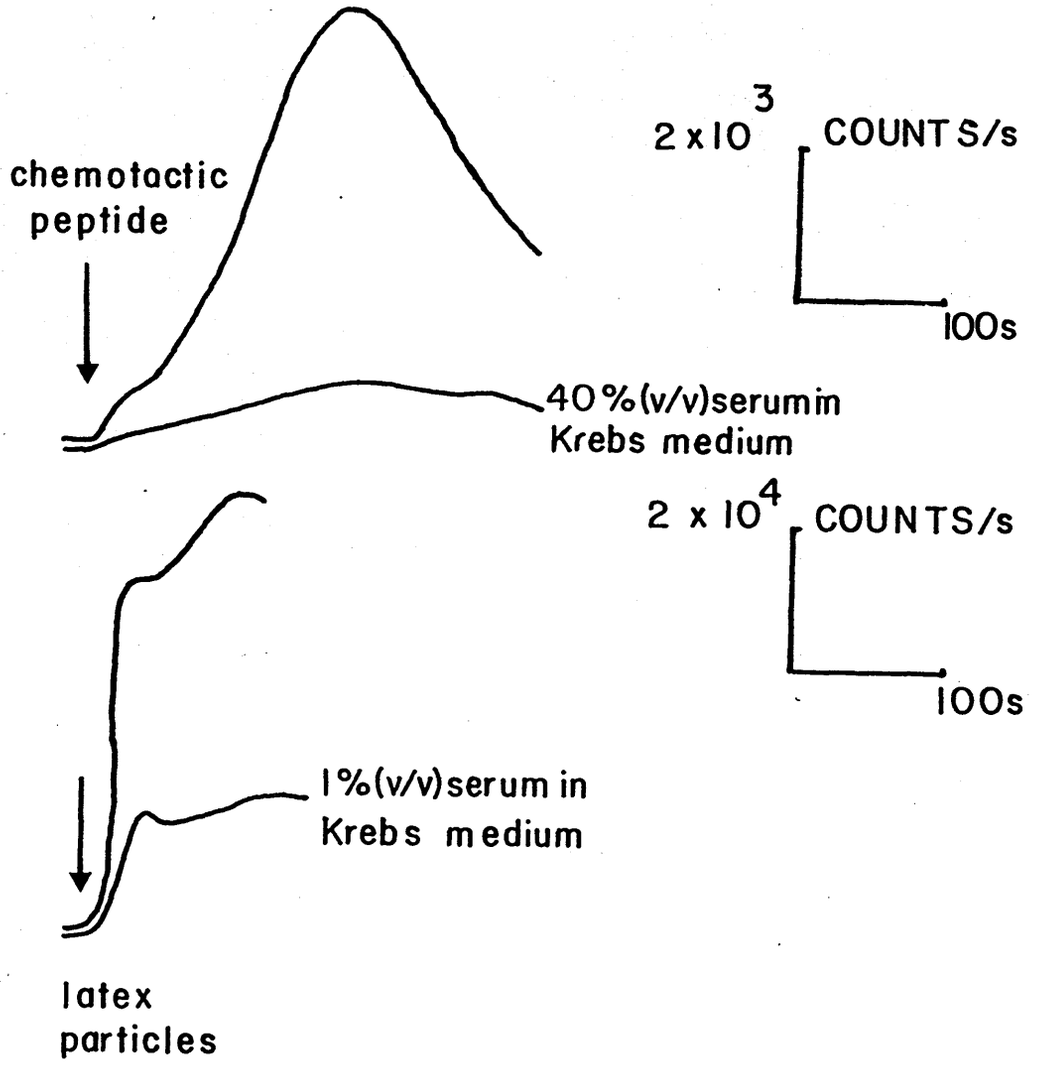


Figure 58 Inhibition of chemotactic peptide and latex particle stimulated PMN chemiluminescence by normal serum

The top pair of chemiluminescence traces were produced by 5×10^5 normal PMN stimulated by addition at arrow of 1μM chemotactic peptide in the presence and absence (as indicated) of 40% (v/v) serum in Krebs medium.

The lower pair of traces were produced by 6×10^5 normal PMN stimulated by addition at arrow of 4×10^9/ml latex particles in the presence and absence (as indicated) of 1% (v/v) serum in Krebs medium.

The sera were heat treated to inactivate complement. pH 7.4, 37°C, 10μM luminol.

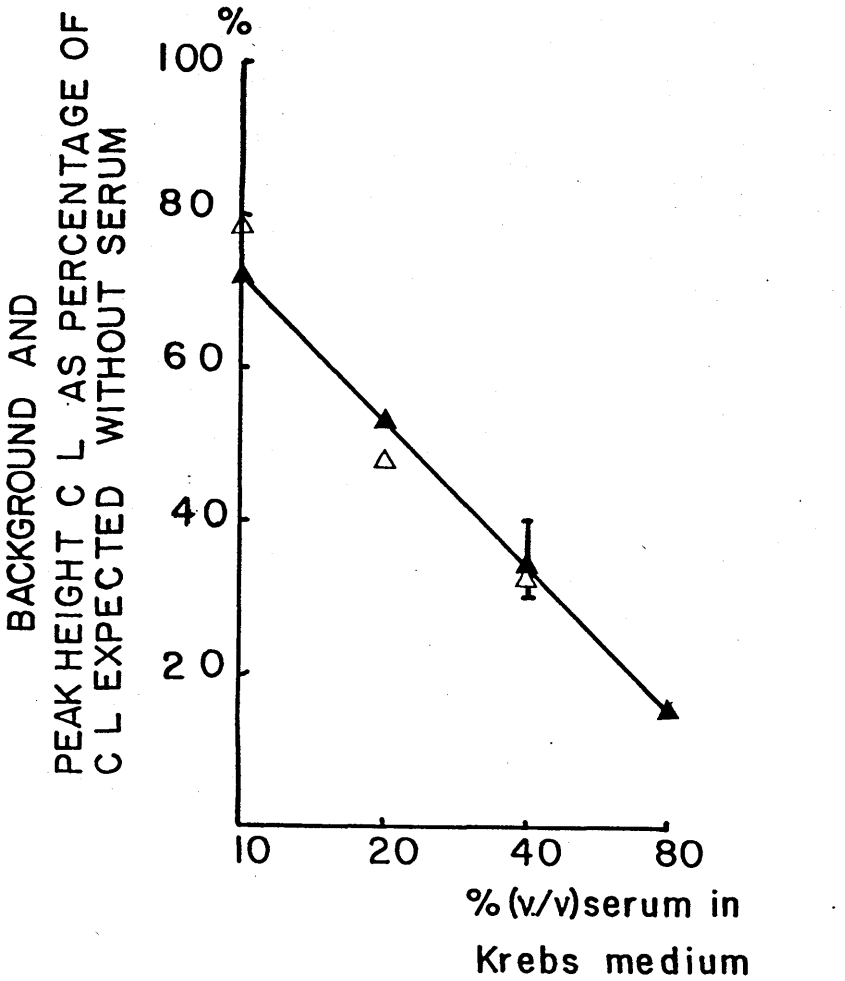


Figure 59 Concentration related inhibition of resting and stimulated PMN chemiluminescence by normal serum

The graph shows the results of one experiment in which 5×10^5 PMN/ml were stilulated by 1mg/ml opsonised zymosan in the presence of 10-80% (v/v) normal serum in Krebs medium. Points represent the values of background (△) and peak height (▲) chemiluminescence obtained for each serum concentration expressed as a percentage of the chemiluminescence expected in the absence of serum, (single or duplicate observations). The serum was heat treated to inactivate complement. pH 7.4, 37°C, 10μM luminol.

TABLE 23

Effect of Dialysis on inhibition of resting and stimulated PMN luminol dependent chemiluminescence caused by serum

	% inhibition of PMN background (resting) chemiluminescence (± SD, n=3)	% inhibition of peak height stimulated PMN chemiluminescence (± SD, n=3)
Non dialysed serum (40% v/v)	50.3 ± 15.3%	75.7 ± 2.1%
Dialysed serum (40% v/v)	28.7 ± 12.3%	53 ± 5.6%

Chemiluminescence of 5×10^5 PMN/ml which were resting, or stimulated by opsonised zymosan (1mg/ml) in the presence of 40%(v/v) serum which was either undialysed, or had been dialysed against Krebs medium pH 7.4 for 24 hrs. 37°C 10μM luminol.

4.4 Discussion

The experiments reported in this chapter demonstrated that both normal and rheumatoid serum, and synovial fluid from rheumatoid and other inflammatory arthropathies exerted a potent inhibitory effect on the luminol chemiluminescence of PMN which were resting or stimulated by 'model' chemotactic or phagocytic stimuli. Only one of four rheumatoid synovial fluids tested caused stimulation of PMN luminol chemiluminescence suggesting that either the remaining fluids did not contain stimuli at all, or that stimuli were in insufficient concentrations to overcome the inhibitory effect of the fluid on luminol chemiluminescence. The nature of the inhibition was not defined by these experiments but it was found to be dose dependent and to be retained following dialysis.

An important prelude to the experiments was the definition of conditions (using luminol dependent chemiluminescence as an assay of live cells) required to denude viscous synovial fluid of all suspended cells and thereby remove a potential source of artefact in any subsequent examination of the fluid for stimuli of PMN luminol dependent chemiluminescence (4.2.2). It was thus shown that to remove all live cells, synovial fluid should be centrifuged at a minimum of 10,000g for 10 min.

Of the two papers published subsequently by other authors reporting stimulation of PMN luminol dependent chemiluminescence by rheumatoid synovial fluid, one (Gale et al 1984) did not detail the speed at which the fluid was centrifuged, and the other (James, Betts and Cleland 1983) centrifuged the fluid diluted 1:1 in buffer medium at 200g for 30 minutes. Removal of all PMN is clearly a sine qua non for the measurement of any cell product (such as a proteolytic enzyme) in synovial fluid, where the presence of PMN as a persistent source of

the product would also be a potential source of artefact.

A method was also established for the isolation of viable PMN from synovial fluid samples which allowed subsequent examination of the luminol dependent chemiluminescence responses of the cells not only to model stimuli but also to synovial fluid itself. The unexpected quantitative variation in the resting and peak height values of normal blood PMN exposed to a fixed dosage of latex particles prevented the establishment of a 'normal range' and meaningful quantitative comparison with the chemiluminescence of rheumatoid blood or synovial fluid PMN. Nicholson and Sugars (1982) had experienced similar difficulty in establishing a 'normal range' for the luminol dependent chemiluminescence of PMN stimulated by the calcium ionophore A23187.

The higher background chemiluminescence of rheumatoid synovial fluid PMN when compared to that of rheumatoid blood PMN (Table 22, Fig 48) was concurrently demonstrated by James et al (1983) who also found that the synovial fluid PMN achieved a higher peak height of chemiluminescence on subsequent stimulation with heat aggregated IgG (c.f. table 22). Both observations would be compatible with the prior in vivo stimulation of synovial fluid PMN (Turner et al 1973, Vann Epps and Garcia 1980, English, Roloff and Lukens, 1981). By contrast Van de Stadt, Van de Voorde-Vissers and Feltkamp-Vroom (1980) were unable to demonstrate any difference in the rate of oxygen consumption by resting or stimulated synovial fluid PMN when compared with that of normal or rheumatoid peripheral blood PMN, but correlated this observation with the low frequency of immunoglobulin inclusions in their rheumatoid PMN samples. Chiu et al (1983) reported no significant differences between normal and (non Felty) rheumatoid peripheral blood PMN ferricytochrome c reduction in response to

stimulation with n formyl met leu phe. Youssef and Baron (1983), however, found increased 'superoxide dismutase activity' in their rheumatoid peripheral blood PMN and mononuclear cells when compared to normals.

Concomitant with the work of this thesis, Gale et al 1984) described stimulation of normal peripheral blood PMN luminol dependent chemiluminescence by 16 of their 21 samples of rheumatoid synovial fluid and by none of their 10 non-rheumatoid samples. They were unable to correlate their results with assays of Clq binding for immune complexes or with rheumatoid factor titres. James et al (1983) also described enhancement of normal peripheral blood PMN luminol dependent chemiluminescence from the patients with rheumatoid arthritis. In one instance stimulation of autologous synovial fluid PMN yielded a higher peak height chemiluminescence response. As indicated previously, however it is possible that both groups centrifuged their synovial fluid samples at insufficient speeds to remove all live cells or cell debris.

In the experiments of this chapter, the one rheumatoid synovial fluid sample which stimulated normal blood PMN chemiluminescence also stimulated chemiluminescence in PMN isolated from the synovial fluid of a patient with a seronegative inflammatory monoarthropathy, but failed to stimulate an increase in chemiluminescence when added back to autologous rheumatoid synoival fluid PMN. The latter may have represented an example of 'desensitisation' of oxidative initiation occurring as a result of multiple exposures of the cells to chemotaxins in vivo (English et al 1981, James et al 1983). The minimal inhibition of the stimulatory capacity of this sample by incubation at 56^{o}C for 30 minutes indicated that it was not caused by complement (Fig 50). Similarly its failure to withstand storage at -

20°C for 5 days was suggestive of at least partial dependence of the stimulatory capacity on non-covalent molecular bonding which was disrupted by freeze-thawing.

Although Starkebaum et al (1981) and James et al (1983) both described stimulation of PMN luminol chemiluminescence by rheumatoid sera, in the experiments of this chapter, even in the presence of the low concentrtions of sera (5% (v/v) serum in Krebs medium) used by Starkebaum et al (1981), an inhibition rather than a stimulation of PMN luminol chemiluminescence occurred. The inhibition of resting PMN luminol chemiluminescence effected by normal serum was found to be concentration dependent such that 80% (v/v) serum caused >80% inhibition. Normal serum similarly inhibited opsonised zymosan and chemotactic peptide stimulated PMN luminol dependent chemiluminescence and was a particularly potent inhibitor of latex particle stimulated PMN chemiluminescence.

The previously undescribed inhibition of resting and stimulated PMN luminol chemiluminescence by serum and synovial fluid demonstrated in this chapter clearly required further investigation if the role of oxygen radicals in the pathogenesis of rheumatoid arthritis was to be established. Whatever the known stimuli of PMN oxygen radical production within the synovial fluid and sera of patients with this disease the experiments of this chapter had provided preliminary evidence of the co-existence of potent extracellular oxygen radical scavenging mechanisms. Other potential explanations for the inhibitory effects of the synovial fluid and serum on PMN chemiluminescence also, included interference with light transmission, quenching of the luminol excited state or inhibiiton of cell stimulus interaction. Evidence in favour of the latter had previously been provided by Turner et al (1973) and Wilton et al (1979).

Persistence of the PMN chemiluminescence inhibiting effect in dialysed serum suggested that a high molecular weight component was responsible. Identification of the component and investigation of the mechanism by which PMN chemiluminescence was inhibited provided the basis of the next two chapters of this thesis (cf 1.6).

Summary

Serum and synovial fluid were found to be potent inhibitors of resting and stimulated PMN luminol chemiluminescence. A possible explanation for this inhibition was oxygen radical scavenging.

(a) Luminol dependent chemiluminescence and differential centrifugation were used to define the conditions required to denude synovial fluid of all cells (centrifugation of the fluid at 10,000g for 10 minutes).

(b) Considerable inter and intra-subject variation in the chemiluminescence responses of normal PMN to a model stimulus impaired quantitative comparison with the chemiluminescence of rheumatoid PMN. No qualitative differences were observed.

(c) Synovial fluid (20% v/v) from three patients with rheumatoid arthritis and two patients with seronegative inflammatory arthropathies, together with serum from three patients with rheumatoid arthritis failed to stimulate normal or autologous PMN luminol dependent chemiluminescnece. One rheumatoid synovial fluid sample was stimulatory, although this was inhibited by storage of the sample at -20°C for 5 days.

(d) Synovial fluid (40% v/v) from three patients with rheumatoid arthritis and one patient with chordrocalcinosis inhibited the

peak height of the luminol chemiluminescence of normal PMN stimulated by chemotactic peptide by up to 95%.

(e) Normal serum caused potent, dose dependent, inhibition of resting and stimulated PMN luminol chemiluminescence. 40% (v/v) normal serum inhibited the peak height chemiluminescence of opsonised zymosan stimulated PMN by 75%. Demonstration that more than 2/3 of this inhibition persisted following dialysis of serum indicated that a high molecular weight component was responsible.

Chapter 5

Investigation of the inhibitory effect of serum and synovial fluid on PMN chemiluminescence: evidence for oxygen radical scavenging by albumin

5.1 Introduction

5.2 Methods

5.2.1 General

5.2.2 Measurement of $O_2^{\cdot -}$ by ferricytochrome c reduction

5.2.3 Acellular luminol chemiluminescence

5.3 Results

5.3.1 Inhibition of PMN chemiluminescence by serum albumin

5.3.2 Lack of effect of albumin on cell viability and light transmission

5.3.3 Lack of effect of albumin on luminol excited state

5.3.4 Effect of dialysed albumin on PMN chemiluminescence

5.3.5 Effect of albumin on PMN oxygen consumption and cytochrome c reduction

5.4 Discussion

5.5 Summary

5.1 Introduction

The low level of 'known' oxygen radical scavengers in the extracellular milieu (1.2.3) had previously formed an integral part of the proposal that oxygen radicals may play an important role in the pathogenesis of rheumatoid arthritis (Del Maestro 1980, Greenwald 1981). The demonstration, in the experiments of the last chapter, that serum and synovial fluid were potent inhibitors of PMN oxygen radical activity, as measured by luminol chemiluminescence, therefore required further investigation. It was important to establish:-

(a) which component of serum and synovial fluid was responsible for the inhibition of chemiluminescence

and

(b) the mechanism of the inhibition and, in particular, what proportion occurred via oxygen radical scavenging.

Persistence of more than two-thirds of the inhibitory effect of normal serum following its dialysis (4.3.3) indicated that a high molecular weight component was responsible for the majority of its inhibition of luminol dependent chemiluminescence. The low extracellular concentrations of superoxide dismutase and catalase (Table 5, 1.2.3) had already been shown to be insufficient to inhibit PMN luminol dependent chemiluminescence (cf Fig 21, 2.11.3 and Fig 43, 3.3.). Of other molecules found in the extracellular space known to be capable of scavenging oxygen radicals (Fig 8, 1.2.3), ascorbic acid, purines and catecholamines were excluded by size, and only transferrin, caeruloplasmin and possibly amino acid residues or transported substances on other high molecular weight proteins, such

as albumin, remained as candidates.

Bovine serum albumin had, in fact, already been identified as an inhibitor of PMN luminol chemiluminescence when the optimum conditions for assay were defined (2.12.). Although albumin (molecular weight 66,000) was known as the major extracellular protein, being found in high concentration in normal serum (40g/l, 600μM) and synovial fluid (20g/l, 300μM) no functions for it, other than transport or maintainance of osmotic pressure had yet been identified (Peters 1975). The concentration of albumin in synoival fluid was known to rise in inflammatory arthropathies such as rheumatoid arthritis (Wilkinson and Jones 1964), and the exciting possibility that this molecule might act as an important scavenger of oxygen radicals in vivo demanded further investigation.

In the experiments described in this chapter the effect of human serum albumin on resting and stimulated normal PMN luminol dependent chemiluminescence was investigated. Before ascribing any inhibitory effect of albumin (or indeed of whole serum) to oxygen radical scavenging it was essential to establish that the inhibition did not arise from impaired cell viability, impaired cell-stimulus interaction or impaired generation of luminol chemiluminescence per se.

5.2 Methods

5.2.1 General

Albumin, source, storage and purity

Unless otherwise stated all albumin used in experiments reported in this chapter was commercially prepared and purified pooled human serum albumin (Boehringer). Stock solutions were prepared by dissolving the albumin in Krebs medium at a concentration of 10% (w/v), readjusting pH to 7.4. The stock solutions were stored in 1ml

aliquots at -20°C and warmed and diluted further in Krebs medium pH 7.4 as indicated on the day of experiment. This further dilution did not result in any pH change.

Polyacrilamide gel electrophoresis confirmed that there were no high molecular weight contaminants in the albumin preparation.

Dialysis of albumin

When indicated albumin was dialysed by suspension of 10-20ml 20% (w/v) human serum albumin in Krebs medium in dialysis tubing, in 300-400ml Krebs medium in a one litre volume flask or beaker stirred by a magnetic stirrer and maintained at 4°C for 12-18 hours. The dialysate was then substituted by an equal volume of Krebs medium pH 7.4 on two occasions. Dialysis was terminated after a total of 48 hours. During this period the volume of fluid in the dialysis bag increased but knowledge of the original weight of albumin in the bag allowed readjustment with additional Krebs medium pH 7.4 to a final concentration of 10% (w/v). The dialysed albumin was then stored in 1ml aliquots at -20°C.

Measurement of free ionised calcium concentration

Free ionised calcium concentrations were measured using a commercially available Ca^{2+} selective electrode (Radiometer, Copenhagen) attached to a voltmeter. The electrode was calibrated using Krebs medium pH 7.4 in which the calcium concentration was adjusted between 0.129mM and 4.22mM (Fig. 60).

5.2.2 Measurement of $O_2^{\cdot -}$ ferricytochrome c reduction

The superoxide dismutase inhibitable reduction of ferricytochrome c exposed to phagocytosing PMN has been used as a measure of superoxide anion production by these cells (Babior et al 1973, Root

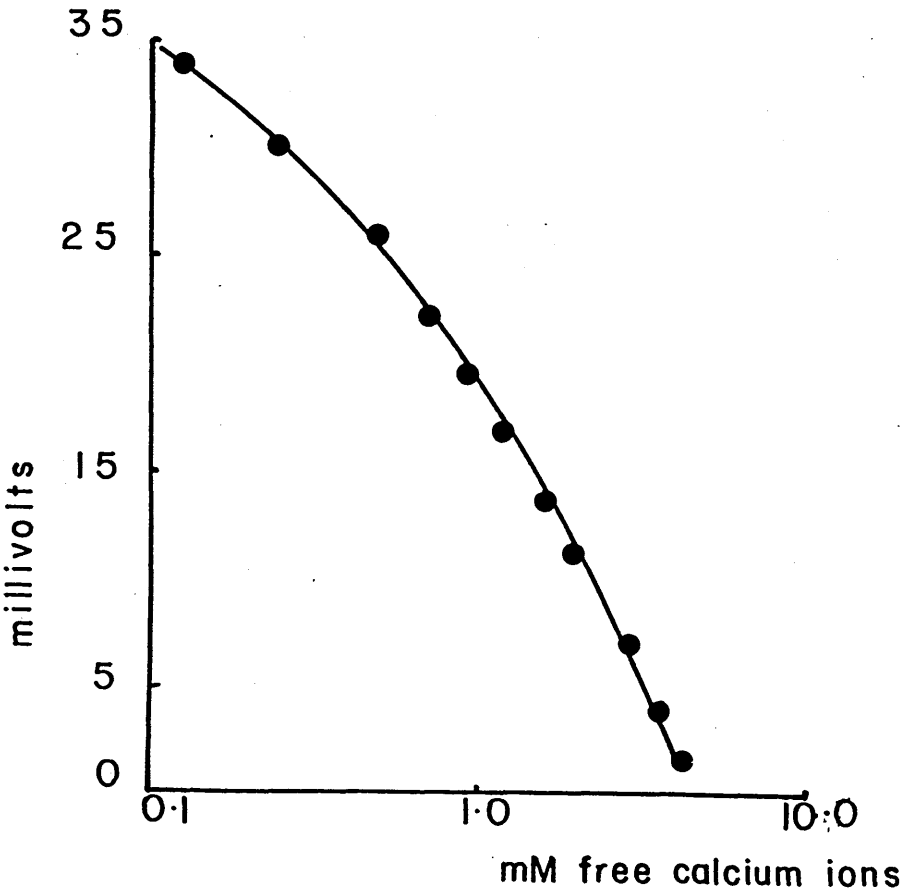


Figure 60 Calibration of electrode for selective measurement of free ionised calcium

The Ca^{2+} selective electrode, which was attached to a voltmeter, was calibrated by immersion in Krebs medium pH 7.4 in which the known Ca^{2+} concentration had been adjusted between 0.129mM and 4.22mM. The points represent the millivoltage reading for each Ca^{2+} concentration. Single observations. Room temperature.

and Metcalf , 1977). Using an adaptation of the method of Babior et al, PMN were preincubated in Krebs medium pH 7.4 at 37°C in an Eppendorf tube for 10 min prior to the simultaneous addition of ferricytochome c and opsonised zymosan stimulus also at 37°C. The cell stimulus suspension was then maintained at 37°C in a shaking water bath for 20 minutes after which reaction was terminated by centrifugation at 10,000g in an Eppendorf centrifuge for 50 seconds. The supernatant was then placed in a plastic spectrophotometer cuvette and the absorption of light at 550nm was measured against that of a matched cuvette containing Krebs medium only in a Beckman dual wavelength spectrophotometer. In control experiments cytochome c was incubated at 37°C with Krebs medium only, and unstimulated cells. The effect of albumin on the reduction of ferricytochome c in the presence and absence of PMN was examined as detailed in 5.3.5.

In order to confirm that measurement of the absorption of light by ferricytochrome c at 550nm provided the most sensitive measure of ferricytochrome c reduction (or conversion to ferrocytochrome c), and to determine what percentage of ferricytochrome c was alredy reduced, prior to its exposure to superoxide anion, the effect of the powerful reducing agent sodium dithionite on the spectral scan of cytochrome c was examined between 565 and 440nm (Fig. 61) in the dual wavelength spectrophotometer.

After a small shoulder at 550nm absorption rose to a larger peak at 517nm and then fell to a trough at 490nm before beginning the ascent to a maximum at 416nm (data not shown) (Fig. 61 (a)). Upon addition of sufficient (2-3mg) sodium dithionite to cause full reduction of 100μM(1ml) ferricytochrome c, a change in absorption occurred resulting in elongation of the absorption peak at 550nm and an increase in the absorption at 517nm (Fig. 61(b)), which confirmed

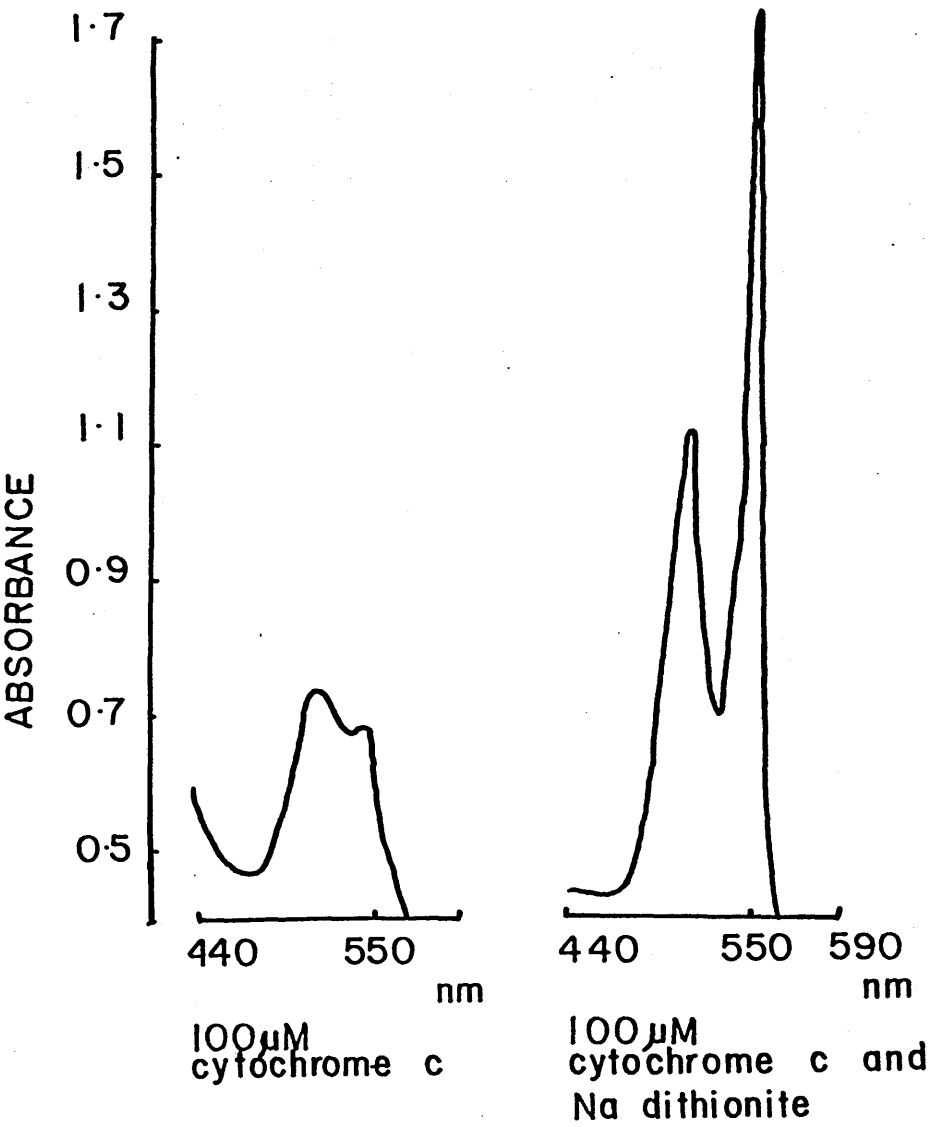


Figure 61 Absorption spectra of cytochrome c

The graphs show the absorption of light by 100µM cytochrome c measured in a scanning spectrophotometer between 565 and 440nm, before (first trace) and after (second trace) its complete reduction by sodium dithionite (2-3mg/ml). Krebs medium, pH 7.4, room temperature. It was thus established that absorption of light at 550nm could be used to indicate the presence of reduced cytochrome c.

the findings of Margoliash and Frohwirt (1959) and Babior et al (1973). The extinction coefficient (Δ EmM) for ferrocytochrome c minus ferricytochrome c at 550nm was taken as 15.5 (Babior et al, 1973).

In Fig 61 (a) and (b) the absorption at 550nm were 0.653 and 1.750 respectively. The concentration of cytochrome c reduced by addition of sodium dithionite was thus

$$\mu M \text{ ferrocytochrome c} = \frac{1.750 - 0.653}{15.5} \times 1000\mu M$$
$$= 70.8\ \mu M$$

The original 100μM ferricytochrome c preparation was thus already 30% reduced.

5.2.3 Acellular luminol chemiluminescence

Luminol chemiluminescence was generated in the absence of PMN in the following systems

(a) Xanthine/xanthine oxidase/luminol

(b) Microperoxidase/hydrogen peroxide/luminol

(a) Xanthine/xanthine oxidase/luminol

The method was adapted from that of Hodgson and Fridovich 1973. Xanthine oxidase (4 units/ml) prepared from milk was supplied by Sigma (Grade IV) in suspension in 2.3M $(NH_4)_2SO_4$ solution containing 0.02% sodium salicylate. Before use, 300μl of the suspension was centrifuged at 10,000g in an Eppendorf centrifuge for 1 min. The supernatant was removed and the pellet was dissolved in 12,000μl Krebs medium pH 7.4 containing 4mM EDTA to give xanthine oxidase 1 unit/ml. Xanthine (2.6 dihydroxypurine) was dissolved in 2mM NaOH at a concentration of 33mM and diluted in Krebs medium pH 7.4 containing 4mM EDTA to a final concentration of 1mM xanthine. EDTA prevented the

formation of a precipitate which was otherwise observed in the presence of divalent cations.

An LP_3 tube containing 0.2mM xanthine and 20µM luminol in 500µl Krebs medium pH 8.0 was placed in the luminometer cell housing and rotated in front of the photomultiplier tube where background chemiluminescence was recorded. Reaction was initiated by the addition of 500µl Krebs medium pH 7.4 containing 0.03 - 0.12 units/ml xanthine oxidase, 4mM EDTA via a syringe through the aperture in the sample housing roof. The pH of the final reaction mixture was 7.9. Serial chemiluminescence counts per 10s were recorded.

(b) Microperoxidase/hydrogen peroxide/luminol

A stock solution of microperoxidases (sodium salt from horse equine cytochrome c) 0.4mg/ml in Tris HCl buffer pH 7.4 was maintained at +4°C for up to six weeks, and was diluted in Krebs medium pH 7.4 to a concentration of 10µg/ml on the day of experiment. Hydrogen peroxide (H_2O_2) (30 volumes) was diluted 0.2% (v/v) and 0.002% (v/v) in Krebs medium pH 7.4 to final concentrations of 35mM and 35µM respectively. Luminol was dissolved in DMSO at a stock concentration of 10mM and was thereafter diluted in Krebs medium pH 7.4 to concentrtions in the range 200pM-2µM.

The background chemiluminescence of 0.5µg/ml microperoxidase and luminol in 500µl Krebs medium at room temperature was measured in the cell housing of luminometer 'A' or 'B' (2.8.1) before initiation of the reaction by injection of hydrogen peroxide in 500µl Krebs medium.

5.3 Results

5.3.1 Inhibition of PMN luminol dependent chemiluminescence by human serum albumin

The main objectives of the experiments carried out in this section were:-

(a) to determine whether human serum albumin would exert an inhibitory effect on normal PMN chemiluminescence.

and

(b) to determine whether the degree of any inhibition observed would vary according to the cell stimulus employed and thereby indicate that the inhibition might, at least in part, result from impairment of cell stimulus interaction by albumin.

Experiments in previous chapters had shown that bovine serum albumin inhibited latex particle stimulated PMN luminol chemiluminescence, (2.12), and that the inhibitory effect of normal human serum on latex particle stimulated PMN chemiluminescence was greater than on the chemiluminescence which resulted from stimulation of the cells by chemotactic peptide or opsonised zymosan (3.3).

Effect of human serum albumin on PMN background chemiluminescence

When human serum albumin in the range 0.02 - 8% (w/v) was incubated at 37°C for 5 min. with PMN and luminol prior to measurement of background (resting) PMN luminol chemiluminescence a dose related inhibition of chemiluminescence occurred such that 4% (w/v) albumin caused a 75 $\pm$ 2.5% (mean $\pm$ SEM, n = 6) inhibition when compared with PMN resting chemiluminescence in the absence of albumin (Fig 62).

Effect of albumin on opsonised zymosan and chemotactic peptide stimulated PMN chemiluminescence

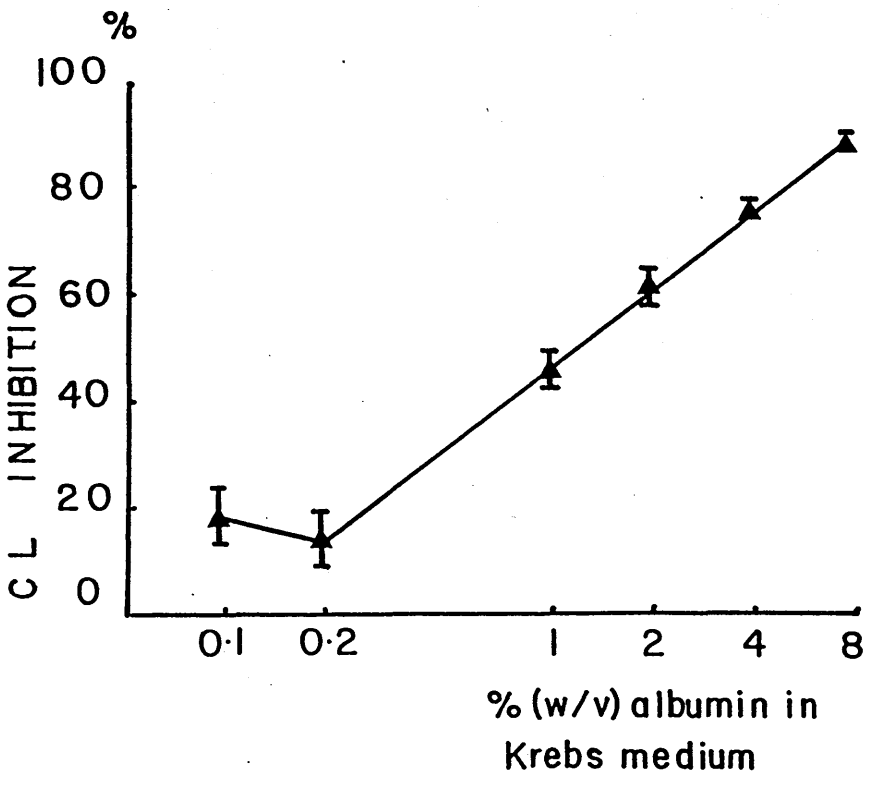


Figure 62 Effect of albumin on luminol chemiluminescence of unstimulated PMN

Normal peripheral blood PMN ($5x10^5$/ml) were incubated for 5 min at 37°C with 0-8% (w/v) human serum albumin before determination of background (unstimulated) PMN chemiluminescence. Results are expressed as the percentage by which albumin inhibited unstimulated PMN chemiluminescence (mean ± SEM, n=9). Krebs medium, pH 7.4, 37°C, 20μM luminol.

Similarly when normal blood PMN were stimulated by opsonised zymosan or chemotactic peptide in the presence of 0.5 - 4% (w/v) human serum albumin the peak height of PMN chemiluminescence was reduced (Fig 63) such that in the presence of 4% (w/v) albumin PMN chemiluminescence was only 14 ± 1% and 10 ± 1% (mean ± S.E.M., n = 3) respectively of chemiluminescence in the absence of albumin.

Effect of albumin on latex particle stimulated PMN chemiluminescence

An even larger inhibition of peak height PMN chemiluminescence occurred when PMN were stimulated by latex particles in the presence of 0.01 - 4% (w/v) human serum albumin, 0.05% (w/v) albumin causing a 74 ± 8% (mean ± S.E.M., n = 3) inhibition and 4% (w/v) albumin causing a 99.5 ± 0.1% (mean ± S.E.M., n = 3) inhibition of peak height PMN chemiluminescence.

The greater inhibition by albumin of latex particle stimulated PMN chemiluminescence when compared with its inhibition of background PMN chemiluminescnece or of opsonised zymosan/chemotactic peptide stimulated PMN chemiluminescence suggested that albumin, via protein coating of the particles, decreased the capacity of latex particles to stimulate PMN chemiluminescence.

The effect of protein coating on the capacity of latex particles to stimulate PMN luminol dependent chemiluminescence was further investigated with the aid of opsonised latex particles and IgG coated latex particles, which via stimulation of C3b and Fc PMN membrane receptors respectively would have been expected to enhance PMN chemiluminesccence; (cf effect of opsonisation on zymosan stimulated PMN chemiluminescence 3.3.2).

Latex particles ($4x10^7$/ml) which were opsonised by incubation in normal human serum at 37°C for 30 min achieved only 4.5% of the PMN

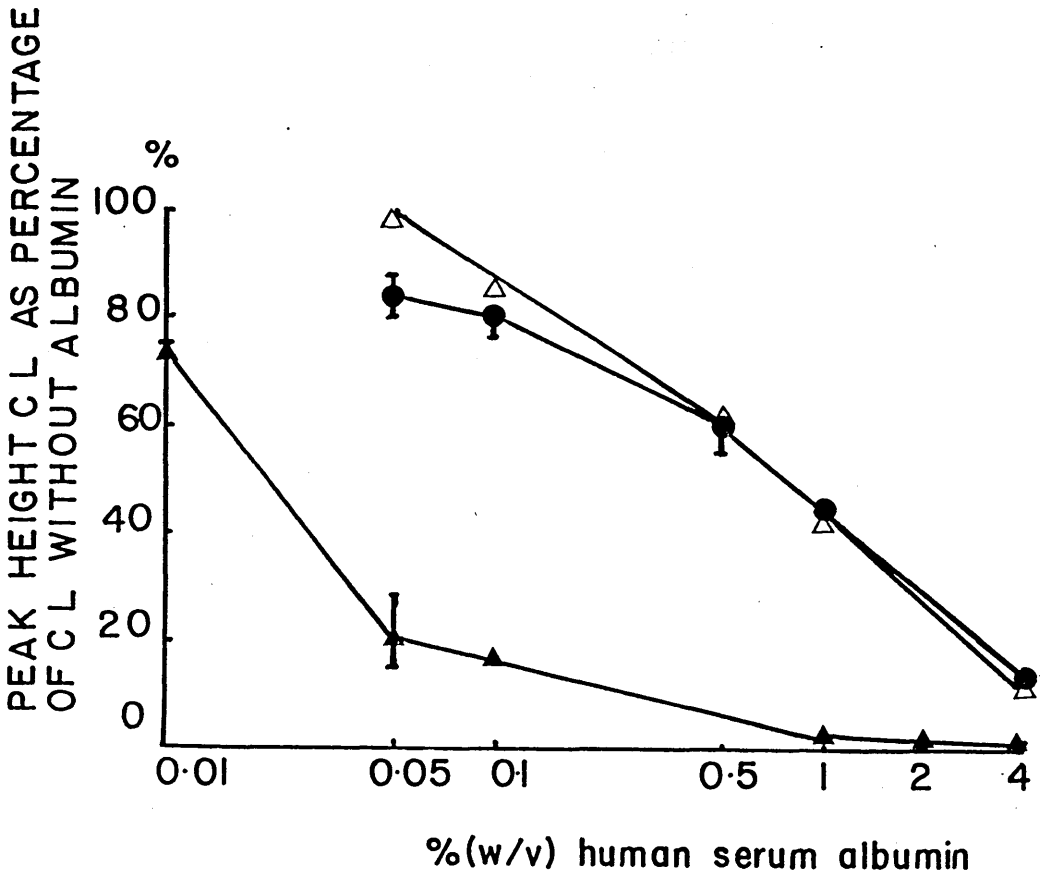


Figure 63 Effect of albumin on peak height PMN luminol chemiluminescence

Normal peripheral blood PMN ($5x10^5$/ml) were stimulated by 1mg/ml opsonised zymosan (●), 0.25μM chemotactic peptide (△), or $2x10^9$/ml latex particles (▲) in the presence of 0.01 - 4% (w/v) human serum albumin. Krebs medium pH 7.4, 37°C, 10μM luminol. The points show the peak height (maximum) chemiluminescence observed expressed as a percentage of control value obtained in the absence of albumin (means ± SEM, n=3).

chemiluminescnece stimulated by an equivalent concentration of ordinary latex particles (Table 24). Commercially prepared 1gG coated latex particles caused an even smaller chemiluminescence. The 1gG coated latex particle suspension contained sodium azide, known to be an inhibitor of PMN chemiluminescence (Hatch et al, 1978), but when an equivalent concentration was included during the stimulation of PMN by ordinary latex particles the chemiluminescence achieved was greater than twenty fold larger than that of the 1gG coated particles. 0.1% (w/v) albumin caused a 92% inhibition of the chemiluminescence provoked by ordinary latex particles at this lower concentration.

These results confirmed that the presence of low concentrations of protein in each case efffected major inhibition of the latex particle stimulated PMN luminol dependent chemiluminescence and supported the hypothesis that the greater inhibitory effect of albumin when cells were activated by this stimulus was attributable to an influence on cell stimulus interaction. For the experiments described in the remainder of this chapter only opsonised zymosan or chemotactic peptide stimuli were used.

5.3.2 Lack of effect of human serum albumin on cell viability and light transmission

Before attributing the inhibitory effect of albumin on the luminol dependent chemiluminescence of PMN, which were resting or stimulated by opsonised zymosan or chemotactic peptide, to oxygen radical scavenging, a number of other possible causes for the inhibition required exclusion (5.1). In this section the effect of human serum albumin on PMN viability and light transmission were examined.

PMN Viability

Assays of PMN lactate dehydrogenase release and of ATP content

Table 24 Comparison of effect of human serum albumin, opsonisation and IgG coating on stimulation of PMN luminol dependent chemiluminescence by latex particles

Stimulus	Additive	PMN chemiluminescence 180 seconds following addition of stimulus
$4x10^7$/ml latex particles	-	63,945 counts/s
$4x10^7$/ml latex particles	0.1%(w/v) human serum albumin	5,018 counts/s
$4x10^7$/ml opsonised latex particles	-	2,901 counts/s
3.75×10^7/ml IgG coated latex particles	0.01mg/ml sodium azide	240 counts/s
$4x10^7$/ml latex particles	0.01mg/ml sodium azide	5,668 count/s

$5x10^5$ PMN/ml Krebs medium pH 7.4, 37°C, 10μM luminol

(2.6 and 2.7) were used to determine whether the inhibition of PMN resting and stimulated chemiluminescence by albumin occurred via a decrease in cell viability (Table 25).

Lactate dehydrogenase release

The presence of 4% (w/v) albumin during PMN stimulation by opsonised zymosan did not increase the rate of lactate dehydrogenese release by PMN, instead a decrease occurred (Table 25) indicating an enhancement of cell viability ($p < 0.05$) (cf. 2.9.2).

ATP content

Albumin had no effect on the ATP content of PMN which had not been stimulated (Table 25). The ATP content of PMN which had been stimulated in the absence of albumin was 50% of that of unstimulated cells. Stimulation of PMN in the presence of 4% (w/v) albumin resulted in only a 42% drop in the ATP content when compared with unstimulated cells. In the presence of albumin cell viability was thus not impaired.

Light transmission

The spectrum of luminol chemiluminescence in aqueous medium occurs over the range 350-525nm with a maximum at 425 nm (Roswell and White, 1978). The absorption of light by 4% (w/v) human serum albumin in this range measured photometrically was found to be only 11.5% of the total emitted by luminol (Fig 64). The greater than 80% inhibitor of PMN resting and stimulated chemiluminescence in the presence of albumin was thus not explained by a reduction in light transmission by albumin.

5.3.3 Lack of effect of albumin on the luminol excited state

The possibility existed that albumin might reduce luminol

Table 25 Effect of Human Serum Albumin on PMN ATP content and lactate dehydrogenase release

Albumin 4% (w/v)	Opsonised zymosan	nmol ATP/10^7 cells	% of total lactate dehydrogenase in supernatant
-	-	18.4 ± 1.5	not assayed
+	-	18.2 ± 0.6 p>0.4 (NS)	not assayed
-	+	9.2 ± 0.5	26.3 ± 3.5%
+	+	10.7 ± 0.7 p>0.03 (N.S.)	19.1 ± 1.2% p>0.05

1.1x106 PMN/ml were allowed to rest in Krebs medium pH 7.4, 37°C or were stimulated by 1mg/ml opsonised zymosan for 5 min in the presence or absence of 4% (w/v) albumin prior to measurement of their ATP content or % release of lactate dehydrogenase to supernatant (mean ± SD, n=3)

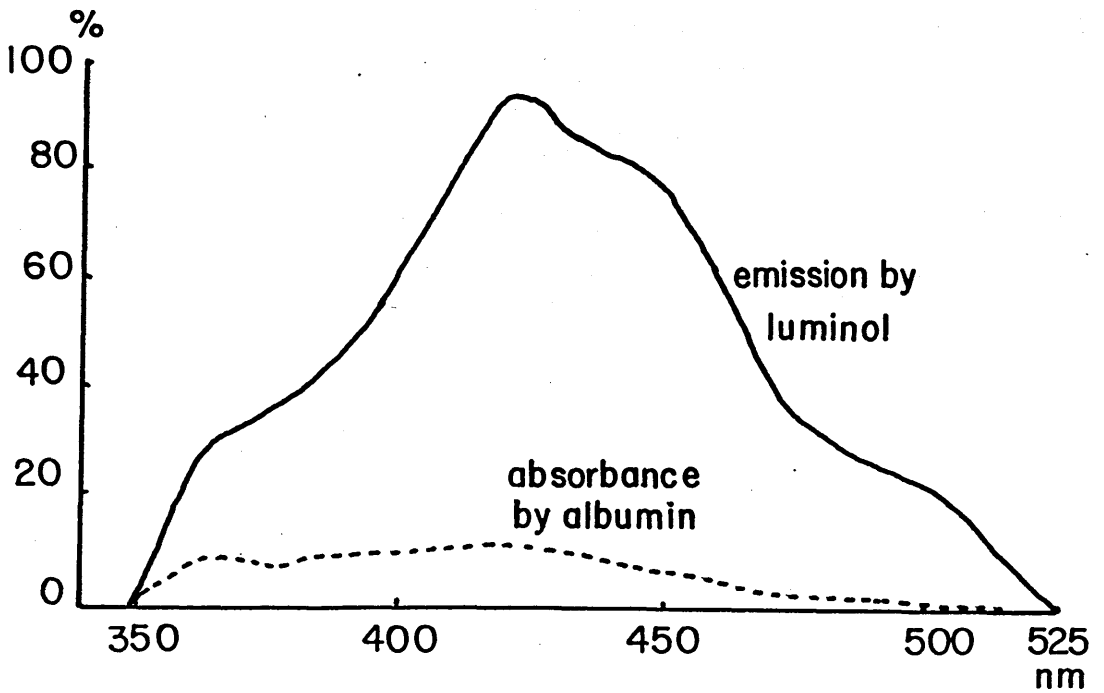


Figure 64 Comparison of light absorption by albumin and light emission by luminol

The absorbance of light by 4% (w/v) human serum albumin between 350 and 525nm was derived from observation of its effect on the transmission of light through Krebs medium pH 7.4, using a dual wavelength spectrophotometer. The % emission of light by luminol in this range was previously established (Roswell and White 1978). When the area under the dotted line (absorbance by albumin) was drawn on tissue paper and weigh ed it was found to be only 11.5% of that under the unbroken line (emission by luminol).

chemiluminescence either by quenching the excited state or by direct binding of luminol. If this were the case albumin would be expected to inhibit luminol chemiluminescence under all conditions. The effect of human serum albumin on luminol chemiluminescence was thus further investigated using two acellular systems of oxygen radical generation and by detailed examination of the effect of albumin on the time course of PMN chemiluminescence.

Effect of albumin on acellular chemiluminescence

Chemiluminescence was produced by the generation of oxygen radicals in the presence of luminol using two acellular systems namely hydrogen peroxide/microperoxidase and xanthine/xanthine oxidase.

Hydrogen peroxide/microperoxidase

Addition of 35mM H_2O_2 to 50µg/ml microperoxidase in the presence of 200pM luminol in Krebs medium pH 7.4 at room temperature resulted in a rapid increase in chemiluminescence counts/s reaching a maximum 10 seconds after addition, and falling to the background rate within 30 seconds (Fig 65(a)). Human serum albumin inhibited this chemiluminescence in a dose related manner, 0.1% (w/v) human serum albumin causing a 34·6 ± 5 % (mean ± S.E.M., n = 3) inhibition of peak height chemiluminescence. When the concentration of hydrogen peroxide was decreased one thousand fold and the concentration of luminol was increased ten thousand fold (Fig 65(b)), chemiluminescence resultant upon the addition of hydrogen peroxide reached a peak 20 seconds following addition and decayed slowly over the subsequent 3-5 minutes. In the presence of 0.1% (w/v) human serum albumin chemiluminescence was enhanced, the peak height at 20 seconds being 2.3 ± 0.16 fold (mean ± S.E.M., n =3) larger that chemiluminescence in the absence of albumin. A condition in which albumin did not inhibit luminol

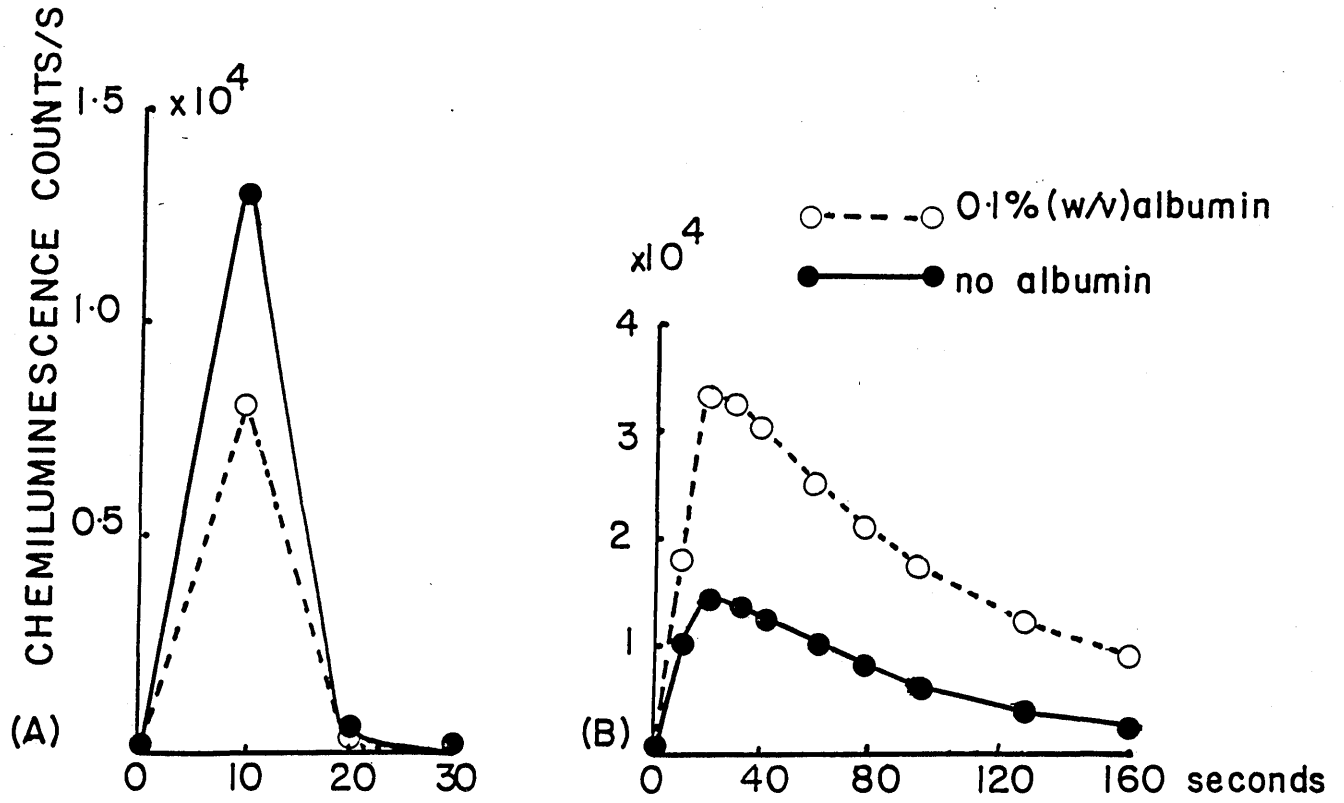


Figure 65 Effect of albumin on acellular hydrogen peroxide/microperoxidase/luminol chemiluminescence

The graphs show the effect of 0.1% (w/v) human serum albumin on the chemiluminescence which resulted from addition of H_2O_2 to luminol in the presence of microperoxidase. (A) 17.5mM H_2O_2, 25μg/ml microperoxidase, 0.2nM luminol. (B) 17.5μM H_2O_2, 25μg/ml microperoxidase, 2μM luminol. Krebs medium, pH 7.4, room temperature. Points represent means of three observations (errors too small to demonstrate).

chemiluminescence had thus been identified.

Xanthine/xanthine oxidase

The oxidation of xanthine to uric acid catalysed by xanthine oxidase is accompanied by the production of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (McCord and Fridovich 1969).

$$\text{xanthine} \xrightarrow[\text{xanthine oxidase}]{} \text{urate} + H_2O_2 + O_2^{\cdot -}$$

Addition of xanthine oxidase to xanthine in the presence of luminol (see 5.2.2) was followed by an increase in chemiluminescence counts/s reaching a maximum at 10 seconds after addition and decaying to background over the following 60 seconds. When reaction was initiated in the presence of physiological concentrations of albumin (3.9%(w/v)) a 27.5 $\pm$ 3.7% (mean $\pm$ S.E.M., n = 3) inhibition of peak height chemiluminescence occurred (Fig. 66), while at lower concentrations there was an increase, 0.5% (w/v) albumin causing a 23.2 $\pm$ 8.45 (mean $\pm$ S.E.M., n = 3) enhancement of peak height cehmiluminescence. A second circumstance in which albumin did not inhibit luminol chemiluminescence had thus been identified.

Effect of albumin on the first phase of chemotactic peptide and opsonised zymosan stimulated PMN chemiluminescence

Having demonstrated that albumin did not inhibit luminol chemiluminescence under all circumstances using acellular systems of oxygen radical generation, it was important to return to PMN luminol chemiluminescence in order to confirm that albumin was not simply a luminol excited state quencher.

Enchancement of the PMN chemiluminescence response to stimulation

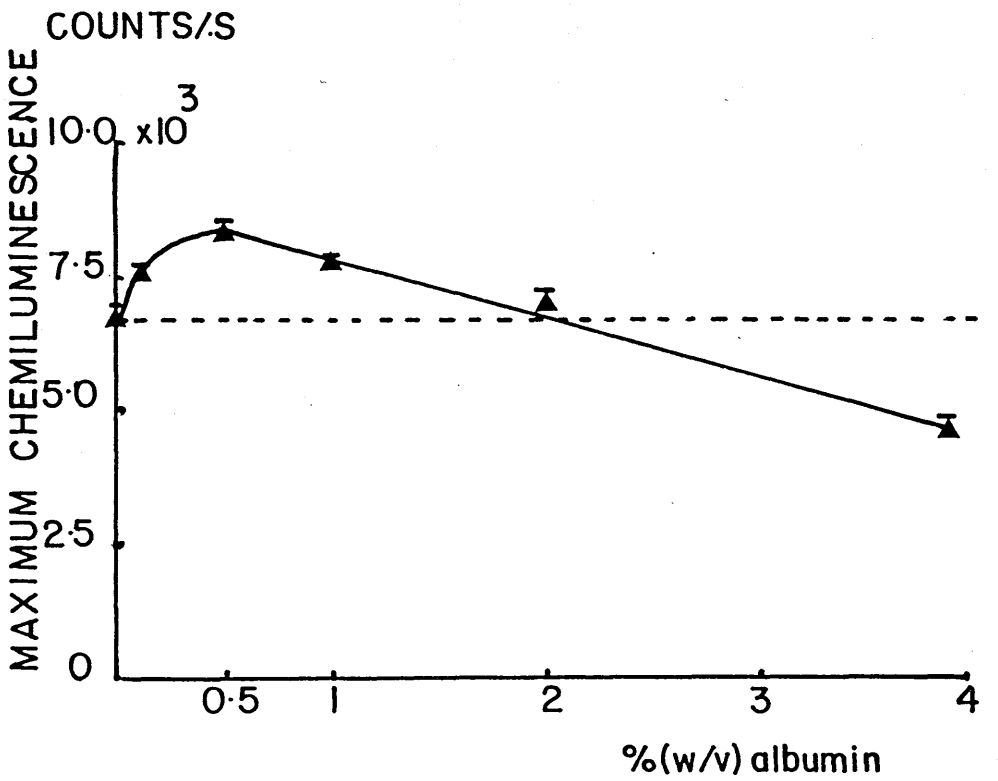


Figure 66 Effect of albumin on acellular xanthine/xanthine oxidase/luminol chemiluminescence

The dotted line represents the maximum value of chemiluminescence (mean, n=3) obtained when 0.1mM xanthine was oxidised by 0.03 units/ml xanthine oxidase in the presence of 10μM luminol. The points show the effect of 0-3.9% (w/v) human serum albumin on this chemiluminescence (mean ± SEM, n=2 or 3). Krebs medium pH 7.9, 4mM EDTA. Room temperature.

by chemotactic peptide and exaggeration of its biphasic nature by the presence of the microfiliament inhibitor cytochalasin B was previously demonstrated in 3.3.5 (Fig. 41). When PMN which had been pre-incubated with cytochalasin B and 2% (w/v) albumin were stimulated by chemotactic peptide a marked inhibition of the second phase occurred while the first phase (maximum 60s) was enhanced (Fig. 67).

Closer analysis of the effect of albumin on PMN chemiluminescence in the absence of cytochalasin B revealed that in the range 0.05 - 0.5% (w/v) albumin, an enhancement of the first phase of chemotactic peptide induced PMN chemiluminescence also occurred (cf 3.3.3) (Table 26) and while higher concentrations were inhibitory, their inhibiton was smaller than those of the second phase.

Enchancement of the first phase and inhibition of the second phase also occurred if albumin was added 20 or 50 seconds following the addition of stimulus (Fig. 68 (a) and (b)). In control experiments an equal volume of Krebs medium without albumin was added at the same time point. When albumin was added at the peak of the second phase (180s) (Fig. 66 (c)) subsequent chemiluminescence was again decreased. The percentage inhibition of the second phase of chemotactic peptide stimulated PMN luminol dependent chemiluminescence did however decrease as the time interval between addition of stimulus and addition of albumin increased. Thus when albumin (1.7% (w/v)) was present from the moment of stimulation (0s) the percentage inhibition at a fixed time in the second phase (220s) was 53%. When albumin was added at 20s, 50s, or 100s (Fig. 68) the percentage inhibition at 220s was 45%, 36% and 26% respectively.

An enhancement of the early phase of opsonised zymosan induced PMN chemiluminescence in the presence of low concentrations (0.05 - 0.5% (w/v)) of albumin was also found (Fig 69, Table 26).

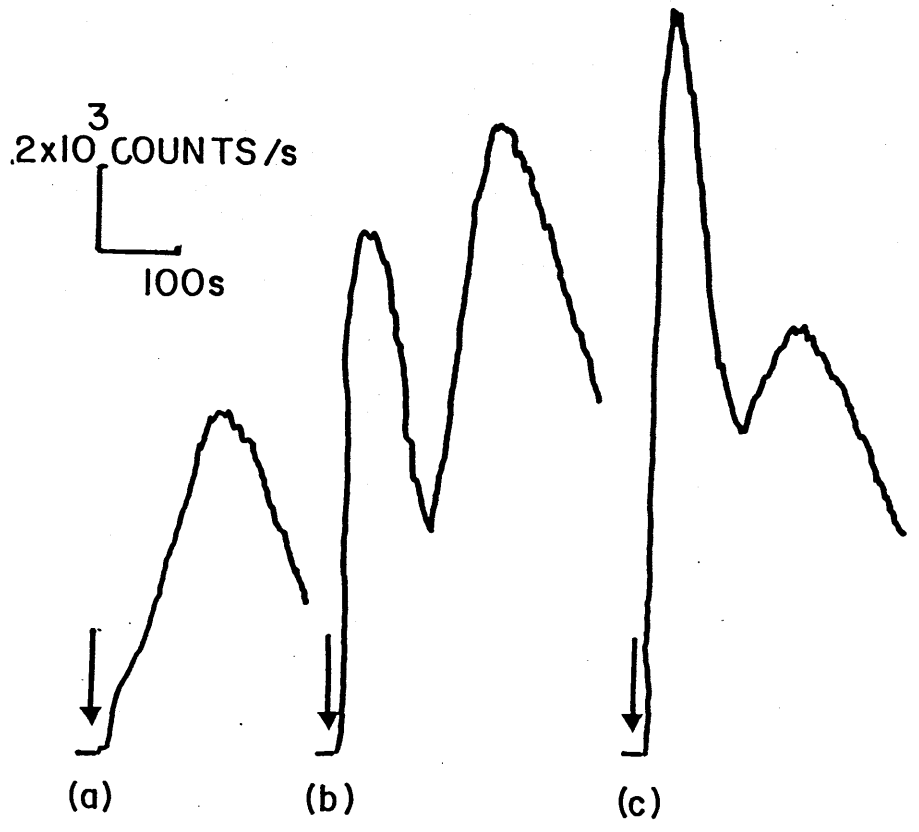


Figure 67 Effect of albumin on the luminol chemiluminescence of PMN stimulated by chemotactic peptide in the presence of cytochalasin B.

In each chemiluminescence trace 1μM chemotactic peptide was added (at arrow) to $5x10^5$/ml PMN. The usual PMN chemiluminescnece response to this stimulus is shown in (a). The response in the presence of 5μg/ml cytochalasin B is shown in (b), and the response in the presence of both cytochalasin B and 2% (w/v) albumin is shown in (c). Krebs medium, pH 7.4, 37°C, 10μM luminol.

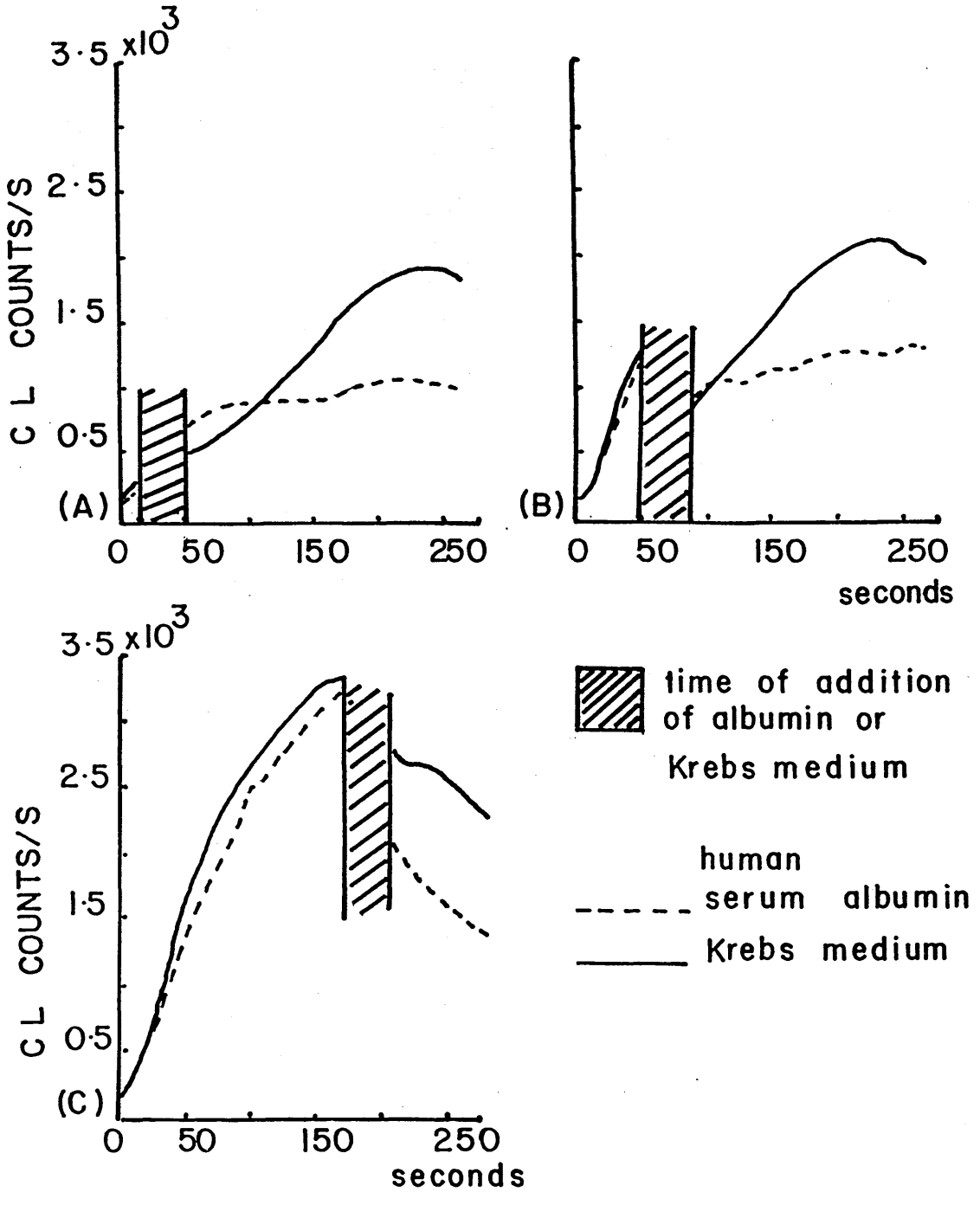


Figure 68 Effect of time of addition of albumin on its modification of chemotactic peptide stimulated PMN chemiluminescence

Chemiluminescnece traces produced by 5×10^5 PMN/ml stimulated by 0.1μM chemotactic peptide in the presence (dotted lines) and absence (unbroken lines) of 1.7% (w/v) albumin. The hatched areas indicate the time of addition of albumin or an equal volume of Krebs medium only at (A) 10s, (B) 50s and (C) 180s following addition of stimulus. Krebs medium, pH 7.4, 37°C, 10μM luminol.

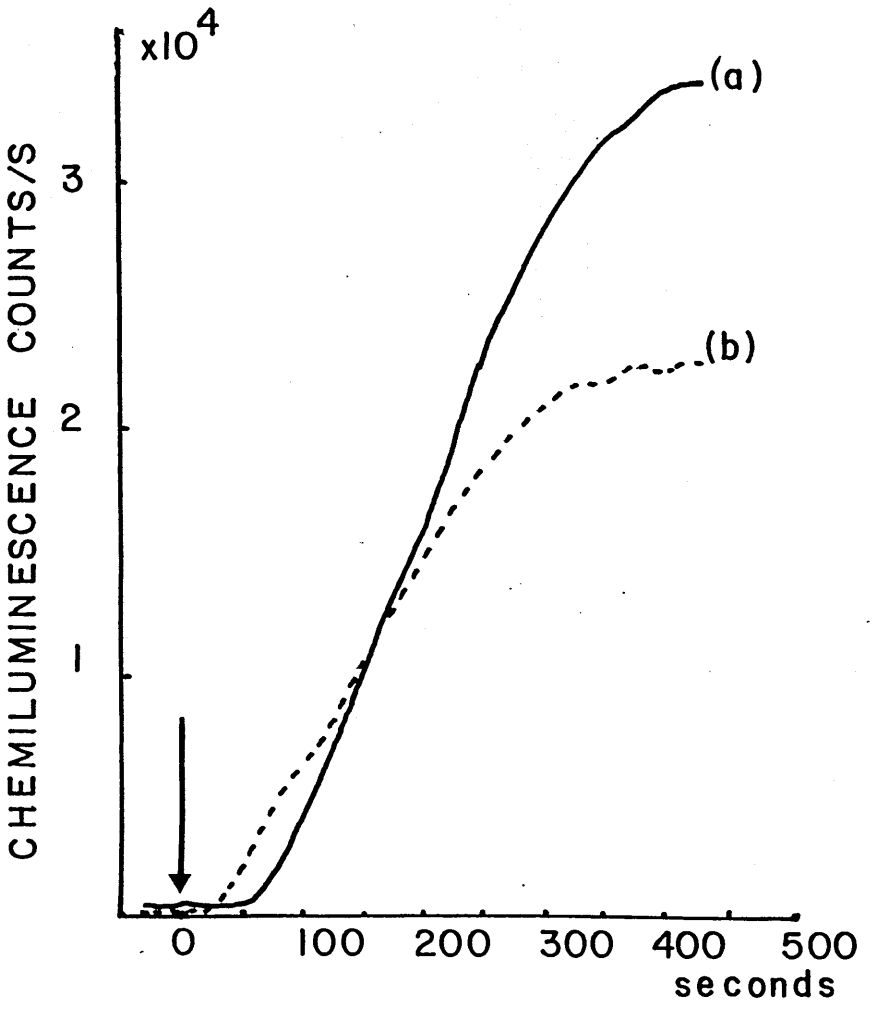


Figure 69 Effect of albumin on the early and late phases of opsonised zymosan stimulated PMN chemiluminescence

Chemiluminescence traces which resulted when 5×10^5 PMN/ml were preincubated for 5 minutes at 37°C in the absence (a) or presence (b) of 1% (w/v) albumin, before the addition (at arrow) of 1mg/ml opsonised zymosan. The final concentration of albumin in (b) was therefore 0.5% (w/v). Krebs medium, pH 7.4, 10μM luminol.

Table 26 Comparison of Effect of Albumin on 1st and 2nd phases of opsonised zymosan and Chemotactic peptide induced PMN chemiluminescence

	Opsonised Zymosan			Chemotactic Peptide		
% (w/v) albumin	1st Phase (60 sec)	2nd Phase (420 sec)	Level of significance	1st Phase (60 sec)	2nd Phase (180 sec)	Level of significance
no albumin	100%	100%	-	100%	100%	-
0.05%	101 ± 12%	84 ± 4%	p<0.3	126 ± 2%	99 ± 1%	p<0.01
0.1%	138 ± 12%	80 ± 4%	p<0.1	122 ± 9%	84 ± 3%	p<0.02
0.5%	178 ± 17%	60 ± 5%	p<0.01	104 ± 4%	61 ± 3%	p<0.01
1%	114 ± 15%	44 ± 4%	p<0.02	92 ± 4%	43 ± 1%	p<0.001
4%	48 ± 15%	14 ± 1%	p<0.01	29 ± 4%	10 ± 1%	p<0.01

5×10^5 PMN were stimulated by the addition of 1mg/ml opsonised zymosan or 0.25uM chemotactic peptide in the presence of 0-4% (w/v) albumin, 37°C, pH 7.4, 10μM luminol. The columns show PMN chemiluminescence in the presence of albumin at the indicated time points as a percentage of PMN chemiluminescence expected in the absence of albumin (means ± SEM, n=3). The effect of albumin on PMN chemiluminescence in the two phases was significantly different as indicated.

For both stimuli the second phase of the response was considerably larger and longer in duration than the first. Thus when the amount of light emitted by PMN over the complete time course of an experiment was integrated to provide a measure of total oxygen radical production an overall inhibitory effect of albumin was still found (Fig. 70). In the presence of 4% (w/v) albumin summated chemiluminescence was 17 ± 1.2% (mean ± S.E.M., n = 3) of opsonised zymosan and 15 ± 1.2% (mean ± S.E.M., n = 3) of chemotactic peptide induced chemiluminescence in the absence of albumin.

Human serum albumin was therefore shown to be capable both of inhibiting and of enhancing acellular and PMN luminol chemiluminescence. The inhibition was thus not attributable to luminol binding or excited state quenching and was likely to be attributable to direct interaction with oxygen radicals. The differential effect of albumin on the two phases of stimulated PMN chemiluminescence further suggested that a different oxygen radical population predominated in each phase. Persistence of the enhancing and inhibitory effects of albumin on the first and second phases of chemotactic peptide induced chemiluminescences when added 10 - 180s after the addition of stimulus suggested that oxygen radicals in both phases were extracellular. Furthermore, the inhibition of the second phase by albumin added at the maximum height of this phase (Fig. 68 (c)) contrasted with the lack of inhibitory effect of superoxide dismutase when added 100 seconds after stimulation of PMN by latex particles (Fig. 43, 3.3.5).

5.3.4 Effect of dialysed albumin on PMN chemiluminescence

It is well established that both intra and extra-cellular free calcium ion concentrations influence the ability of stimuli to provoke

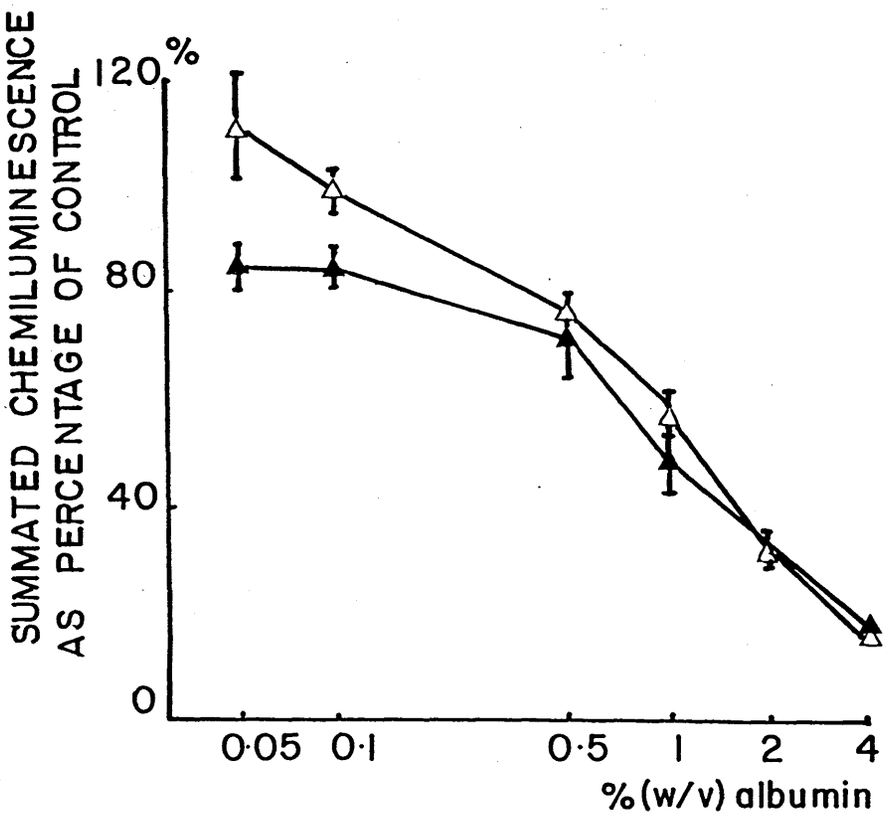


Figure 70 Effect of albumin on summated PMN chemiluminescence

$5x10^5$PMN/ml were stimulated by 1mg/ml opsonised zymosan (▲) or 0.25μM chemotactic peptide (△). Chemiluminescence was summated from time of stimulus addition to 420s (zymosan) and 300s (chemotactic peptide). Points show the effect of 0.05 - 4% (w/v) albumin on summated chemiluminescence expressed as percentage of control value obtained in the absence of albumin (means ± SEM, n=3). Krebs medium, pH 7.4, 37°C, 10μM luminol.

PMN luminol dependent chemiluminescence (1.4.2, Table 10) It is also known that albumin binds calcium ions. In order to investigate whether this property might influence the effect of albumin on PMN chemiluminescence, the effect of human serum albumin which had been extensively dialysed against Krebs medium pH 7.4 was examined (Table 27). Whereas 4% (w/v) albumin which had not been dialysed inhibited background PMN chemiluminescence by 66.2 $\pm$ 3% (mean $\pm$ S.E.M., n = 4) and peak height opsonised zymosan stimulated PMN chemiluminescence by 74.3 $\pm$ 3.7% (mean $\pm$ S.E.M., n = 3), dialysed albumin caused only 31.7 $\pm$ 7.1% and 42 $\pm$ 5.9% inhibition respectively. A similar discrepancy occurred with chemotactic peptide stimulated PMN.

When the free calcium ion concentration of both albumin preparations was measured using the calcium electrode (5.2.2) the free calcium ion concentration of undialysed 4% (w/v) albumin was 1.0mM whereas that of the dialysed 4% (w/v) albumin was 1.3mM, identical with the concentration of free calcium ions in Krebs medium alone. Using the calcium electrode the free calcium ion concentration of undialysed albumin was titrated to 1.3mM by addition of calcium chloride such that both undialysed and dialysed 4% (w/v) albumin gave identical readings in millivolts on the calcium electrode. With the calcium ion concentration corrected the inhibition of resting and opsonsed zymosan stimulated PMN chemiluminescence was reduced to that of dialysed albumin (Table 28). Furthermore the possibility that inhibition of PMN chemiluminescence by albumin was caused by a dialysable low molecular weight impurity in the albumin preparation was thus excluded.

Before characterising the inhibitory effect of albumin on PMN luminol dependent chemiluminescence further, it was now important to establish what proportion of the inhibition of PMN chemiluminescence

TABLE 27

Comparison of the effects of dialysed and undialysed human serum albumin on resting and stimulated PMN chemiluminescence

	Background % inhibited	Opsonised zymosan peak height % inhibited 1mg/ml	Chemotactic peptide peak height % inhibited 0.1μM
4% (w/v) undialysed	66.2 ± 3%	74.3 ± 3.7%	74%
4% (w/v) dialysed	31.7 ± 7.1%	42 ± 5.9%	36%
	mean ± SEM n = 4	Mean ± SEM n = 3	Single observations

5x105 PMN/ml were resting, or stimuated by 1mg/ml opsonised zymosan or 0.1μM chemotactic peptide in the presence of 4% (w/v) albumin which was either undialysed or had been dialysed against Krebs medium pH 7.4 for 48 hours 37oC, 10μM luminol.

Table 28 - Effect of correction of calcium ion concentration on inhibition by albumin of background PMN chemiluminescence and maximum peak height opsonised zymosan stimulated PMN chemiluminescence

	4% (w/v) dialysed albumin	4% (w/v) undialysed albumin	4% (w/v) undialysed albumin with calcium concentration corrected
Background chemiluminescence % inhibition	37 ± 22%	68 ± 4%	37 ± 14.5%
Peak height (2nd phase) chemiluminescence % inhibition	36 ± 9.2%	55 ± 1%	40 ± 7%

$5x10^5$PMN/ml were resting, or were stimulated by 1mg/ml opsonised zymosan in the presence of 4% (w/v) albumin which was either (a) fully dialysed against Krebs medium pH 7.4 or (b) undialysed or (c) undialysed but with free calcium ion concentration corrected to that of native Krebs medium. The chemiluminescence observed was expressed as a % of that obtained in the absence of albumin (means ± SEM, n=3) 37°C. 10μM luminol.

caused by serum, and persisting following dialysis of the serum (4.3.4), could be accounted for by albumin which had been dialysed extensively against Krebs medium pH 7.4 such that the free calcium concentration had re-equilibrated to 1.3mM. The inhibition of background and opsonised zymosan stimulated PMN luminol dependent chemiluminescence caused by 39% (v/v) normal human serum which had been heated at 56°C for 20 min to inactivate complement and dialysed against Krebs medium pH 7.4 was then compared with that caused by dialysed human serum albumin of a concentration calculated to be equivalent to the concentration of albumin in the diluted serum sample (Table 29).

All of the inhibition of peak height opsonised zymosan stimulated PMN chemiluminescence could thus be accounted for by albumin. Background PMN chemiluminescence was as usual observed with PMN suspended in 500µl Krebs medium containing the diluted serum or albumin. The final concentration of serum was thus 78% (v/v) and of albumin was 3.2% (w/v). The difference in percentage inhibition at these concentrations (apparent greater inhibition by albumin) was not significant.

5.3.5 Effect of albumin on PMN oxygen consumption and-cytochrome c reduction

It had thus been shown that all of the inhibitory effect of dialysed serum on PMN luminol dependent chemiluminescence was attributable to albumin. As 70% of the inhibitory effect of serum persisted following dialysis (4.3.3), albumin was confirmed as the major cause of its inhibition. The inhibitory effects of albumin could not be explained by decreased cell viability, a reduction in light transmission, low or high molecular weight contaminants in the albumin preparation or quenching of the luminol excited state. Part

Table 29 - Comparison of inhibition of background and peak height opsonised zymosan stimulated PMN chemiluminescence by dialysed whole (heat treated) serum and dialysed human serum albumin

	39% (v/v) dialysed heat treated serum	1.6% (w/v) dialysed albumin	p value
Background chemiluminescence % inhibition	28.7 ± 12.3%	42 ± 11.5%	p > 0.1 (NS)
Peak height (2nd phase) % inhibition	53 ± 5.6%	53.3 ± 5.8%	p > 0.05

5x10^{5}PMN/ml were resting, or stimulated by 1mg/ml opsonised zymosan in the presence either of 39% (v/v) heat treated serum or 1.6% (w/v) human serum albumin, both of which had been dialysed against Krebs medium pH 7.4 for 48 hours. Chemiluminescence is expressed as a % of the value expected in the absence of serum or albumin (means ± SEM, n=3). The effect of dialysed serum and albumin in these concentrations was not significantly different (student's t test).

of the inhibition by albumin of latex particle induced PMN chemiluminescence was explained by protein coating of the particles leading to reduced stimulatory capacity (Table 23, 5.3.1). Whilst interference with stimulus and cell interaction could not be entirely ruled out for chemotactic peptide and opsonised zymosan, the comparable inhibitory effect of albumin on resting and stimulated PMN chemiluminescence, taken together with the enhancement of the early phase effected by low concentrations of albumin indicated that the overall decrease in PMN luminol dependent chemiluminescence (Fig. 70) was not attributable to impaired activation of the cells by these stimuli. The most likely explanation for this inhibition was thus modification of the oxygen radical generating pathway by albumin via oxygen radical scavenging.

In order to provide direct evidence of modification of the PMN oxygen radical generating pathway by albumin, the effect of albumin on stimulated PMN oxygen consumption and cytochrome c reduction was examined.

Oxygen consumption

It was predicted that if the inhibition by albumin of PMN opsonised zymosan and chemotactic peptide stimulated chemiluminescence was not mediated by modification of the cell stimulus interaction, the presence of albumin would not affect the rate of oxygen consumption of PMN exposed to the stimuli. The large diameter of the zymosan particles and hence the larger diameter needle required for their injection caused difficulties in the standardisation of stimulus addition and mixing. Experiments were therefore carried out using only the chemotactic peptide stimulus.

In the presence of a low concentration of albumin (0.1% (w/v)),

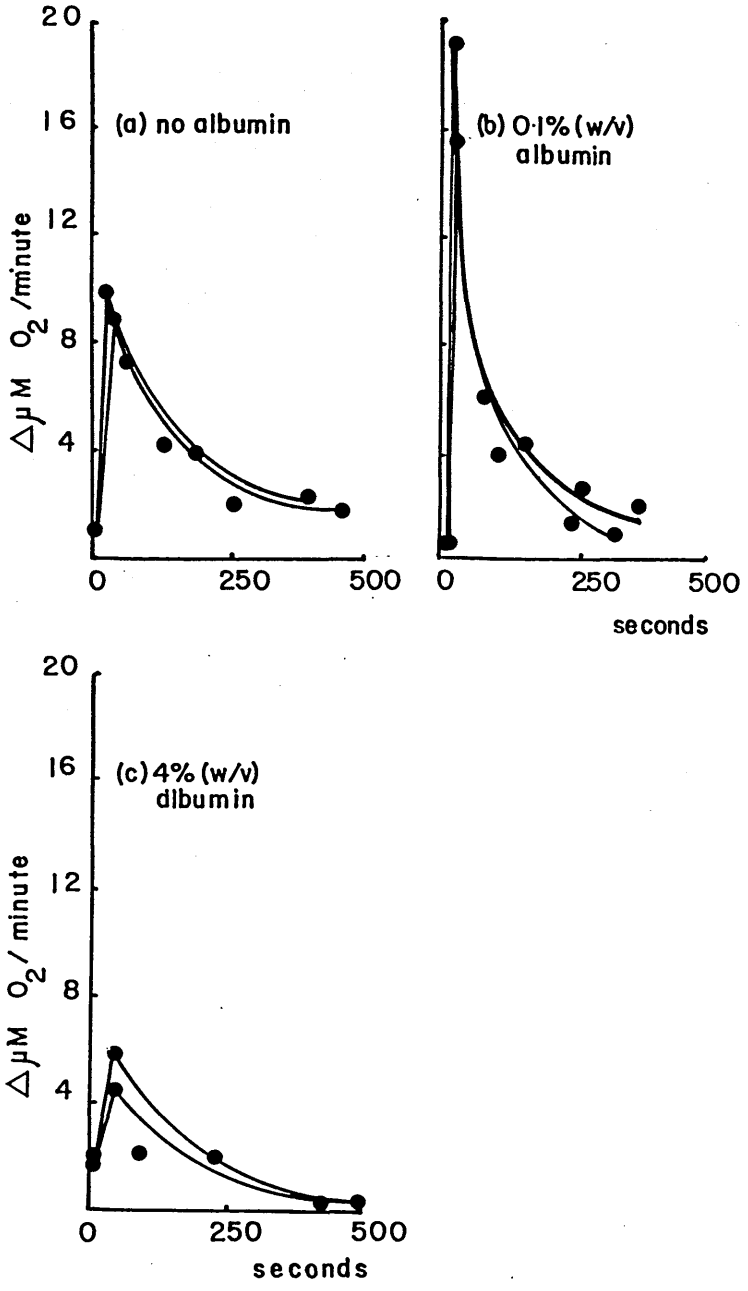


Figure 71 Effect of albumin on PMN oxygen consumption

The oxygen consumption of 5×10^6 PMN/ml was measured following the addition (at time 0 seconds) of 1μM chemotactic peptide. Krebs medium pH 7.4, human serum albumin 0-4%(w/v) as shown (a) - (c) 37°C,

Fig 71(b) the maximum rate of oxygen consumption, which occurred at 18-21 seconds was increased by 75%. In the presence of 4% (w/v) albumin the maximum rate of oxygen consumption, delayed to 30 and 42 sec was decreased by a mean of 47% (Fig 71(c)) when compared with the oxygen consumption of PMN stimulated by chemotactic peptide in the absence of albumin (Fig 71(a)).

The maximum rate of oxygen consumption of PMN stimulated by chemotactic peptide was temporally related to the peak height of the first phase of chemotactic peptide stimulated PMN chemiluminescence (Fig 33, 3.3.3). Increase of the maximum rate of oxygen consumption in the presence of a low concentration of albumin appeared similarly to correspond with the enhancement of the first phase of chemotactic peptide induced luminol chemiluminescence caused by 0.05 - 0.5% (w/v) albumin (Table 25, 5.3.3). If this increase was explained by a greater efficiency in the carriage of chemotactic peptide to the cell membrane by albumin, the inhibition of oxygen consumption in the presence of 4% (w/v) albumin was either explained by modification of the oxygen radical generating pathway by albumin with return of native oxygen to the environment and hence a net reduction in oxygen consumption (Roos et al 1977) or by an effect if high concentrations of protein on the efficiency of the oxygen electrode membrane (Davies 1962).

Cytochrome c reduction

The ability of superoxide anion ($O_2^{\cdot -}$) to reduce cytochrome c (McCord and Fridovich, 1969) has been employed as a monitor of superoxide anion production by phagocytosing PMN (Babior et al, 1973), the reduction of cytochrome c following exposure to the PMN being inhibitable by superoxide dismutase. If the inhibition of luminol dependent PMN chemiluminescence occurred via scavenging of

superoxide anion by albumin a similar effect of albumin on the reduction of cytochrome c by PMN would be expected to occur.

Incubation of 20μM ferricytochrome c with resting PMN for 20 minutes at 37°C resulted in a small reduction of the cytochrome as shown by the absorption at 550nm in spectral scan (d) when compared with the scan of cytochrome c alone (a) Fig. 72 (Table 30). When the cells were stimulated by opsonised zymosan the absorption at 550nm was greatly increased (Fig. 72(e)), the magnitude of the absorption being unaffected by the presence of 4% (w/v) human serum albumin (Fig 72(f), Table 29). Incubation of 4% (w/v) albumin and 20μM cytochrome c alone however also resulted in a small reduction of the cytochrome manifested by an increased absorption at 550nm (Fig. 72(b), Table 29) and the absorption at 550nm of 20μM cytochrome c exposed to both unstimulated cells and albumin was greater than that of the cytochrome exposed to cells alone (Fig. 72(c), Table 29). The increase in absorption at 550nm following stimulation of the cells by opsonised zymosan in the presence of 4% (w/v) albumin was thus only 78% of that which occurred in the absence of albumin (Table 29), thus indicating an inhibitory effect of albumin on cytochrome c reduction by phagocytosing PMN.

This ability of albumin to cause direct reduction of ferricytochrome c (Table 30 (a) and (b)) was further examined using human serum albumin, bovine serum albumin, dog serum albumin and bovine serum albumin which had been exposed to a metal chelating agent (Fig. 73(a)). In each case a dose related reduction of cytochrome c occurred after exposure to the albumin preparation for a mean of 50 ± 5.6 minutes (mean ± S.E.M., n = 32), the greatest reduction occurring with the bovine serum albumin which had been exposed to a chelating agent. No significant absorption occurred at 550nm for 4% (w/v) human

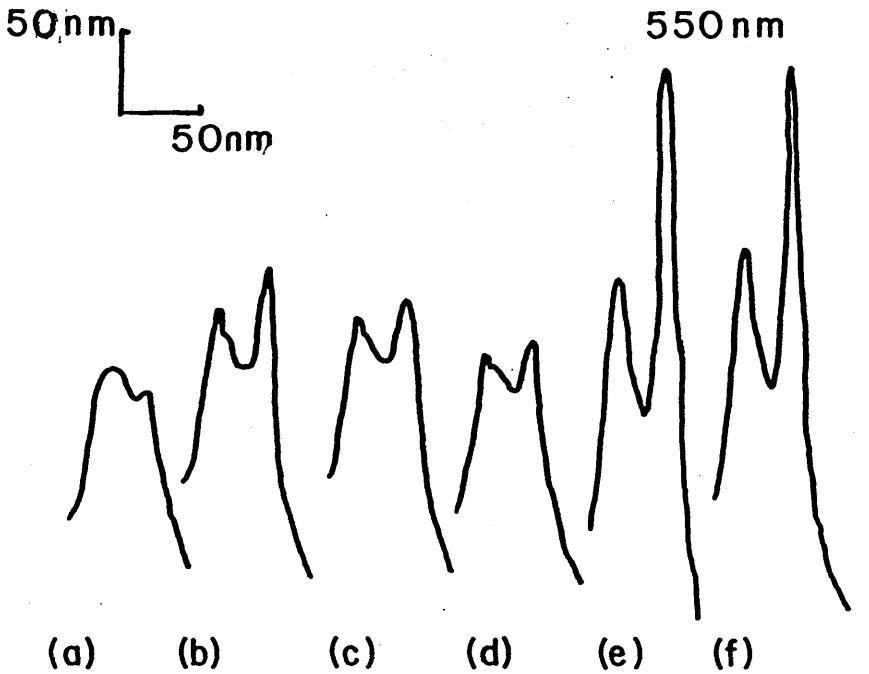


Figure 72 Effect of albumin on the reduction of cytochrome c by PMN

The traces are the absorption spectra of 20μM ferricytochrome c measured in a scanning spectrophotometer between 565 and 440nm (cf Fig 61). The cytochrome had previously been incubated at 37°C in Krebs medium for 20 minutes as follows:-

(a) ferricytochrome c alone
(b) + 4% (w/v) human serum albumin (HSA)
(c) + 4% (w/v) HSA, $2x10^6$ PMN/ml
(d) + $2x10^6$ PMN/ml
(e) + $2x10^6$ PMN/ml, 2mg/ml opsonised zymosan
(f) + $2x10^6$ PMN/ml, 2mg/ml opsonised zymosan, 4% (w/v) HSA.

and then centrifuged at 10,000g for 50 seconds to remove cells and particles, before the absorption was measured. The amount of reduced (ferro)cytochrome c in each was denoted by the height of the absorption peak at 550nm (cf Fig 61).

Table 30

	Mean absorption 550nM ± SEM (n = 2)	µM cytochrome c reduced (ΔEmM = 15.5mM)	Legend
(a)	0.108 ± 0.003	3.0µM	reduction of cytochrome c by 4% (w/v) albumin alone (no cells).
(b)	0.154 ± 0.001		
(d)	0.134 ± 0.003	8.3µM	reduction of cytochrome c by stimulated PMN (no albumin)
(e)	0.262 ± 0.001		
(c)	0.165 ± 0.001	6.5µM	reduction of cytochrome c by stimulated PMN (4% (w/v) albumin)
(f)	0.266 ± 0.006		

The reduction of cytochrome c by albumin and PMN

The reduction of 20µM cytochrome c achieved either by 4% (w/v) albumin alone (b)-(a)

or by

$2x10^6$PMN/ml stimulated by 2mg/ml opsonised zymosan ((e) - (d))

or by

$2x10^6$PMN/ml stimulated by 2mg/ml opsonised zymosan in the presence of 4% (w/v) albumin ((f) -(c))

The cytochrome was exposed to each condition at 37°C for 20 mins after which reaction was terminated by centrifugation

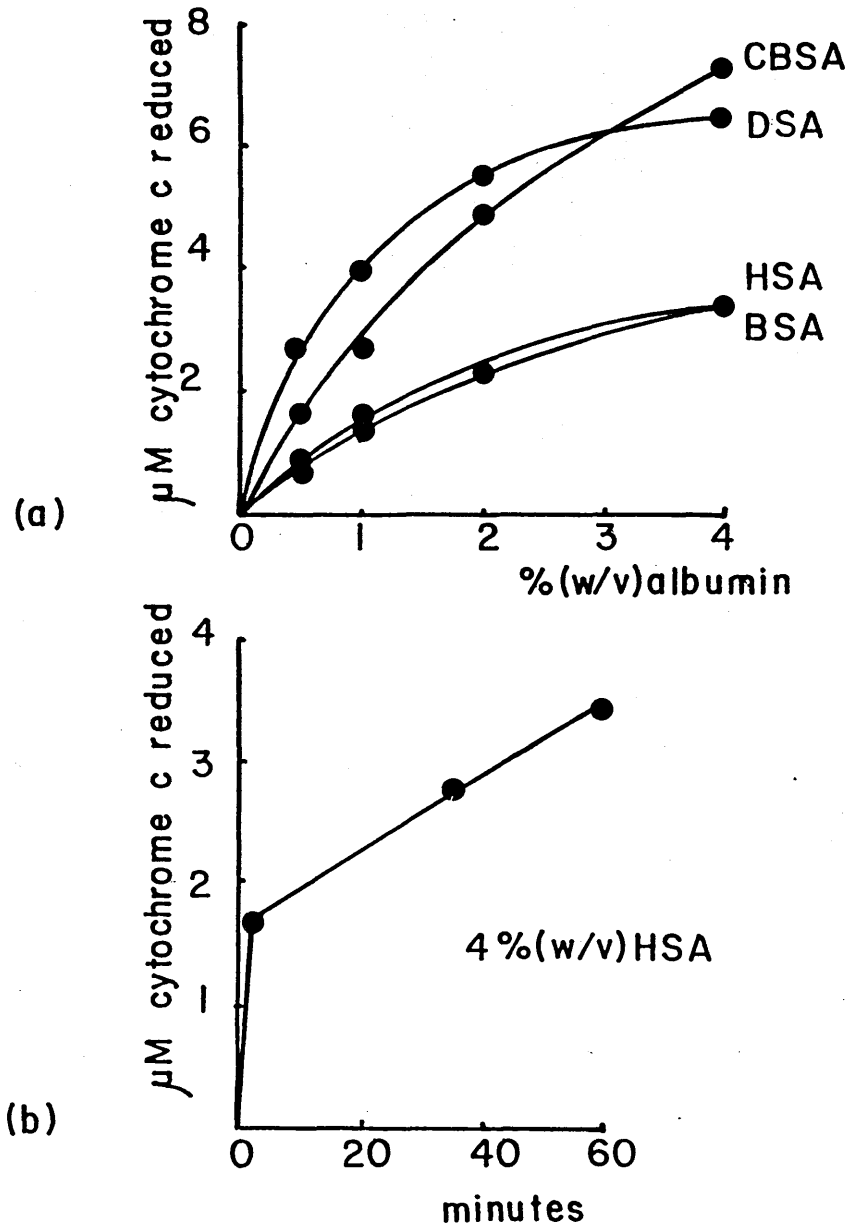


Figure 73 Reduction of cytochrome c by albumin (in the absence of PMN)

(a) 20uM ferricytochrome c was mixed with 0.5-4% (w/v) albumin. The amount of reduced (ferro)cytochrome c which resulted in each condition was measured in random order 40-100minutes following the additions, by measurement of the absorption at 550nm (albumin blanks subtracted). Four varieties of albumin were used:- BSA (bovine serum albumin) HSA (human serum albumin), DSA (dog serum albumin), CBSA (chelox treated bovine serum albumin - Ca^{2+} < 5nmoles/g), Krebs medium, pH 7.4, room temperature.

(b) This graph shows the rate of appearance of reduced (ferro) cytochrome c following the addition to 20uM ferricytochrome c of 4% (w/v) HSA at time 0 minutes. Albumin blanks subtracted. Krebs medium, pH 7.4, room temperature.

or bovine serum albumin in the absence of cytochrome c (range -0.001 to - 0.006), while for dog serum albumin absorption in the range (-0.014 to + 0.126) occurred for 0.5% - 4% (w/v) albumin and figures were therefore adjusted prior to the calculation of μM cytochrome c reduced.

The rate of reduction of ferricytochrome c by 4% (w/v) human serum albumin at room temperature was also examined (Fig. 73 (b)). Greater than 50% of the reduction achieved in 60 minutes occurred in the first two minutes.

Thus albumin was shown not only to decrease the rate of reduction of cytochrome c by phagocytosing PMN but also to be capable of reducing cytochrome c directly. Direct reduction of cytochrome c by albumin was enhanced by the prior removal of cations from the albumin. These observations were unified by the hypothesis that albumin was able to donate or receive electrons and thus adopt the role of an oxidoreducing or redox agent and thereby act as an oxygen radical scavenger.

5.4 Discussion

The experiments reported in this chapter identified a new role for albumin as an oxygen radical scavenger, firstly by showing that its potent inhibitory effect on PMN luminol dependent chemiluminescence could not be fully explained on any other basis, and secondly by providing evidence for the modification of oxygen radical generating pathways by albumin in cellular and acellular systems.

Luminol dependent chemiluminescence of PMN, which were resting or stimulated by latex particles, opsonised zymosan or the chemotactic peptide n formyl met leu phe, was inhibited in a dose dependent manner by increasing concentrations of human serum albumin such that physiological concentrations of albumin achieved greater than 80%

inhibition (Figs 62, 63 and 70, Table 26). The inhibition was most apparent in the second phase of the chemiluminescence response, the first phase being enhanced by albumin under certain conditions (Fig. 69, Table 26). The biphasic nature of the chemotactic peptide induced luminol dependent chemiluminescence, and the dual effect of albumin was even more marked in the presence of cytochalasin B (Fig 67).

Inhibition of opsonised zymosan stimulated PMN luminol dependent chemiluminescence by bovine serum albumin (0.1 - 2% (w/v)) was also observed by Andersen and Amirault (1979) and by Hastings et al (1982). Both groups discounted interference by albumin in light transmission as a mechanism, but neither had investigated the phenomenon further. Yanai and Quie (1981) demonstrated that albumin inhibited the chemiluminescence of PMN adhering to surfaces.

In the experiments reported in this chapter the inhibitory effects of albumin could not be explained by decreased cell viability, quenching of luminol excited state or a reduction in light transmission. Part of the inhibition by albumin of latex particle induced PMN chemiluminescene was explained by protein coating of the particles leading to reduced stimulatory capacity.

Whilst interference with stimulus and cell interaction could not be entirely ruled out for chemotactic peptide and opsonised zymosan, the comparable inhibitory effect of albumin on resting (unstimulated) PMN chemiluminescence, taken together with the enhancement of the early phase effected by low concentrations of albumin indicated that the overall decrease in PMN luminol dependent chemiluminescence (Fig 70) was not attributable to impairment of activation of the cells by these stimuli. Modification of the oxygen radical generating pathway by albumin via oxygen radical scavenging thus remained the most likely explanation for its inhibition of PMN chemiluminescence.

Superoxide anion and myeloperoxidase dependent mechanisms have been implicated in both opsonised zymosan and chemotactic peptide induced chemiluminescence (1.4.2, 3.4.) (Rosen and Klebanoff 1976, Hatch et al 1978). The differential effect of albumin on the two phases of the stimulated PMN chemiluminescence response further suggested that a different oxygen radical population predominates in each phase. A prediction that oxygen radicals in the first phase are related to the presence of $O_2^{\cdot -}$ and H_2O_2 (or products of their reaction $OH^{\cdot}$ and $^1\Delta_g O_2$) may be made from the observed similarlity of the enhancing and inhibitory effects of albumin on this phase and on the chemiluminescence resulting from the xanthine oxidase catalysed oxidation of xanthine in the presence of luminol (Table 26, Fig 64).

Persistence of an inhibitory effect of albumin on the second phase of chemotactic peptide induced PMN chemiluminescence, irrespective of its time of addition (Fig 68) similarity allowed the conclusion that the second phase oxygen radical pool is also readily accessible to albumin and is therefore likely to be extracellular (cf 3.3.5). These observations contrast with those of Briheim, Stendahl and Dahlgren (1984) published subsequently. Unfortunately performance by this group of their experiments at 22°C so distorted the time courses of their chemiluminescence responses that meaningful comparison of results is not possible.

Some evidence for the dependence of the second phase of chemotactic peptide induced PMN chemiluminescence on myeloperoxidase catalysed reactions was provided in this chapter by demonstration of an inhibitory effect of albumin on chemiluminescence resulting from reaction of the synthetic microperoxidase and hydrogen peroxide in high concentration in the presence of luminol (Fig 65(a)). The enhancing effect of albumin on chemiluminescence produced by this

system in the presence of low concentrations of H_2O_2 however provided yet another circumstance in which albumin was shown to be capable of both increasing and decreasing the magnitude of luminol dependent chemiluminescence.

The relative concentrations of reactive oxygen metabolites, luminol and albumin are likely to have determined not only the efficiency but also the mechanism by which luminol was dioxygenated or peroxidised (1.4.2). Hodgson and Fridovich have shown that superoxide dismutase can both inhibit xanthine/xanthine oxidase luminol chemiluminescence (by accelerating removal of $O_2^{\bullet -}$ from the oxygen radical pool by dismutation) or in fact enhance the chemiluminescence by directly catalysing the peroxidation of luminol (Hodgson and Fridovich 1975). Misra and Squatrito (1982) attributed the enhancing effect of known hydroxyl radical scavengers on horse radish peroxidase/H_2O_2/luminol chemiluminescence to removal of $OH^{\bullet}$ from the pool thereby preventing its reaction with $O_2^{\bullet -}$ and freeing more of the latter for the dioxygenation of luminol (1.4.2).

While high albumin concentrations are likely to have interfered with the performance of the oxygen electrode membrane (Fig 71) (Davies 1962), the similarity in effect of low and high concentrations of albumin on the maximum rate of PMN oxygen consumption and on the temporally related first phase of PMN chemiluminescence provided further evidence for modification of the oxygen radical generating pathway (or scavenging) by albumin. Oxygen consumption by stimulated PMN cannot be accurately measured in the presence of a scavenger which is returning O_2 to the environment (Roos et al 1977).

Albumin minimally inhibited the reduction of cytochrome c by stimulated PMN (Fig 72) (attributed to $O_2^{\bullet -}$). Scrutiny of the effect of albumin on cytochrome c in the absence of cells however led to the

novel observation that albumin was itself able to reduce cytochrome c in a dose and time dependent manner, the magnitude of which was increased by prior treatment of the albumin by a metal chelating agent (Fig 71). Albumin was thus able to participate in oxidation/reduction reactions and did so more efficiently following the removal of metal ions. Cytochrome c is known to be reduced by thiol groups such as reduced glutathione (Jocelyn 1972). The free thiol group on albumin (Peters 1975) may similarly be responsible for its reduction of cytochrome c. PMN chemiluminescence by dialysed albumin when compared to that of undialysed albumin (Table 28) merely reflected a beneficial effect of restoration of extracellular free calcium ions (Campbell and Hallett 1983, Williams and Cole 1981) or was mediated by binding of divalent cations to the protein during dialysis thereby shielding its reactive groups from later encounter with oxygen radicals.

Comparison of the effects of dialysed albumin and dialysed whole serum on resting and stimulated PMN luminol chemiluminescence showed that the former accounted for all of the inhibition caused by the latter (Table 29) and thus at least 70% of the inhibition of chemiluminescence effected by undialysed serum (Table 23, 4.3.3).

Albumin had hitherto been assigned several physiological roles including maintainance of blood osmotic pressure, fatty acid transport, sequestration and transportation of bilirubin and conveyance of certain amino acids and hormones (Peters 1975). Oxygen radical scavenging had not previously been demonstrated. Further examination of this property in the major extracellular protein was therefore required if the role of reactive oxygen metabolites in the pathogenesis of rheumatoid arthritis was to be established.

5.5 Summary

The potent inhibitory effect of normal serum on PMN luminol chemiluminescence was further investigated and was found to be entirely attributable to albumin. The major share of the inhibitory effects of albumin on PMN luminol chemiluminescence could in turn only be explained by oxygen radical scavenging and a new role for albumin was thus identified:-

(a) Luminol chemiluminescence of normal PMN which were resting or stimulated by latex beads, opsonised zymosan or the chemotactic peptide n formyl met leu phe was inhibited in a dose dependent manner such that physiological concentrations of albumin achieved greater than 80% inhibition.

(b) The inhibitory effects of albumin could not be explained by decreased cell viability, quenching of luminol excited state or a reduction in light transmission. Part of the inhibition by albumin of latex particle induced PMN chemiluminescence was explained by protein coating of the particles leading to decreased stimulating capacity.

(c) The inhibitory effect of albumin was most apparent in the second phase of chemotactic peptide and opsonised zymosan stimulated chemiluminescence. Albumin actually enhanced the first phase of stimulated PMN luminol chemiluminescence and luminol chemiluminescence resulting from two acellular radical generating systems. Low concentration of albumin also increased oxygen consumption by chemotactic peptide stimulated PMN.

(d) Direct evidence for albumin's capability as an oxidoreductant was provided by the observation of its direct reducing effect on cytochrome c, the magnitude of which was increased by prior treatment of albumin with a metal chelator.

Chapter 6

Investigation of the mechanism by which albumin scavenges oxygen radicals: the role of thiol group oxidation

6.1 Introduction

6.2 Methods

6.2.1 General

6.2.2 Assay for thiol groups.

6.2.3 Assay for protein concentrations in synovial fluid.

6.3 Results

6.3.1 Oxidation of free and protein bound thiol groups exposed to PMN.

6.3.2 Comparison of the effects of reduced glutathione and albumin on PMN luminol chemiluminescence.

6.3.3 Serum and synovial fluid thiol levels in patients with rheumatoid arthritis.

6.4 Discussion

6.5 Summary

6.1 Introduction

In the previous chapter the predominantly inhibitory effect of albumin on luminol dependent chemiluminescence was used to identify this major extracellular protein as a scavenger of oxygen radicals. Furthermore, albumin was found to be responsible for the major share of the potent inhibitory effect of normal human serum on PMN luminol dependent chemiluminescence.

Scavenging of an oxygen radical implies a 'termination reaction' resulting in the removal of an oxygen radical from the propagating pool (Del Maestro 1980). Molecules which act as scavengers must themselves be structurally altered by the termination reaction, however transiently. Conclusive proof of albumin's role as an oxygen radical scavenger would therefore only be provided by the demonstration of modification of its structure by exposure to oxygen radicals and other reactive oxygen metabolites in vitro and in vivo.

The protein, human serum albumin, molecular weight 69,000 (Cohn et al 1947), is composed of 610 peptides, comprising 19 different amino acids. Potential sites for oxygen radical reaction (and thus scavenging by albumin) include the amino groups, tryptophan, histidine and tyrosine residues and the lone thiol (-SH) group on cysteine in the thirty fourth position (cf 1.2.3). Lipids transported by albumin and accounting for 0.3% of its weight (Schultze and Heremans 1966) may also contribute to albumin's scavenging capability by undergoing peroxidation (Petrone et al 1980) (1.2.3).

Arrangement of albumin's polypeptide chain into a convoluted structure of 4 Svedberg units determined by intramolecular disulphide bonds (Schultse and Heremans 1966) is likely to limit the availability of some of the above mentioned sites for oxygen radical reaction. While methodical examination of each possible scavenging site for

evidence of modification following exposure to oxygen radicals was required, constraints of time during the work of this thesis demanded the selection of only one site.

Attention was directed to the lone thiol group whose potential for oxidation to disulphides, sulphenic or sulphonic acids (Jocelyn 1970) may have accounted for the ability of albumin to gain an electron in the reduction of cytochrome c demonstrated in the last chapter (5.3.5). Further, albumin is the major source of thiol group in serum (Jocelyn 1972) and supporting evidence for involvement of thiol group oxidation in the pathogenesis of rheumatoid arthritis has been afforded by the observations by Lorber (1964) and Thomas and Evans (1975) of low serum thiol levels in patients with active disese. Serum thiol levels had also been shown to rise in patients undergoing improvement in disease activity following treatment with the thiol containing D-penicillamine (Dixon et al 1980).

The aim of the experiments described in this chapter was therefore to determine whether exposure of albumin to oxygen radicals generated by stimulated PMN in vitro and in vivo within the synovial fluid and whole blood of patients with rheumatoid arthritis, resulted in oxidation of the lone thiol group.

6.2 Methods

6.2.1 General Methods

For luminol dependent chemiluminescence and thiol oxidation experiments, PMN were isolated from normal whole blood as previously described (2.9.1). Opsonised zymosan was prepared, and chemotactic peptide and luminol were diluted in Krebs medium from stock solutions in D.M.S.O. as previously described (2.2). Reduced glutathione and sodium azide were disolved in Krebs medium with pH maintained at 7.4.

For use in PMN thiol oxidation experiments, normal human serum was heated at 56°C for 30 minutes to inactivate complement and was then stored at -20°C for a minimum of 48 hours.

For in vivo thiol oxidation studies synovial fluid was obtained from the knee joints of patients with rheumatoid arthritis, osteoarthritis and post menisectomy joint effusions as indicated. The samples were centrifuged at 10,000g for 10 min to remove all live cells (4.2.2) and were then stored at -20°C for 2-6 months prior to use. Before centrifugation, where indicated, sample aliquots were taken for total cell counting using phase contrast microscopy. Differential white cell counts were not prepared. Serum samples were obtained form normal subjects and patients with rheumatoid arthritis with their informed consent.

6.2.2 Assay for thiol groups

Free and protein bound sulphydryl group concentrations in the range 40-600μM were assayed by a modification of the method of Janatova et al (1968) which was in turn based on the method of Ellman, (1959).

A stock solution of 10mM dithiobis-2-nitrobenzoate (DTNB) in 37mM phosphate buffer pH 8.0 was freshly prepared on the day of assay. The DTNB was not completely soluble under these conditions, and after rigorous mixing the solution was centrifuged at 1000g in a bench centrifuge to remove any particulate matter. A stock solution of 25mM EDTA (disodium Salt) in 7.4mM phosphate buffer was also prepared, pH readjusted to 7.9.

The assay was carried out in a dual wavelength spectrophotometer (Beckman) at room temperature using matched plastic cuvettes (Sarstedt). The reagent blank was prepared by mixing 50μl of the 25mM

EDTA stock solution with 750µl 7.4mM phosphate buffer pH 7.9 in a plastic cuvette. The reaction mixture was prepared by mixing 50µl of the 25mM EDTA stock solution with 50-100µl of the test substance dissolved in Krebs medium pH 7.4 and 700-650µl 7.4mM phosphate buffer pH 7.9 in a matched cuvette. Reaction was initiated by the addition with, thorough mixing, of 200µl of the 10mM DTNB stock solution to both reagent blank and reaction mixture. The absorbance of the reaction mixture was read against that of the reagent blank in the spectrophotometer at 412nm (Fig 74) and documented on a pen chart recorder until the maximum absorbance was determined. This occurred following the addition of DTNB for low molecullar weight thiol and at 10-15minutes for protein bound thiol, thereafter decaying (Fig 75).

A test solution blank was prepared by mixing 50-100µl of the test solution with 200µl 37mM phosphate buffer pH 8.0, 50µl of the 25mM EDTA stock solution and 700-650µl 0.0074M phosphate buffer pH 7.9. The absorbance of this solution at 412nm was subtracted from the absorbance of the reaction mixture.

The concentration of thiol groups in the original test solution was calculated according to the following formula:-

$$Co = \frac{A}{E} D \text{ moles/litre}$$

where Co = concentrations of thiol groups in test solution

A = maximum absorbance at 412nm

E = extinction coefficient (13,600/M/cm)

D = dilution factor

There was a linear relationship between absorbancce at 412nm and concentration of albumin in the range 2-10% (w/v) human serum albumin (Fig 76), corresponding to 92-486µM thiol group concentration. The lower limit of detection for this assay therefore was an absorbance of

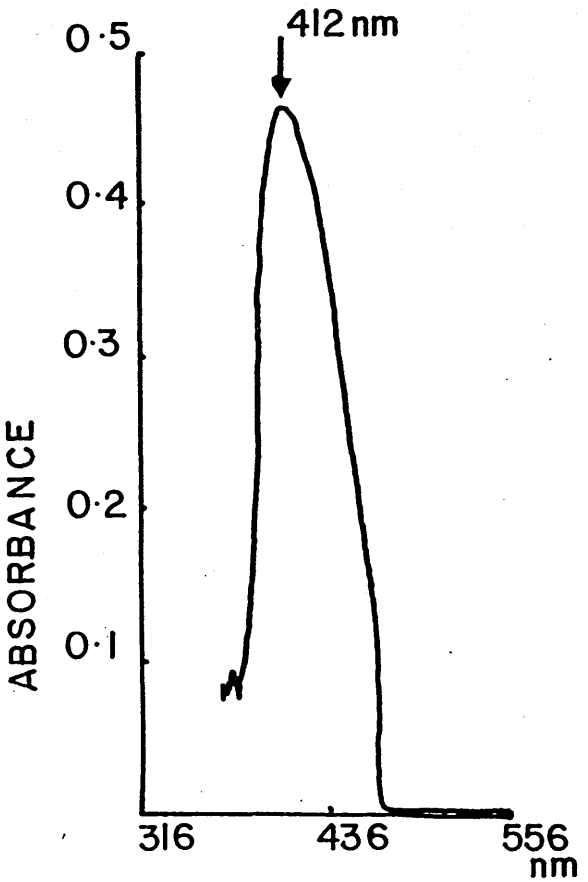


Figure 74 Spectrophotometric assay for thiol groups using dithiobis-2-nitrobenzoate (DTNB)

This absorbance trace was obtained in a dual wavelength spectrophotometer scanning between 556 and 356nm and shows the absorbance peak at 412nm of the benzoate anion liberated by reaction of 100µl of 400µM reduced glutathione (diluted in Krebs medium pH 7.4) and 200µl 10mM DTNB (in 37mM phosphate buffer pH 7.9) diluted in 7.4mM phosphate buffer pH 7.9 with EDTH as described in 6.2.2. The absorbance of the "reagent blank" containing 100µl Krebs medium only was simultaneously subtracted.

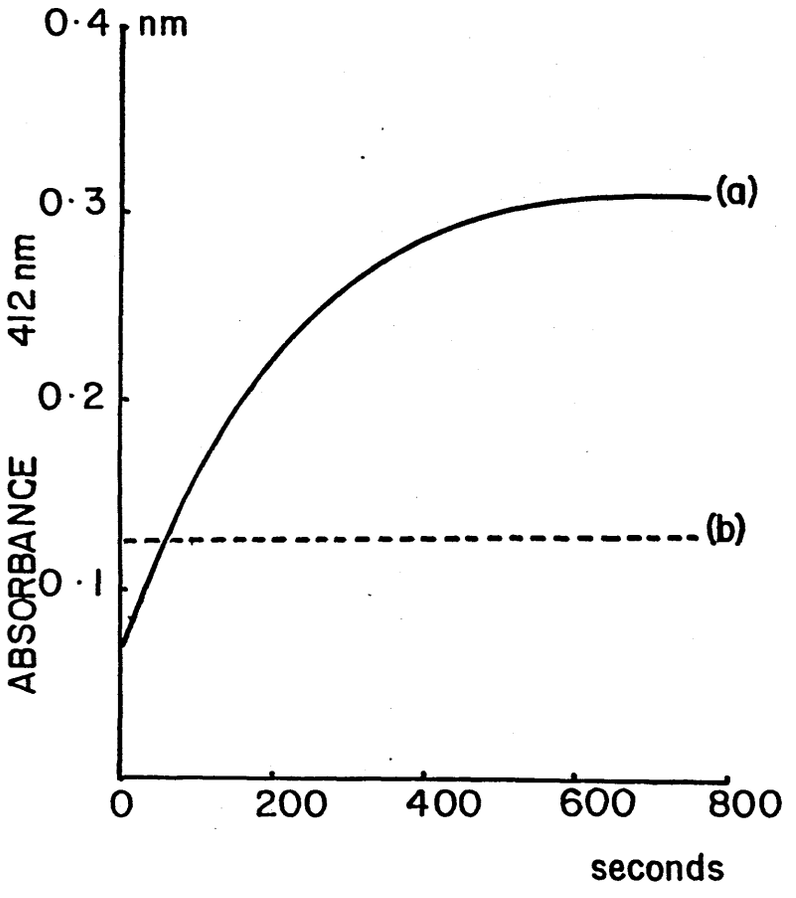


Figure 75 - Comparison of the rate of reaction of low molecular weight and protein bound thiol groups with DTNB in the thiol group assay.

At time 0 seconds 200ul 0.01M DTNB was added to the reaction mixture and to a reagent blank in matched plastic cu-vettes. The reaction mixture contained 700ul 0.0074M phosphate buffer, 0.025M EDTA stock solution (see 6.2.2), pH 7.9 and either (a) 50µl 10% (w/v) human serum albumin or (b) 50µl 200µM reduced glutathione, Krebs medium pH 7.4, room temperature. The absorbance of reaction mixture against blank at 412nm was monitored continuously in a dual wavelength spectrophotometer.

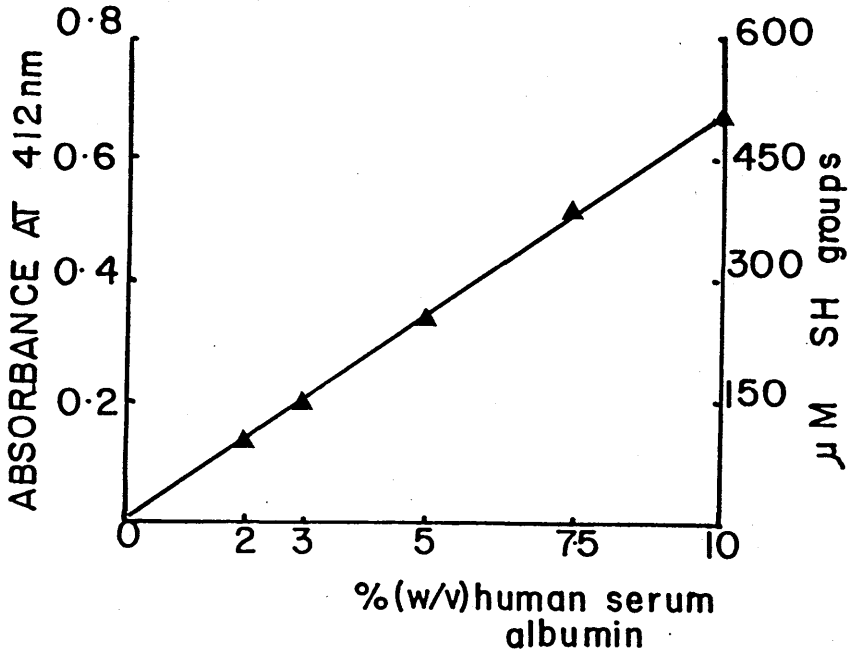


Figure 76 Dose response relationship for human serum albumin in DTNB assay for thiol groups

The graph shows the absorbance 15 minutes following reaction of 100µl of 2-10% (w/v) human serum albumin in Krebs medium pH 7.4 with DTNB as described in 6.2.2 and Fig 74. Reagent and test blanks were subtracted. The known extinction coefficient (13,600/M/cm) allowed calculation of the thiol group concentration, for each albumin concentration, from the absorbance at 412nm.

0.050, corresponding to a thiol group concentration of 37μM.

6.2.3 Assay for protein concentrations in synovial fluid

The total protein concentration in aspirated synovial fluid samples, denuded of cells, was measured according to the method of Lowry (1955) detailed below. As this assay only allowed measurements in the ranges 12.5 - 100μg/100μl, all synovial fluid samples were diluted 1:00 in Krebs medium prior to estimation. The results were calculated as the mean of duplicate samples.

Stock solutions:-

Reagent I:- 2% (w/v) Na_2CO_3

0.1M NaOH

0.04% (w/v) Na tartrate

Reagent II:- 1% (w/v) $CuSO_4$ in double distilled water

Lowry Reagent:- 100ml I and 2ml II

Folin Giocalteau's reagent:-

10ml diluted to 20ml in double distilled water

3ml of Lowry reagent was thoroughly mixed with 100μl of test sample and allowed to stand for 10 min., 300μl of the diluted Folin's reagent was then added to the mixture which was allowed to stand for 30 min. The absorbance of this mixture at 750nm was read in the dual wavelength spectrophotometer (Beckman). The assay was calibrated with bovine serum albumin standards of known concentration in double distilled water (Fig. 77).

The albumin concentrations in aspirated synovial fluid samples were measured in the Department of Medical Biochemistry clinical laboratory, University Hospital of Wales, Cardiff. The synovial fluid albumin concentrations were measured in a Technicon SMAC using

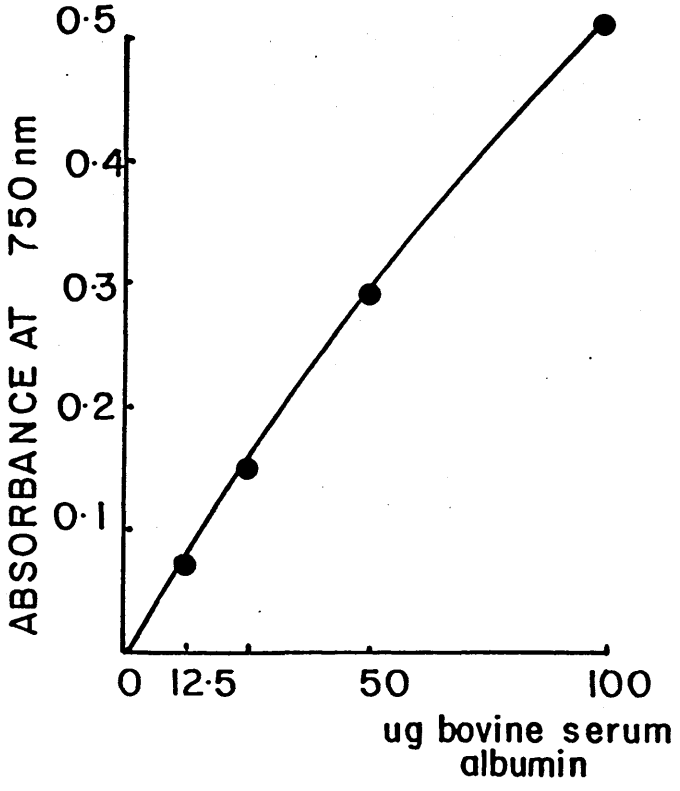


Figure 77 Lowry assay for protein - calibration with bovine serum albumin

The graph shows the absorbances at 750nm which resulted when 12.5-100µg bovine serum albumin in 100ul double distilled water and mixed with 3ml Lowry reagent was added to 300µl diluted Folin's reagent and allowed to stand for 30 minutes (6.2.3). Single observations. Room temperature.

Bromocresol green as an indicator in a colourimetric assay.

6.3 Results

6.3.1 Oxidation of free and protein bound thiol groups exposed to PMN

In order to determine whether scavenging of oxygen radicals by albumin occurred via thiol group oxidation, the total number of thiol groups in the albumin preparation detectable by the thiol group assay (6.2.2) was measured before and after its exposure to PMN which were resting or stimulated by opsonised zymosan or chemotactic peptide. The effects of exposure to resting or stimulated PMN on the measurable thiol content of reduced glutathione and of normal serum which had been heated at 56°C for 30 minutes to inactivate complement were similarly examined.

Stimulation of PMN by opsonised zymosan - effect on thiol group oxidation

Comparison was made of the effect of incubation at 37°C for seventy minutes with PMN stimulated by opsonised zymosan on the thiol content of (a) 4% (w/v) human serum albumin, (b) 50% (v/v) heat treated serum and (c) 300μM reduced glutathione (Table 31). In control experiments the albumin, serum, or gluathione were incubated with unstimulated PMN or with opsonised zymosan alone. Following incubation with the stimulated PMN the measurable thiol content of the albumin preparation was 17.9 ± 1.9% (mean ± SEM, n = 3) lower than that of albumin exposed only to opsonised zymosan but not cells. An even larger reduction in the measurable thiol content occurred for serum (46.6 ± 1.5%) and for glutathione (57.1 ± 0.9%) (mean ± SEM, n = 3). In all three preparations therefore, exposure to stimulated PMN resulted in thiol group oxidation. Unstimulated PMN caused a (4.5 ±

Table 31 - Oxidation of free and protein bound thiol groups by opsonised zymosan stimulated PMN

	No PMN 2mg/ml opsonised zymosan	5x10⁶PMN/ml 2mg/ml opsonised zymosan	5x10⁶PMN/ml no zymosan
4% (w/v) human serum albumin	196.8 ± 4.8µM	162.7 ± 3.9µM ($p > 0.01$)	197.3 ± 0.9µM ($p > 0.4$, NS)
50% (v/v) heat treated serum	196.3 ± 23.3µM	104.7 ± 2.9µM ($p < 0.02$)	187.3 ± 2.3µM ($p > 0.3$, NS)
300uM reduced glutathione	279.3 ± 3.2µM	127.3 ± 2.6µM ($p < 0.0001$)	299.7 ± 2.4µM ($p > 0.2$, NS)

Using the DTNB assay the reduced thiol contents of 4% (w/v) human serum albumin, 50% (v/v) heat treated serum or 300µM reduced glutathione were measured before and after their exposure to 5x10⁶ PMN/ml stimulated by 2mg/ml opsonised zymosan for 70 min or the stimulus or cells alone (means + SEM, n=3). The probabilities with which values obtained with cells were different from those without cells are shown (student's t test). 37oC, Krebs medium pH 7.4

2.1%) (mean ± SEM, n = 3) reduction in the measurable thiol content of heat treated serum but had no significant effect on the thiol content of albumin or reduced glutathione.

The rate at which the measurable thiol content of 300μM reduced glutathione was decreased by exposure to opsonised zymosan stimulated PMN was examined in a separate experiment (Fig 78), comparison being made with the measurable thiol content of glutathione exposed to the zymosan but not to cells. The thiol content decreased by 124μM (48%) in the first 30 minutes of exposure to the stimulated PMN and thereafter decreased more slowly such that after 70 seconds there was a reduction of 167μM (69%) in the measurable thiol content. The disparity between the measured and calculated thiol content of the reduced glutathione not exposed to cells was accounted for by spontaneous oxidation of the glutathione preparation, maintained at 25°C for two hours prior to use.

Stimulation of PMN by chemotactic peptide - effect on thiol group oxidation

Having demonstrated oxidation of free and protein bound thiol groups following their exposure to PMN stimulated by opsonised zymosan, the effect of PMN stimulated by chemotactic peptide on the measurable thiol content of 4% (w/v) human serum albumin and 50% (v/v) heat treated serum was similarly examined (Table 32). Following incubation with the stimulated PMN the measurable thiol content of the serum preparation was 23.2 ± 6.4% (n = 3, mean ± SEM) lower than that of serum exposed only to chemotactic peptide, but not to cells ($p<0.05$). For 4% (w/v) albumin a decrease by only 8.9 ± 1.6% (n = 3, mean ± SEM) occurred ($p > 0.3$) (not significant). Unstimulated PMN caused a 10.7 ± 2.6% (n = 3, mean ± SEM) reduction in the measurable

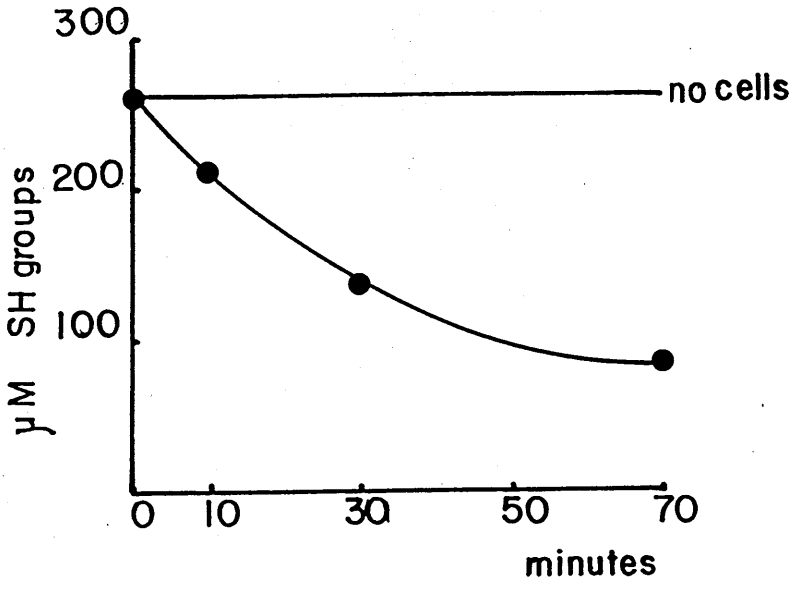


Figure 78 Rate of decrease in measurable thiol group concentration of reduced glutathione following exposure to opsonised zymosan stimulated PMN

The graph shows the thiol group concentration (measured in DTNB assay, 6.2.2) in the supernatants (obtained by centrifugation at 10,000g for 2 mins) from 5×10^6 PMN/ml stimulated by 2mg/ml opsonised zymosan in the presence of 300μM reduced glutathione at 37°C for 0-70 minutes. Comparison is made with the measurable thiol content of glutathione exposed to zymosan, but not cells. Points represent the means of three observations (errors too small to demonstrate). Krebs medium, pH 7.4.

Table 32 - Oxidation of protein bound thiol groups by chemotactic peptide stimulated PMN

	No PMN 1μM chemotactic peptide	$5.3x10^6$PMN/ml 1uM chemotactic peptide	$5.3x10^6$PMN/ml no chemotactic peptide
4% (w/v) human serum albumin	187 ± 6.4μM	170 ± 2.9μM ($p > 0.3$)	186 ± 22μM ($p > 0.2$)
50% (v/v) heat treated serum	236 ± 3.5μM	181 ± 15μM $p < 0.05$)	211 ± 6.4μM ($p < 0.05$)

Using the DTNB assay the reduced thiol contents of 4% (w/v) human serum albumin and 50% (v/v) heat treated serum were measured before and after their exposure to $5.3x10^6$PMN/ml stimulated b y 1μM chemotactic peptide for 70 min, or the stimulus or cells alone (means ± SEM, n=3). 37oC Krebs medium pH 7.4

thiol content of heat treated serum ($p < 0.05$) and had no significant effect on the thiol content of albumin.

Thus PMN, stimulated either by opsonised zymosan or by chemotactic peptide, oxidised serum and albumin thiol groups. Opsonised zymosan stimulation caused more thiol oxidation than chemotactic peptide. This was attributed to the relative size of the respiratory burst as monitored by luminol chemiluminescence. In traces produced by the same concentration of stimuli, the maximum peak height of the opsonised zymosan trace was six fold larger than that of the chemotactic peptide (Fig 79). Unstimulated PMN caused no oxidation of reduced glutathione or albumin thiol groups (Tables 31 and 32) but a 5% and 11% decrease in measurable thiol groups in serum did occur suggesting that the heat treated serum, with complement inactivated, itself caused PMN stimulation possibly via aggregated IgG. (cf. 3.3.4).

Demonstration of oxidation of albumin thiol groups by stimulated PMN thus provided one probable mechanism by which oxygen radical scavenging and hence inhibition of luminol chemiluminescence by albumin could occur.

Effect of superoxide dismutase and sodium azide on thiol oxidation

In order to demonstrate that oxidation of thiol groups by stimulated PMN resulted from oxygen radical scavenging it was necessary to demonstrate the dependence of the oxidation on at least one reactive oxygen metabolite known to result from the respiratory burst. The effects of superoxide dismutase, and myeloperoxidase (catalyst of hypochlorite anion and singlet oxygen formation 1.2.2) on gluthathione thiol oxidation were therefore examined, (Table 33).

Superoxide dismutase (10-200µg/ml) had no effect on either opsonised zymosan or chemotactic peptide stimulated thiol oxidation.

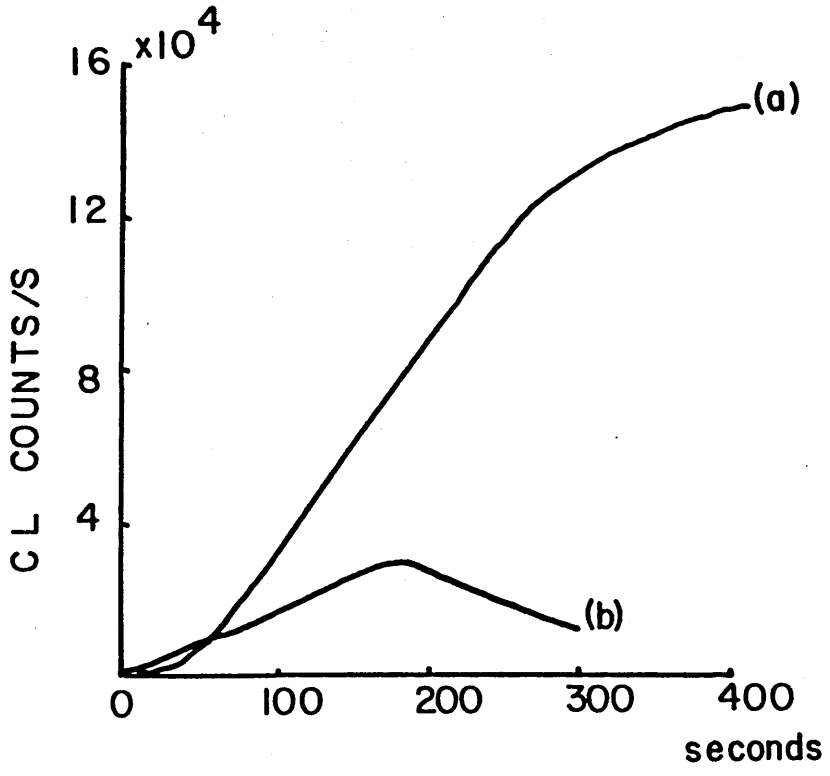


Figure 79 - Relative sizes of opsonised zymosan and chemotactic peptide stimulated PMN luminol chemiluminescence traces

$5x10^5$PMN/ml were stimulated by addition, at 0 seconds, of (a) 1mg/ml opsonised zymosan or (b) 0.1μM chemotactic peptide. Krebs medium pH 7.4 (10% (v/v) phosphate buffered saline) 37°C, 10μM luminol.

Table 33 - Effect-of superoxide dismutase and azide on opsonised zymosan and chemotactic peptide stimulated PMN oxidation of reduced glutathione

		Opsonised Zymosan	Chemotactic Peptide
Sodium Azide	0μM	42 ± 2.2%	34 ± 2.8%
	5μM	39 ± 1.6%	17 ± 3.9%
	50μM	7 ± 4.6%	0 ± 1.2%
	500μM	6 ± 1.6%	0 ± 1.2%
Superoxide dismutase	0μg/ml	37 ± 0.7%	39 ± 1.1%
	10μg/ml	28 ± 1.0%	29 ± 3.1%
	50μg/ml	28 ± 2.2%	34 ± 1.4%
	100μg/ml	26 ± 0.2%	34 ± 0.8%
	200ug/ml	31 ± 1.7%	38 ± 3.1%

1.25×10^6 PMN/ml were stimulated by 2mg/ml opsonised zymosan or 0.1uM chemotactic peptide in the presence of 300μM reduced glutathione for 70 min., 37°C, pH 7.4, Krebs medium. The concentration of free thiol in the supernatant was then measured by reaction with DTNB and expressed as a percentage of that obtained when glutathione was exposed to stimulus alone. (This experiment was performed by Mr. D. E. Jenner, Research Assistant, Department of Medical Biochemistry, University of Wales College of Medicine).

Sodium azide (> 5μM) provided potent inhibition of both (Table 33).

6.3.2 Comparison of the effects of reduced glutathione and albumin on PMN luminol chemiluminescence

If the inhibition of resting and stimulated PMN chemiluminescence by albumin was to be explained entirely via oxygen radical scavenging by oxidation of its thiol groups, it was to be expected that low molecular weight thiol groups would have a similar effect on PMN luminol chemiluminescence. The low molecular weight thiol compound reduced glutathione was therefore examined.

When PMN were stimulated by chemotactic peptide in the presence of 2% (w/v) dialysed human serum albumin (92.6μM thiol groups) minimal inhibition of first phase and marked inhibition of second phase luminol chemiluminescence occurred (Fig. 80 (a) and (b)) as in previous experiments. When stimulation occurred in the presence of reduced glutathione, however, it was the first phase which was markedly inhibited, while the second was relatively spared (Fig. 81 (c)). This preferential inhibition of the first phase of chemotactic peptide induced PMN chemiluminescence occurred throughout the range 0.1μM - 1000μM reduced glutathione (Table 34). Lower concentrations (0.1μM - 10μM glutathione) caused a small inhibition of background PMN chemiluminescence (20-23%) while in the range 100μM - 1000μM glutathione an enhancement of up to 30% occurred.

Thus the effect of reduced glutathione on PMN luminol chemiluminescence was the reverse of the effect of albumin even though thiol groups on both glutathione and albumin had been shown to be oxidised by stimulated PMN. While this apparent discrepancy may be explicable by the relative inaccessibility of the thiol group on albumin on account of its convoluted quarternary structure, the

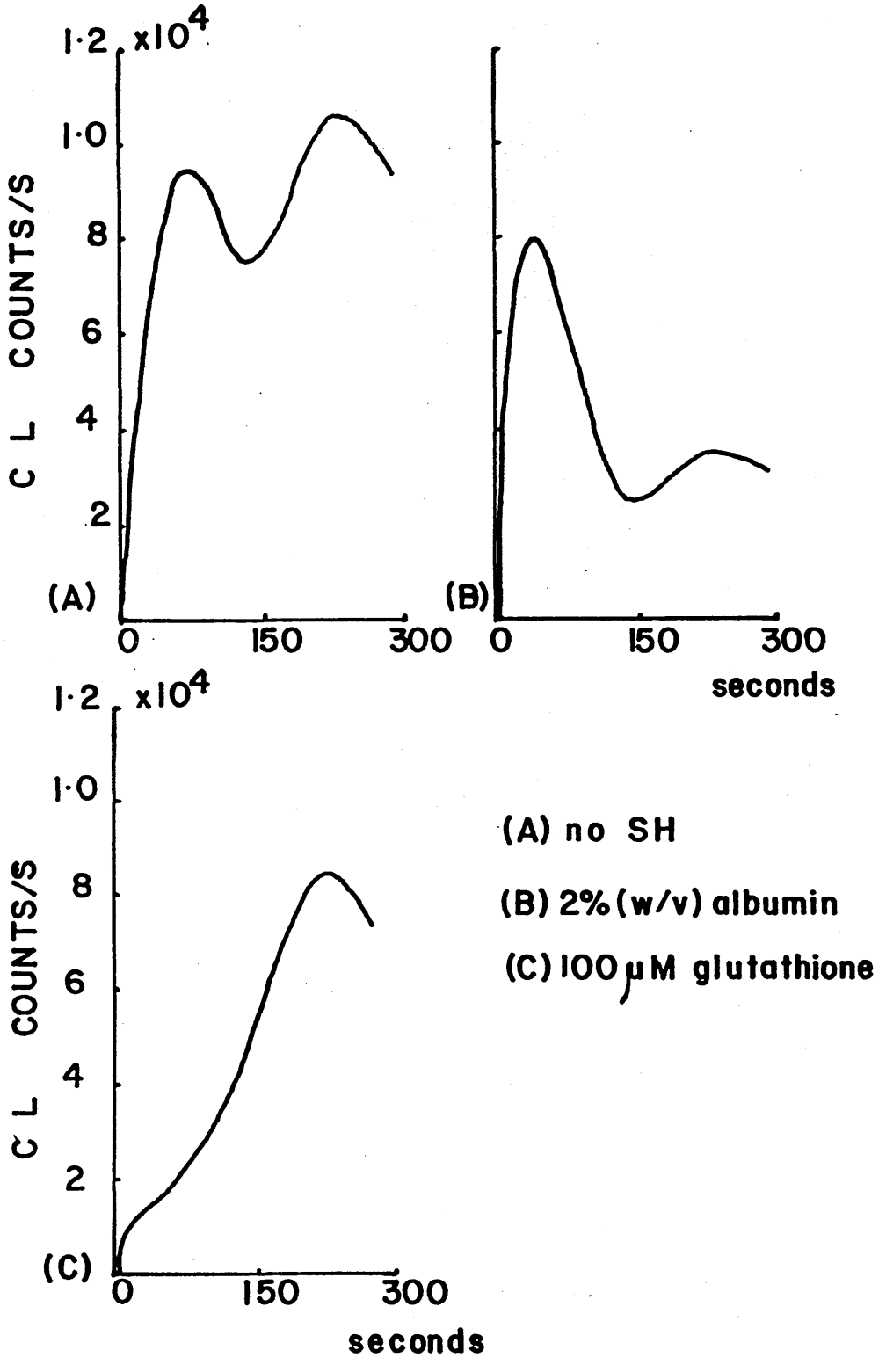


Figure 80 Effects of albumin and reduced glutathione on chemotactic peptide induced PMN luminol chemiluminescence

In each trace 1μM chemotactic peptide was added at time 0 seconds to 5×10^5 PMN/ml. The first and second phases of the normal chemiluminescence response are clearly shown in (A). The response in the presence of 2% (w/v) human serum albumin is shown in (B) and that in the presence of 100μM reduced glutathione in (C). Krebs medium, pH 7.4, 37°C, 10μM luminol.

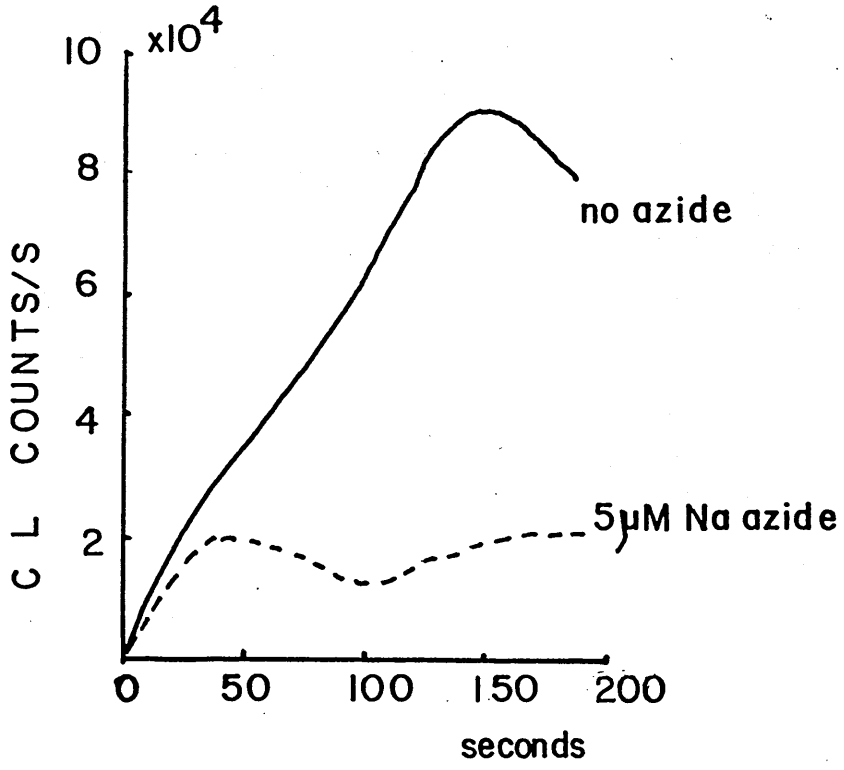


Figure 81 Effect of sodium azide on chemotactic peptide stimulated PMN chemiluminescence

Chemiluminescence traces produced by $5x10^5$ PMN/ml stimulated by 1μM chemotactic peptide in the presence (dotted line) and absence (unbroken line) of 5uM sodium azide. Krebs medium, pH 7.4, 37°C, 10μM luminol.

Table 34 - Effect of reduced glutathione on luminol chemiluminescence of PMN resting or stimulated by chemotactic peptide

µM reduced glutathione	Background Chemiluminescence	1st Phase (60 sec)	2nd Phase (240 sec)
0.1µm	77%	66%	93%
1µM	79%	34%	93%
10µM	80%	28%	95%
100µm	123%	22%	78%
300µM	130%	21%	85%
1000µM	110%	19%	88%

$5x10^5$PMN/ml were resting or stimulated by 1uM chemotactic peptide in the presence of 0.1 - 100µM reduced glutathione. The columns indicate the chemiluminescence observed expressed as a % of that expected in the absence of glutathione. 37°C. Krebs medium pH 7.4 10µM luminol.

possibility also existed that albumin's inhibitory effect on luminol chemiluminescnce may have been mediated by reaction of the oxygen radicals with another moiety (6.1.).

In order to determine whether the oxygen radical or reactive oxygen metabolite with which albumin reacted to inhibit luminol chemiluminescence resulted from a myeloperoxidase catalysed reaction, the effect of the peroxidase inhibitor sodium azide on PMN chemiluminescence was investigated. When PMN were pre-incubated with sodium azide (1-5μM) prior to stimulation with chemotactic peptide, preferential inhibition of background PMN chemiluminescence and of the second phases of the luminol chemiluminescence response occurred (Table 35, Fig. 81), although at all concentrations of azide in this range some inhibition of the first phase also occurred (cf Fig. 81 and Fig 65 and Table 26 5.3.3 - effect of albumin). By contrast, when PMN were pre-incubated with superoxide dismutase, preferential inhibition of the first phase occurred (Fig. 82).

6.3.3 Serum and Synovial fluid thiol levels in patients with rheumatoid arthritis

Having demonstrated oxidation of thiol groups on albumin following exposure of the molecule to oxygen radicals produced by stimulated PMN in vitro, it was now necessary to determine whether albumin underwent similar structural modification by scavenging of oxygen radicals in inflammatory disease in vivo.

Depression of serum thiol levels in patients with rheumatoid arthritis was well recognised (Lorber et al 1964; Thomas and Evans 1975) and as albumin was known to account for the majority of serum thiol levels (Jocelyn 1970) circumstantial evidence of albumin thiol oxidation in rheumatoid arthritis already existed. The most likely

TABLE 35

Effect of Sodium Azide on Luminol Chemiluminescnece of PMN resting, or stimulated by chemotactic peptide

μM sodium azide	Background Chemiluminescence	1st Phase (60 sec)	2nd Phase (180 sec)
1μM	50%	84%	48%
2μM	39%	70%	32%
4μM	24%	66%	28%
5μM	30%	76%	25%
6μM	25%	71%	23%
7μM	24%	60%	20%
10μM	22%	38%	21%
50μM	11%	21%	12%

$5x10^5$PMN/ml were resting or stimulated by 1uM chemotactic peptide in the presence of 1-50μM sodium azide. The columns indicate the chemiluminescence observed expressed as a percentage of that expected in the absence of azide. 37°C Krebs medium pH 7.4 10μM luminol.

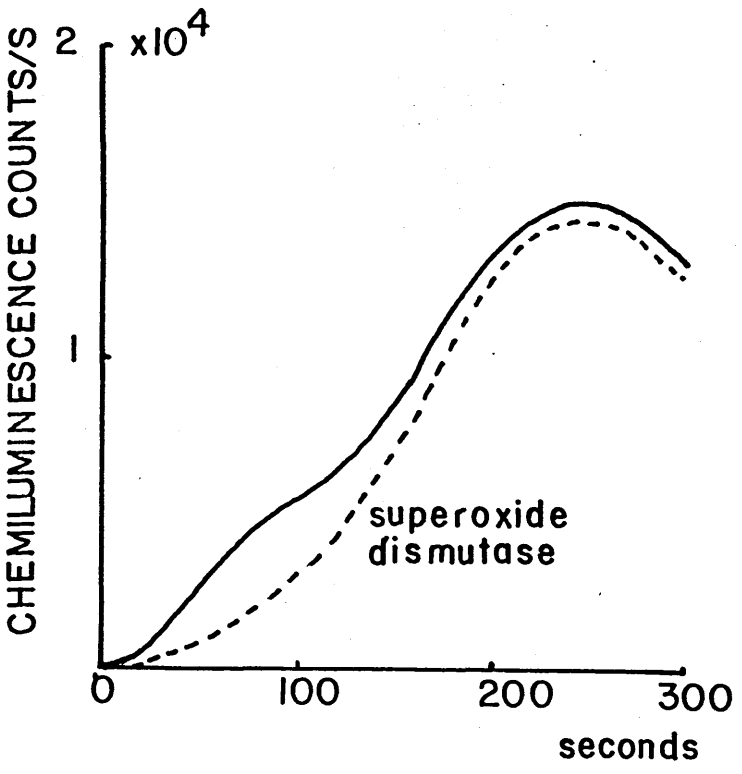


Figure 82 Effect of superoxide dismutase on chemotactic peptide stimulated PMN chemiluminescence

Chemiluminescence traces produced when 5×10^5 PMN/ml were stimulated by 0.1μM chemotactic peptide in the presence (dotted line) and absence (unbroken line) of 200ug/ml superoxide dismutase. Krebs medium, pH 7.4, 37°C, 10μM luminol.

anatomical location for albumin to encounter oxygen radicals was however the synovial fluid of affected joints where PMN and their stimuli are in plentiful supply (1.3.1).

The aim of the preliminary study described in this section was therefore to examine rheumatoid synovial fluid (with fluid from patients with non-inflammatory arthritis as controls) for evidence of albumin thiol oxidation by attempting to correlate any depression in alumin thiol levels with the synovial fluid cell count and, where available, other markers of intra articular disease activity.

Synovial fluid was available from four patients with osteoarthritis/post menisectomy effusions, and thirteen patients with seropositive erosive rheumatoid arthritis, seven of whom received only anti-inflammatory drug therapy and six of whom also received the thiol containing antirheumatic drugs gold and D-penicillamine. In addition serum was available from six patients with rheumatoid arthritis and from eleven, non age matched normal controls.

The distribution of total serum and synovial fluid thiol levels in these categories is shown in Fig. 83. While, in agreement with the observations of others, serum thiol levels in patients with rheumatoid arthritis were lower than those of normal subjects, within the synovial fluids the lowest values were found unexpectedly in the non-inflammatory osteoarthritis/post menisectomy groups. A similar distribution was observed when the thiol concentration was expressed as μmoles thiol/gramme of albumin in the synovial fluid or serum samples (Fig 84). When total synovial fluid thiol levels were correlated with the synovial fluid cell counts (which were available in the four osteoarthritic/post menisectomy patients and six patients with rheumatoid arthritis) a weakly positive correlation was obtained (Fig. 85) rather than the predicted negative correlation.

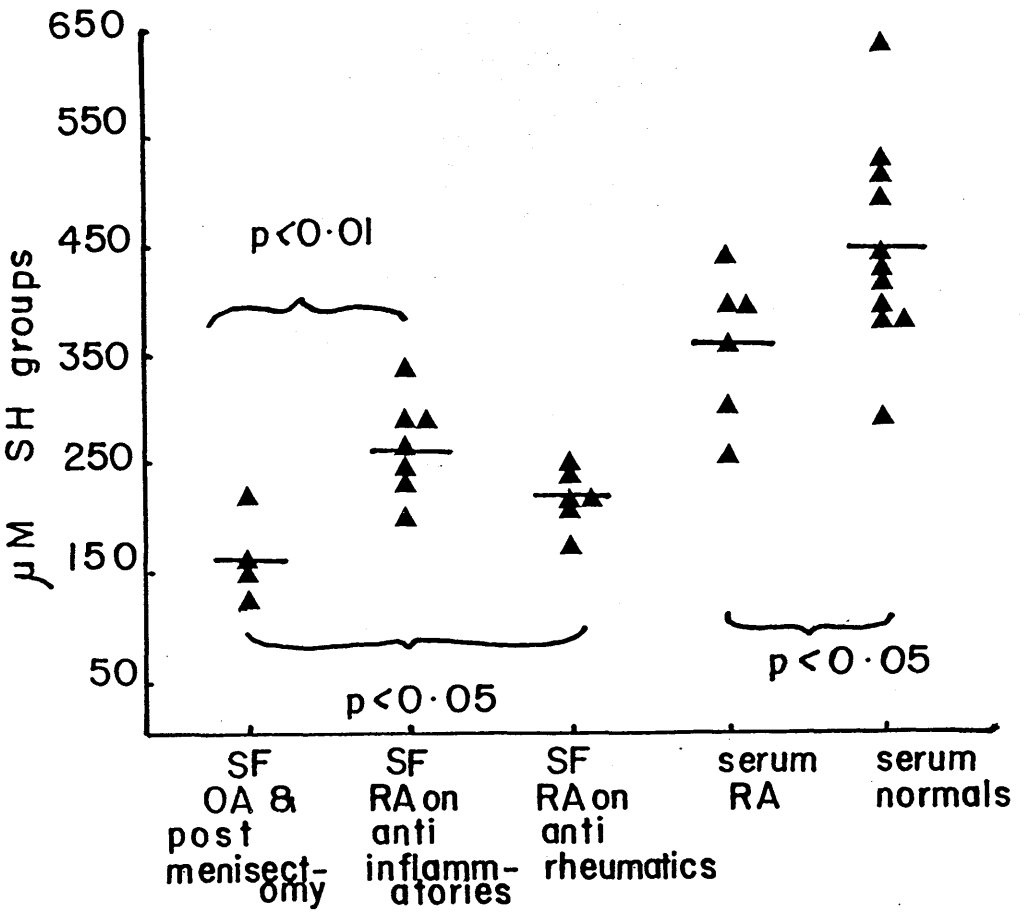


Figure 83 - Comparison of synovial fluid and serum total thiol levels in rheumatoid patients, normals and non inflammatory arthropathy controls.

DTNB was used to measure the reduced thiol concentrations in serum or synovial fluid (SF) samples obtained from normal subjects, patients with rheumatoid arthritis (RA) and patients with osteoarthritis (OA) or who had recently undergone knee menisectomy. Patients with RA were subdivided by therapy as indicated. Points represent the mean of duplicate observations.

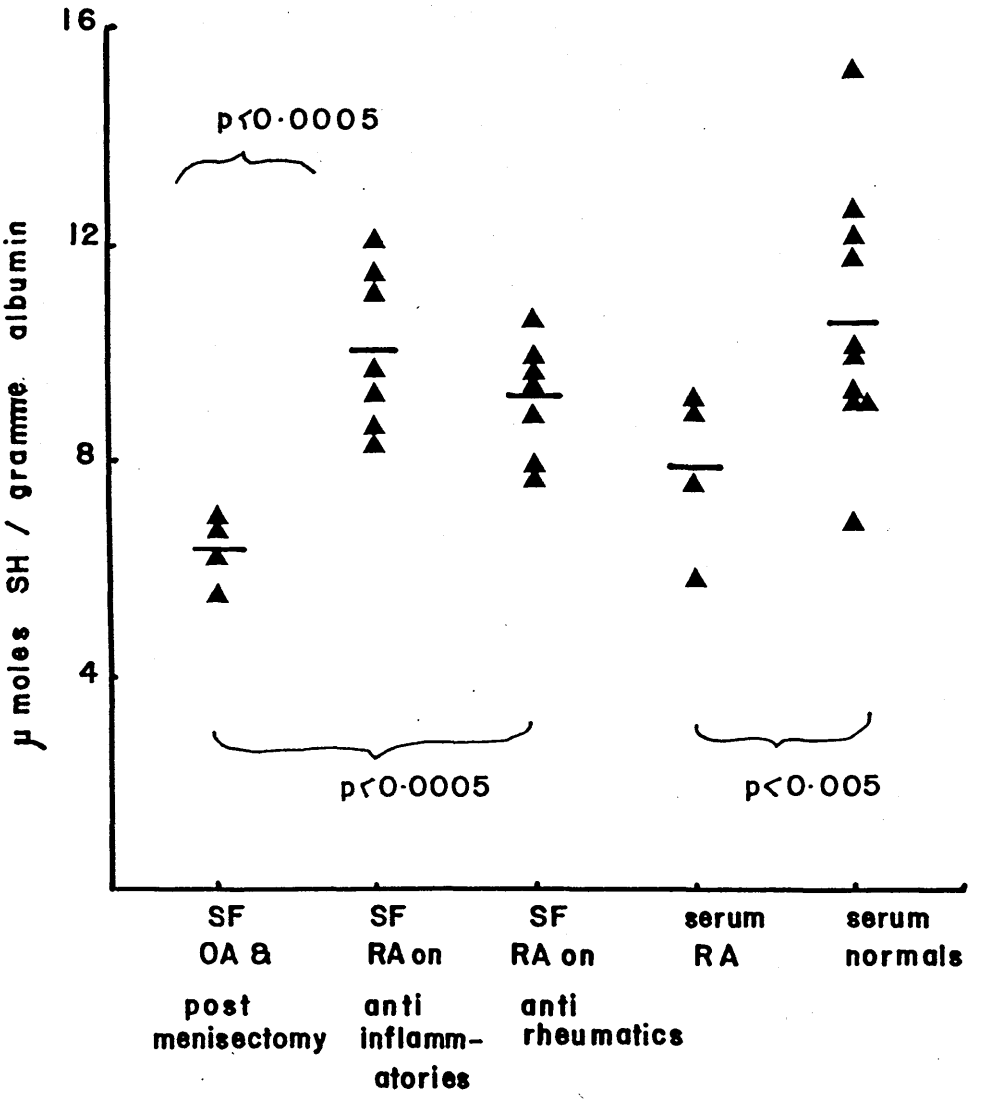


Figure 84 - Comparison of the concentration of thiol groups/gramme albumin in synovial fluid and serum from rheumatoid patients, normals and non-inflammatory arthropathy controls.

(SF - synovial fluid)
RA - rheumatoid arthritis
OA - osteoarthritis)

Points represent mean of duplicate samples.

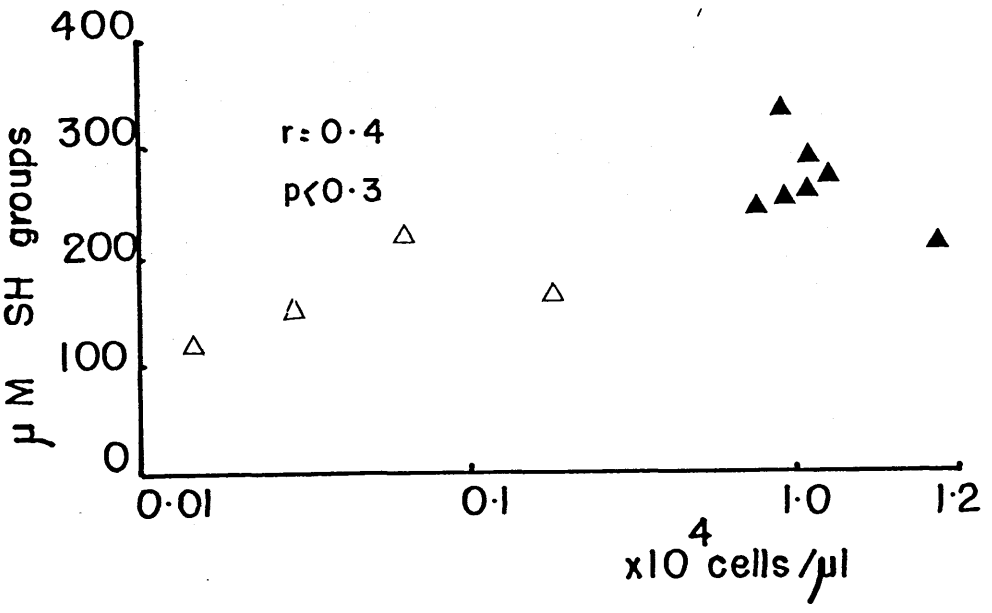


Figure 85 - Comparison of synovial fluid thiol levels and cell counts in rheumatoid patients and non-inflammatory arthropathy controls.

(△) - non-inflammatory (post menisectomy or osteoarthritis).

(▲) - rheumatoid arthritis

Some explanation of these findings was provided by the observation that only a weakly positive correlation existed between synovial fluid thiol levels and synovial fluid albumin or total protein concentrations (Fig. 86 (a) and (b)). Further, when absolute values of synovial fluid albumin in the three groups were compared (Fig. 87 (b)) no significant differences were found. When the concentrations of non-albumin protein were compared, however, the concentration in the non-inflammatory osteoarthritic/post menisectomy group was found to be significantly lower than that of the rheumatoid on anti-inflammatory therapy group ($p << 0.001$). (Fig. 87 (a)). The concentration of non-albumin protein in the synovial fluid of patients with rheumatoid arthritis receiving anti rheumatic therapy was of intermediate value.

It therefore appeared likely that a variable proportion of the synovial fluid thiol levels was being contributed to by non albumin protein (perhaps aggregated IgG). If this variability was in turn superimposed upon a variable degree of albumin thiol oxidation within the three groups, the failure to find evidence of albumin's scavenging role by this mechanism in vivo would be explained.

6.4 Discussion

The main achievement of the experiments reported in this chapter was to confirm the previous identification (Chapter 5) of albumin's oxygen radical scavenging capability by demonstrating that following incubation with stimulated PMN in vitro up to 18% of its thiol groups were oxidised. This oxidation was inhibitable by azide, but not by superoxide dismutase. The low molecular weight thiol compounds did not inhibit luminol dependent PMN chemiluminescence in the same manner as albumin, however, although the inhibition achieved by the latter

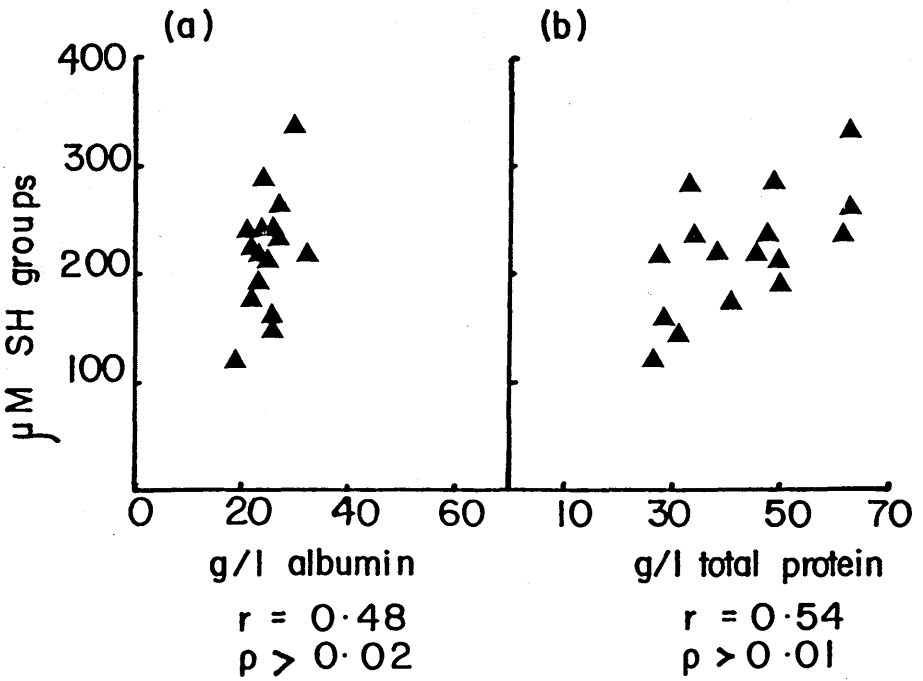


Figure 86 - Relationship between synovial fluid thiol concentrations and (a) albumin concentrations and (b) total protein concentrations in rheumatoid patients and non inflammatory arthropathy controls.

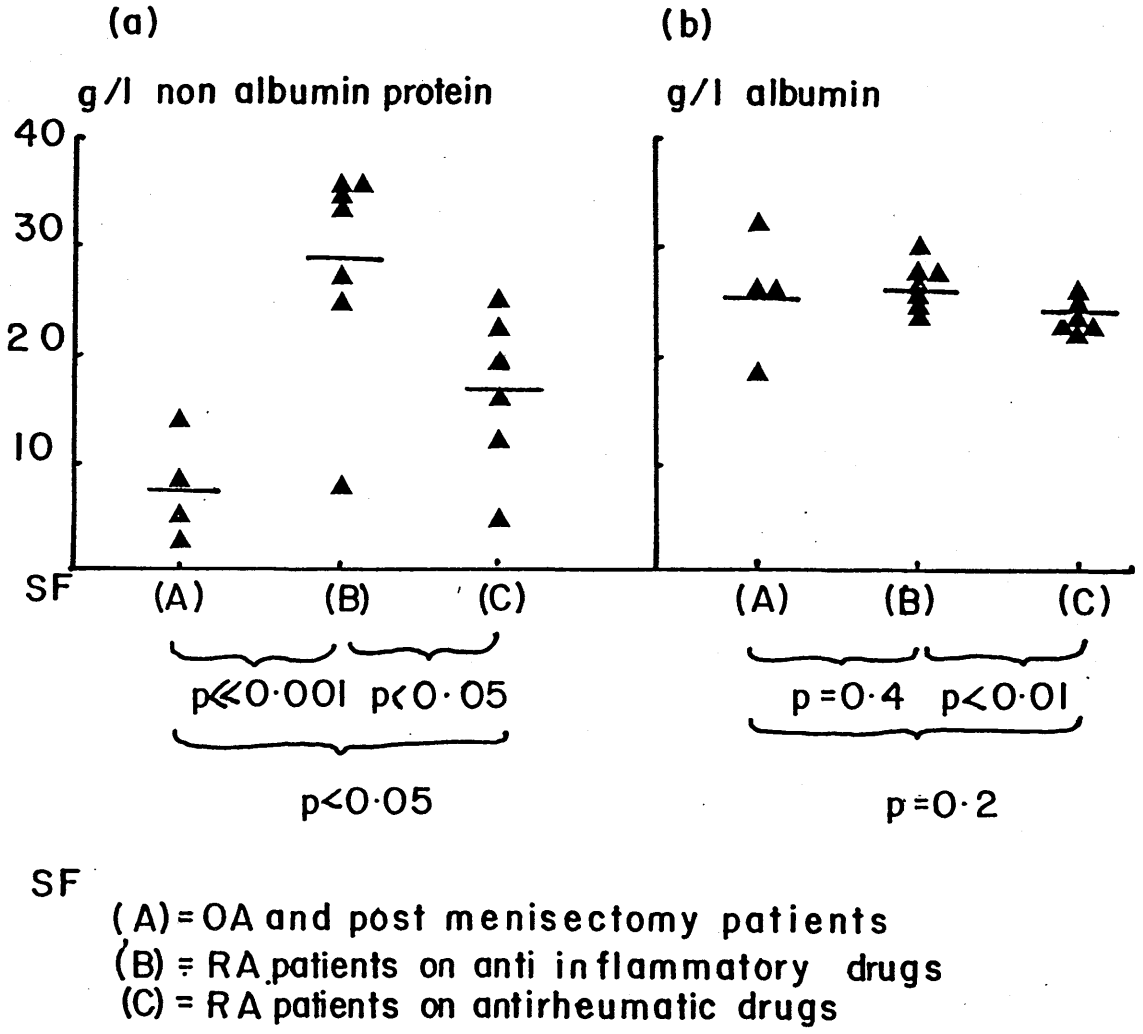


Figure 87 - Comparison of albumin concentrations with 'non-albumin' protein concentrations in synovial fluid from rheumatoid patients and non-inflammatory arthropathy controls

was reproduced when PMN were stimulated in the presence of azide.

Three questions immediately arise:-

(a) which reactive oxygen metabolite(s) does albumin's thiol group scavenge?

(b) what products of this oxidation result?

(c) what evidence is there for a protective role for albumin as an oxygen radical scavenger in vivo in rheumatoid arthritis or in other inflammatory diseases?

Inhibition of stimulated PMN thiol oxidation by azide, but not by superoxide dismutase suggests that the reactive oxygen metabolite responsible for the oxidation resulted from a myeloperoxidase catalysed reaction rather than from the presence of $O_2^{\cdot -}$ or its dismutation products (1.2.2). The principal reactive oxygen metabolites produced by PMN myeloperoxidase catalysed reactions comprise hypochlorite anion (OCl^-) and singlet oxygen ($^1\Delta g\ O_2$) (1.2.2, Fig 5).

The picture is complicated, however, by the fact that sodium azide, in addition to being a peroxidase enzyme inhibitor (Rosen and Klebanoff 1976) is known to quench certain ($^1\Delta g\ O_2$) dependent reactions (Hasty et al 1972). If ($^1\Delta g\ O_2$) is the radical responsible for thiol oxidation, superoxide dismutase would be expected to have an inhibitory effect by limiting the availability of $O_2^{\cdot -}$ to react with H_2O_2 and $OH^{\cdot}$, and thereby produce $^1\Delta g\ O_2$ (reactions (8) and (9) cf 1.2.2).

$$O_2^{\cdot -} + H_2O_2 \text{ ------> } (^1\Delta g\ O_2) + OH^- + OH^{\cdot} \text{ -------- (8)}$$

$$O_2^{\cdot -} + OH^{\cdot} \text{ ------> } (^1\Delta g\ O_2) + OH^- \text{ --------(9)}$$

As superoxide dismutase has also been shown however to catalyse certain peroxidatic reactions (Hodgson and Fridovich 1975), its apparent failure to inhibit ($^1\Delta g\ O_2$) mediated thiol oxidation may

result from a compensatory direct catalysis of thiol peroxidation. Subsequent to completion of the work of this thesis, Hall et al (1984) demonstrated inhibition of aggregated gamma globulin stimulated PMN serum thiol oxidation by catalase. By removal of H_2O_2, catalase would, of course, inhibit the formation of ($^1\Delta g$ O_2) by reaction (8).

The different rates of reaction of DTNB with thiol groups on glutathione and albumin (Fig. 75) presumably reflected the relative inaccessibility of the thiol group on albumin consequent upon its convoluted quaternary structure. The latter may similarly determine which reactive oxygen metabolite the albumin thiol can react with.

The differing effects of the low molecular weight thiol glutathione and albumin on PMN luminol chemiluminescence (Fig. 80) may therefore be explained if (i) a different reactive oxygen metabolite reacts with the thiol groups on each, and/or (ii) alternative moieties on the thiol containing molecules are responsible for their inhibitory actions. The demonstration that superoxide dismutase preferentially inhibits the first phase (Fig. 82) while azide preferentially inhibits the second phase (Fig. 81) of PMN luminol dependent chemiluminescence strongly suggests that a different oxygen radical predominates in each but unfortunately does not help to distinguish between (i) and (ii).

The precise reactive oxygen metabolite which albumin scavenges via thiol oxidation has therefore not been determined. The inter related effects of superoxide dismutase, azide and luminol leave little doubt of the dependence of the thiol oxidation on the PMN respiratory burst. Probable candidates as above include OCl^-, ($^1\Delta g$ O_2) and H_2O_2. Lipid peroxides produced as a consequence of oxygen radical dependent cell membrane lipid peroxidation (cf 1.2.3) also have the potential to oxidise thiol groups however (Little and O'Brien 1968). CLearly further investigation is required, and may be

facilitated by the study of myeloperoxidase deficient PMN.

Leaving aside the question of which reactive oxygen metabolite albumin scavenges, what of the consequences of thiol oxidation to the albumin molecule? The product corresponding the the highest oxidation state of the sulphur atom is sulphonic acid (RSO_3H) (Fig. 88). There are many intermediates of which the disulphide (RSSR) is the least resistant to further oxidation and thus under mild oxidising conditions is the major if not the only product. (Jocelyn 1972). Peroxidase catalysed oxidation of enzyme thiol groups to disulphides, sulphenic or sulphonic acids may occur (Green and Stumpf 1946).

Oxidation of albumin's thiol groups to a disulphide could result in (a) intermolecular disulphide rearrangement with one of the other cysteine residues, (b) dimerisation of albumin or (c) mixed disulphide formation with non albumin cysteine or glutathione. Supporting evidence for the occurrence of (b) and (c) in vivo is provided firstly by the demonstration by Poulik et al (1961) of a protein in 50% of normal human sera, having the antigenic and chromatographic characteristics of an albumin dimer. Other workers have h owever shown that dimerisation of albumin increases with in vitro storage time (Sogami et al 1969). Secondly only two thirds of serum albumin has a free thiol group (described as mercaptalbumin) (Hughes 1947), the remainder does circulate as a mixed disulphide, principally with cysteine (Thomas and Evans 1975). Even allowing for this the measured thiol content of the commercially prepared human serum albumin used in this thesis (Table 31), was only 50% of the predicted value suggesting that further thiol oxidation had occurred during its production.

Increased molecular heterogeneity of albumin has been demonstrated in the sera of patients with rheumatoid arthritis (Reid and Farr 1964). Rheumatoid arthritis was amongst the group of

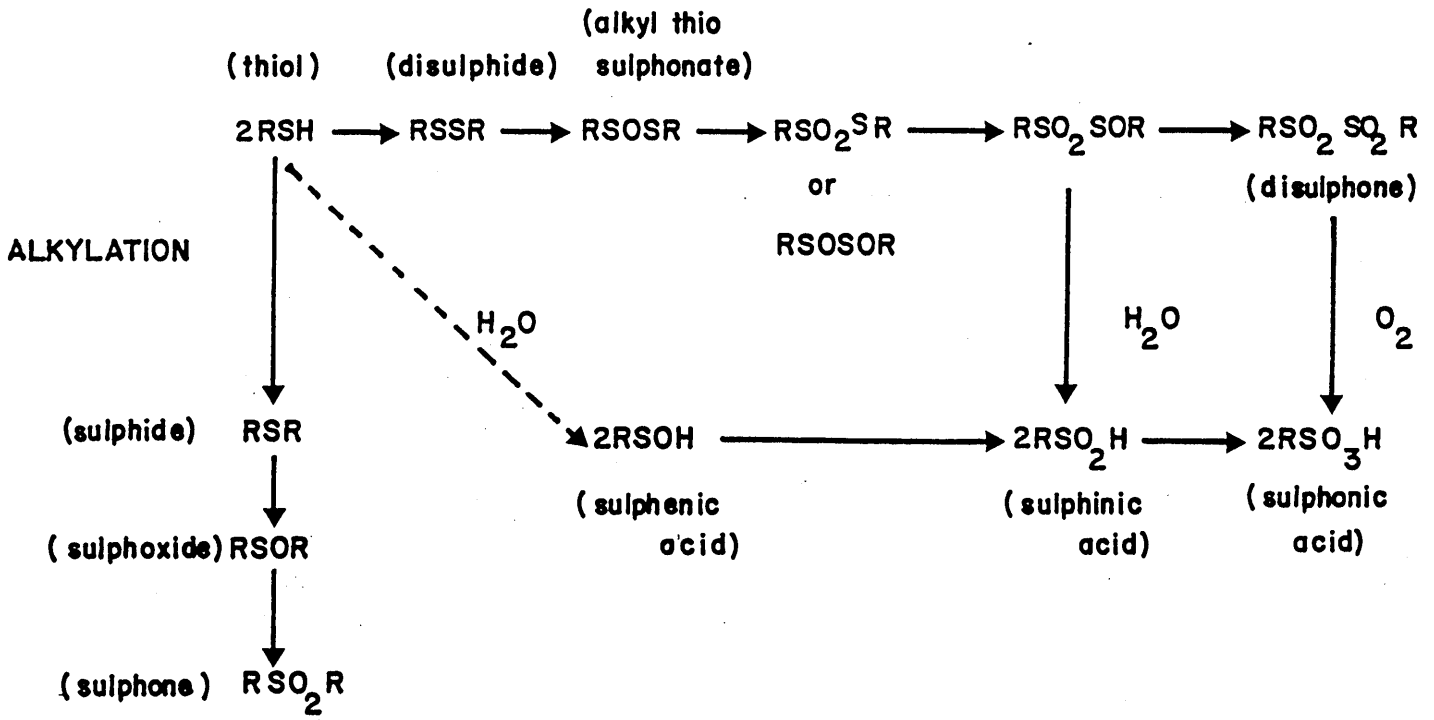


Figure 88 CONSEQUENCES OF THIOL OXIDATION

(adapted from Jocelyn, 1972)

inflammatory arthritides and connective tissues diseases first shown by Lorber et al (1964) to be associated with decreased levels of serum thiol. This observation was later confirmed by Thomas and Evans (1975) whose results further demonstrated that in normal sera albumin accounted for 87% of the total thiol concentration, with the remainder being accounted for in the globulin fractions (α_1 globulin 2%, α_2 globulin 3%, β globulin 4%, γ globulin 4%). This distribution of thiols was maintained in the sera of rheumatoid patients, although the total thiol concentration was reduced.

Thomas and Evans (1975) further demonstrated increased levels of mixed disulphide formation between albumin and cysteine in their rheumatoid sera. Following the demonstration in this thesis of oxygen radical scavenging by albumin via thiol oxidation, it therefore seemed appropriate (6.3.3) to examine rheumatoid synovial fluid for evidence of increased albumin thiol oxidation as it was there, in the presence of plentiful PMN and their stimuli that such oxidation would be most likely to occur.

Unfortunately there were two major constraints on this attempt. Firstly inadequate patient numbers, and secondly the experimental method used did not allow specific measurement of the albumin thiol concentrations. While depression of serum thiol levels in patients with rheumatoid arthritis was demonstrated, no increased thiol oxidation in the synovial fluid was found. An unknown factor was, however, the contribution to the total thiol levels of the non-albumin protein which was considerably increased in the rheumatoid samples (Fig. 87).

The rheumatoid patients were subdivided according to their drug therapy. Antirheumatic therapy with the drugs gold and D-penicillamine can be followed by a fall in the level of rheumatoid

factor in serum (Jaffe 1965, Scott 1984, Gottlieb et al 1975) and a rise in the levels of serum thiols (Dixon et al 1980). D-penicillamine contains a reduced thiol and gold (aurothiomalate) is metabolised to the free thiol containing thiomalate in vivo (Rudge et al 1983, Jellium and Munthe 1980). Both drugs would therefore have the potential to directly scavenge oxygen radicals or to participate in thiol-disulphide exchange reactions with proteins.

The intriguing possibility exists that oxygen radicals produced by stimulated PMN may not only oxidise thiol groups on albumin, but may also oxidise immunoglobulin G thiol groups (0.2 thiol moles/moles, Buchwald and Connell, 1974) and that such structural modification may interfere with the function and immunogencity of the molecule, perhaps giving rise to rheumatoid factor formation (cf 1.3.2 and 1.3.3).

Summary

The results of the experiments described in this chapter provided confirmatory evidence of a role for albumin as an oxygen radical scavenger by demonstrating covalent structural modification of the molecule, via thiol group oxidation, following its exposure in vitro to stimulated PMN. Analysis of the effects of albumin, glutathione, superoxide dismutase and azide on the first and second phases of chemotactic peptide induced PMN luminol chemiluminescence additionally afforded new perspectives on the oxygen radical generating pathways responsible for each phase:-

(a) Up to 57% oxidation of thiol groups on 300μM reduced glutathione 4% (v/v) albumin and 50% (v/v) serum occurred following exposure to normal PMN stimulated by opsonised zymosan or chemotactic peptide in vitro.

(b) This oxidation was inhibited by azide but not by superoxide

dismutase.

(c) Reduced glutathione inhibited the first phase of chemotactic peptide stimulated PMN luminol chemiluminescence whereas albumin preferentially inhibited the second phase.

(d) Superoxide dismutase preferentially inhibited the first phase of chemotactic peptide stimulated PMN luminol chemiluminescence whereas azide preferentially inhibited the second phase.

(e) Examination of thiol levels in rheumatoid synovial fluid samples failed to provide evidence of in vivo albumin thiol group oxidation in the disease. The contribution to the total synovial fluid thiol levels afforded by non-albumin protein (such as IgG) was, however, unknown.

Chapter 7

Effect of anti-inflammatory and antirheumatic drugs on the generation of reactive oxygen metabolites by the PMN respiratory burst

7.1 Introduction

Experiments reported in the previous two chapters have demonstrated that a number of agents (namely albumin, glutathione, azide and superoxide dismutase) preferentially inhibit either the first or the second phase of PMN luminol dependent chemiluminescence. While global inhibition of both phases may be attributable to oxygen radical scavenging, a number of alternative explanations remain (Chapter 5). These include impairment of cell viability and function, impaired cell stimulus interaction and quenching of the luminol excited state. A selective inhibition (or enhancement) of only one phase of stimulated PMN chemiluminescence is, however, indicative of oxygen radical scavenging or other modification of oxygen radical generating pathways. A mechanism for demonstrating similar capabilities in other compounds, such as drugs, is therefore provided.

Drugs commonly used in the treatment of patients with rheumatoid arthritis are conventionally divided into 'first line' non-steroidal anti inflammatory drugs and 'second line' anti rheumatic drugs. While many potentially beneficial actions of these drugs have been identified in vivo (including prostaglandin synthetase inhibition and stabilisation of lysosomal membranes (Tomlinson et al 1972, Peters 1973) the bases of their actions in vivo which contribute to any observed therapeutic effect have not been delineated. If oxygen radicals do play an important role in the pathogenesis of rheumatoid arthritis it may be expected that a reduction in the release or subsequent availability of oxygen radicals produced by stimulated PMN may contribute to the efficacy of some or all of the drugs in vivo.

The anti rheumatic drugs (such as gold, D-penicillamine and hydroxychloroquine) have been grouped together on account of their slow acting, disease modifying actions in vivo (Goddard and Butler,

1984). In recent years increasing interest has been paid to the observation that all such drugs either contain a thiol group within their structure or can be readily metabolised to one in vivo (Drury et al 1984). Experiments in Chapters 5 and 6 of this thesis have highlighted the potential importance of thiol groups as scavengers of oxygen radicals in vitro and in vivo.

The aim of the experiments described in this chapter was to use PMN luminol chemiluminescence to investigate the potential for three 'non-thiol containing' nonsteroidal anti-inflammatory drugs and three 'thiol containing' anti-rheumatic drugs to decrease oxygen radical production by stimulated PMN and in particular to do so by oxygen radical scavenging.

The three anti-inflammatory drugs comprised ibuprofen, indomethacin and fenclofenac (the latter was subsequently withdrawn from prescription in the United Kingdom by the Committee of Safety of Medicines in July 1984). The three antirheumatic drugs comprised D-penicillamine, and two preparations of gold namely aurothiomalate (myocrisin) for intramuscular administration, and the new oral triethyl phosphine gold (auranofin) (Figs. 89 and 90).

7.2 Methods

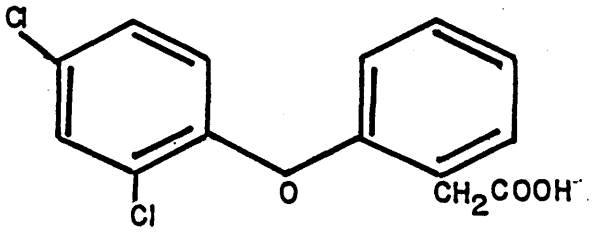
7.2.1 General

For luminol dependent chemiluminescence experiments PMN were isolated from normal whole blood as previously described (2.9). Opsonised zymosan was prepared, and chemotactic peptide and luminol were diluted in Krebs medium from their stock solutions in D.M.S.O. as previously described (2.5, 2.2).

In order to investigate the effects of the chosen drugs on luminol dependent PMN chemiluminescence, cells were pre incubated with

Figure 89 CHEMICAL STRUCTURE OF NON STEROIDAL ANTI INFLAMMATORY DRUGS

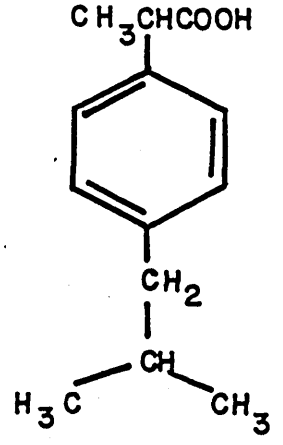
(1)



Fenclofenac MW 297·1

2-(2,4-dichlorophenoxy) phenyl acetic acid

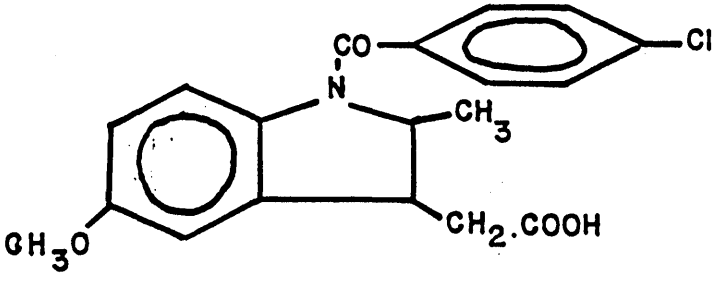
(2)



Ibuprofen MW 206·3

2-(4-isobutylphenyl) propionic acid

(3)



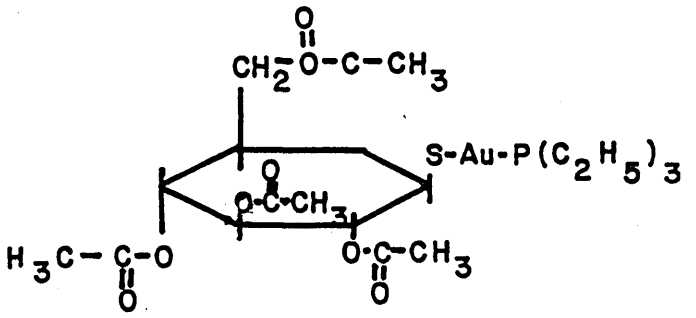
Indomethacin MW 357·8

1-(4-Chlorobenzoyl)-5-methoxy

-2-methylindol-3-yl-acetic acid

Figure 90 CHEMICAL STRUCTURE OF ANTIRHEUMATIC DRUGS

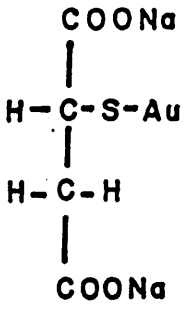
(4)



Auranofin MW 678·5

triethylphosphine gold

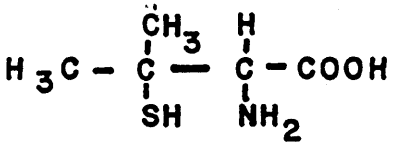
(5)



Aurothiomalate MW 390

disodium aurothiomalate

(6)



D-Penicillamine MW 149·21

D-isomer of 3-mercaptovaline

each drug (or drug diluent alone for control purposes) for 5 min at 37°C prior to addition of stimulus. Cells were stimulated by the concentration of chemotactic peptide or opsonised zymosan previously found to give half maximal luminol chemiluminescence responses (3.3.2, 3.3.3) in order that any enhancing as well as inhibitory effects might be demonstrated. The effects of the drugs on resting (background) PMN luminol chemiluminescence and on the first and second phases of stimulated PMN chemiluminescence were determined and where indicated were expressed as a percentage of the mean of values obtained in the absence of drug or of the chemiluminescence value which would be expected in the absence of drug derived by interpolation of the time course (2.12).

7.2.2 Drug Solubilisation

Available data on in vivo plasma and synovial fluid levels of the drugs following their oral or parenteral administration, yielded values in the range 0.18-62μg/ml (0.3-219μM) (Table 36). In order to attain these concentrations in vitro, the conditions for full solubilisation of each drug in Krebs medium were carefully defined. D-penicillamine, soluble in water at 10% (w/v) at 20°C (Dista, 1983) was freely soluble in Krebs medium allowing the formation of a 'stock solution' in the range 0.22 - 223μg/ml (15 - 1.5mM) pH7.4. Ibuprofen, normally insoluble in water (Boots, 1983) was kindly provided by Boots PLC as the dihydrate salt which was also found to be freely soluble in Krebs medium, allowing the formation of 'stock solutions' in the range 5 - 500μg/ml (2.42-2420μM) pH 7.4.

The remaining drugs were either insoluble in Krebs medium alone, or caused marked calcium/magnesium salt precipitation. Following the recommendations of Van Dyke et al (1979) full solubility of indomethacin in Krebs medium was achieved by first dissolving the

TABLE 36

Plasma and synovial fluid concentration of anti inflammatory and anti rheumatic drugs

Drug	Dose	Mean Plasma Conc.	Range	Synovial Fluid Conc.	Reference
1. Fenclofenac	600mg oral		5 -65µg/ml (17-219µM)		Reckitt & Colman (1978)
2. Ibuprofen	400mg oral	25.8µg/ml	2.5-32µg/ml	8.2µg/ml	Boots (1983) Glass & Swannell (1980)
3. Indomethacin	50mg oral	0.5µg/ml (1.4µM)	2 - 4µg/ml (5.6-11.2µM)	0.5µg/ml (after 5 hours) (1.4µM)	Wade (1977) Goodman Gilman et al (1980)
4. Auranofin	6mg oral	0.49µg/ml (0.7µM)	-	0-0.18µg/ml (0-0.3µM)	Furst et al (1982) Gottlieb (1982)
5. Aurothiomalate	50mg i.m.	0.75-1.25µg/ml (1.92-3.2µM)	0.75-7µg/ml (1.92-17.9µM)	-	Gottlieb (1982)
6. D-penicillamine	750 oral	-	10-20µg/ml (67-134µM)	-	Van der Korst et al (1981)

drugs in Ca^{2+} and Mg^{2+} free phosphate buffered saline pH 7.4, containing 1% (v/v) DMSO, allowing the formation of stock solutions in the ranges 5 - 500µg/ml (1.4 - 1400µM). Stock solutions of aurothiomalate, freely soluble in water (May and Baker, 1983) were formed in Ca^{2+} and Mg^{2+} free phosphate buffered saline alone at concentrations in the range 0.5 - 500µg/ml (1.3 - 128mM). During experiments both indomethacin and aurothiomalate were further diluted in Krebs medium pH 7.4 without visible calcium or magnesium precipitation.

Fenclofenac and auranofin were initially solubilised in 100% DMSO giving stock concentrations in the ranges 0.5 - 50mg/ml (1.7 - 168mM and 0.7 - 74mM respectively). On further dilution 1:100 in Krebs medium pH 7.4 no calcium or magnesium precipitation was visible.

During examination of the effects of individual drugs on PMN luminol dependent chemiluminescence the concentration of DMSO and/or phosphate buffered saline was maintained constant (0.1% (v/v)DMSO, 10% (v/v) phosphate buffered saline) irrespective of changes in the drug concentration. While the presence of phosphate buffered saline caused only minimal inhibition of the first phases of chemotactic peptide induced PMN luminol dependent chemiluminescence (Table 37) the simultaneous presence of 0.1% DMSO (v/v), a known singlet oxygen scavenger, caused inhibition of resting and stimulated PMN chemiluminescence with preferential inhibition of the first phase by 50% (Table 37). The pH of all drug solutions was rigorously maintained at pH 7.4. None of the drugs was found to impair light transmission over the spectrum of that emitted by luminol (c.f. Fig 62, 5.3.2) (data not shown).

TABLE 37 Effect of control drug diluents on chemotactic peptide stimulated luminol dependent PMN chemiluminescence

Drug Diluent	Background CL counts/s	1st Phase (60s) CL counts/s	2nd Phase (220s) CL counts/s	Probability that effect of 2 phases the same ($p<0.05$)
10% (v/v) phosphate buffered saline	108.5 ± 10.6%	95 ± 1.4%	100.5 ± 0.7%	$p < 0.01$
0.1% (v/v) DMSO and 10% (v/v) phosphate buffered saline	74.3 ± 11.5%	47.5 ± 11.8%	77.5 ± 10.4%	$p < 0.05$

5×10^5 PMN/ml were stimulated by 0.1μM chemotactic peptide. Results expressed as percentage of result obtained in absence of diluent. Krebs medium, pH 7.4, 37°C, 10μM luminol (means ± SD, n = 3)

7.3 Results

7.3.1 Non steroidal anti-inflammatory drugs

The aim of the experiments described in this section was to determine whether any of the three non steroidal anti inflammatory drugs (fenclofenac, ibuprofen or indomethecin) would inhibit (or enhance) the luminol chemiluminescence of PMN which were resting or stimulated by chemotactic peptide or opsonised zymosan in half maximal dosage (3.3.2). The concentrations of drugs chosen were both those found in plasma and synovial fluid following oral or parenteral administration (Table 36), and higher concentrations which may occur at cellular microlocations in vivo (Penneys et al 1976). The drugs were pre-incubated with the cells at 37°C for 5 minutes prior to stimulation and any inhibitory or enhancing effects on PMN chemiluminescence were assessed:-

(a) by the percentage change in the value of resting PMN chemiluminescence when compared with that occurring in the absence of drug (=100%)

(b) by percentage change in the peak height of the stimulated PMN chemiluminescence trace (equivalent to peak height of second phase)

and

(c) by comparison of the degree of any inhibiting or enhancing effect of a drug on the first and second phases of stimulated PMN chemiluminescence.

Resting (background) PMN Chemiluminescence

Ibuprofen had no consistent effect (Table 40 and 41). Fenclofenac caused an enhancement which was not dose dependent (Tables 38 and 39). Indomethacin was inhibitory only at high concentrations

TABLE 38 Effect of fenclofenac on luminol dependent chemilumienscence of PMN resting or stimulated by chemotactic peptide

Concentration Drug	Background	1st Phase (60s)	2nd Phase (220s)	Probability that effect on two phases the same ($p<0.05$)
0.5µg/ml(1.7µM)	142.6 ± 6.7%	109.7 ± 31.9%	103 ± 0%	p = 0.36 (NS)
5.0µg/ml(17µM)	136 ± 26.5%	76.7 ± 15.6%	65 ± 8.5%	p = 0.16 (NS)
50.0µg/ml	123.3 ± 29.2%	52.3 ± 9.6%	31.7 ± 3.2%	p = 0.013

$5x10^5$PMN/ml stimulated by 0.1uM chemotactic peptide
Results expressed as percentage of values obtained in absence of fenclofenac (but presence of 0.1% (v/v) DMSO). Krebs medium, pH 7.4, 37°C, 10µM luminol.
Means ± SD, n = 3. Figures within dotted lines indicate concentration of drug found in vivo.

TABLE 39 Effect of Fenclofenac or luminol dependent chemiluminescence of PMN resting or stimulated by opsonised zymosan

Concentration Drug	Background	1st Phase (60s)	2nd Phase (420s)	Probability that effect on two phases the same ($p<0.05$)
0.5µg/ml (1.7µM)	101.5 ± 3.5%	106.5 ± 4.9%	116.5 ± 4.9%*	p = 0.09 (NS)
5.0µg/ml (17µM)	112.3 ± 9.5%	99.7 ± 16.2%	125 ± 11.5%	p = 0.046
50.0µg/ml (170µM)	132.6 ± 8.6%	92.7 ± 14.6%	80 ± 6.9%	p = 0.12

5x10^{5}PMN/ml stimulated by 1mg/ml opsonised zymosan.
Results expressed as percentage of values obtained in absence of fenclofenac (but presence of 0.1% (v/v) DMSO). Krebs medium, pH 7.4, 37°C, 10µM luminol (Means ± SD, n = 3 or *n = 2). Figures within dotted lines indicate concentrations of drug found in vivo.

TABLE 40 Effect of ibuprofen on luminol dependent chemiluminescence of PMN resting or stimulated by chemotactic peptide

Concentration Drug	Background	1st Phase (60s)	2nd Phase (220s)	Probability that effect on two phases the same ($p<0.05$)
0.5µg/ml (2.42µM)	88.3 ± 15.6%	117.0 ± 36.9%	104.7 ± 14.7%	p = 0.31 (NS)
5.0µg/ml (24.2µM)	114.0 ± 20.9%	99.3 ± 18.9%	94.7 ± 14.0%	p = 0.37 (NS)
25.0µg/ml (121µM)	115.3 ± 30.3%	66 ± 24.1%	69.0 ± 7.2%	p = 0.49 (NS)
100.0µg/ml (484µM)	79.3 ± 14.5%	48 ± 12.5%	39.3 ± 6.4%	p = 0.17 (NS)
500.0µg/ml (2420µM)	38.0 ± 10.5%	14.5 ± 4.7%	8.4 ± 1.6%	p = 0.048

5x10^{5}PMN/ml stimulated by 0.1µM chemotactic peptide.
Results expressed as percentage of values expected in absence of ibuprofen.
Krebs medium, pH 7.4, 37°C, 10µM luminol.
Means ± SD, n = 3. Figures within dotted lines indicate concentrations of drug found in vivo.

TABLE 41 Effect of ibuprofen on luminol dependent chemiluminescence of PMN resting or stimulated by opsonised zymosan (1mg/ml)

Concentration Drug	Background CL counts/s	1st Phase (60s) CL counts/s	2nd Phase (420s) CL counts/s	
0µg/ml	530 ± 50	3025 ± 193	63277 ± 5280	(n = 3)
5µg/ml (24.2µM)	652 ± 47 (123%)	3376 ± 459 (112%)	72074 ± 8712	(n = 2)
50µg/ml (242µM)	682 ± 164 (129%)	2832 ± 360 (94%)	69713 ± 14192	(n = 3)
500µg/ml (2420µM)	462 ± 18 (87%)	1973 ± 158 (65%)	48787 ± 6867	(n = 2)

5x10^{5}PMN/ml stimulated by 1mg/ml opsonised zymosan.
Chemiluminescence counts/s in presence and absence of ibuprofen.
Krebs medium, pH 7.4, 37oC, 10µM luminol.
(Means ± SD, n = 2 or 3). Figures within dotted lines indicate concentrations of drug found in vivo.

not found in vivo (Table 42, 43 and 36).

Stimulated PMN chemiluminescence

Each of the non steroidal anti inflammatory drugs when pre-incubated with PMN at concentrations found in vivo (Table 44) inhibited the peak height of chemotactic peptide stimulated PMN chemiluminescence by approximately 30%. When cells were stimulated by opsonised zymosan only fenclofenac inhibited - by 20% (Table 44).

When the degrees of inhibiton of both phases were compared for cells stimulated by chemotactic peptide, indomethacin and fenclofenac were found to inhibit both phases, the inhibition of the second phase being slightly in excess of that of the first phase for both drugs ($p<0.05$) (Tables 38 and 42). With ibuprofen, preferential inhibiiton of the second phase was only observed at high concentrations of the drug (500μg/ml) not found in vivo in plasma or synovial fluid ($p<0.05$, Table 40). When cells were stimulated by opsonised zymosan preferential inhibition of the second phase only occurred with indomethacin and at a concentration (50μg/ml, 140μM) higher than that found in plasma or synovial fluid (Tables 39, 41, 43).

7.3.2 Anti-rheumatic drugs

The effects of the anti rheumatic drugs D-penicillamine, auranofin and aurothiomalate on resting and stimulated PMN luminol chemiluminescence were similarly assessed.

Resting (background) PMN chemiluminescence

Although not inhibitory at concentrations of the drug found in vivo (Table 45), 150 and 1500μM D-penicillamine enhanced PMN resting chemiluminescence by up to 130%. Both at concentrations of the drug found in vivo and at higher concentrations (Tables 46 and 47) aurothiomalate showed no significant enhancement or inhibition of PMN

TABLE 42 Effect of indomethacin on luminol dependent chemiluminescence of PMN stimulated by chemotactic peptide

Concentration Drug	Background	1st Phase (60s)	2nd Phase (220s)	Probability that effect on two phases the same ($p<0.05$)
0.5µg/ml (1.4uM)	99.5 ± 10.1%	80.5 ± 25.1%	89.8 ± 10.5%	p = 0.29 (NS)
5.0µg/ml (14uM)	116.3 ± 15.4%	87.5 ± 5.8%	71.0 ± 3%	p = 0.006
25.0µg/ml (70uM)	75.3 ± 15%	31.2 ± 7.0%	28.2 ± 5.4%	p = 0.29 (NS)
50.0µg/ml (140uM)	66.0 ± 15%	13.9 ± 3.4%	9.0 ± 1.2%	p = 0.038

$5x10^5$PMN/ml stimulated by 0.1µM chemotactic peptide
Results expressed as percentage of chemiluminescence obtained in absence of indomethacin.

TABLE 43 Effect of Indomethacin on luminol dependent chemiluminescence of PMN resting and stimulated by opsonised zymosan

Concentration Drug	Background	1st Phase (60s)	2nd Phase (420s)	Probability that effect on two phases the same ($p<0.05$)
5µg/ml (14uM)	122.9 ± 10.7%	106 ± 21.9%	94 ± 21%	p = 0.27 (NS)
50µg/ml (140uM)	98.3 ± 20.5%	94 ± 9.5%	54.5 ± 3.7%	p = 0.002

$5x10^5$PMN/ml stimulated by 1mg/ml opsonised zymosan
Results expressed as percentage of values obtained in absence of indomethacin but presence of 0.1% (v/v) DMSO.
(Means ± SD, n = 3)
Krebs medium, pH 7.4, 37°C, 10µM luminol.
Figures within dotted lines indicate concentration of drug found in vivo.

Table 44 Maximum inhibition of peak height second phase chemiluminescence by concentrations of drugs found in vivo

Drug	Chemotactic Peptide (0.1μM	Opsonised Zymosan (1mg/ml)
Fenclofenac (17-170μM)	29.3 ± 3.2%	20 ± 2.0%
Ibuprofen	31.0 ± 7.2%	no inhibition
Indomethacin (1.4-14μM)	29.0 ± 3.0%	no inhibition
D-penicillamine (15-150μM)	no inhibition 2nd phase	------
Aurothiomalate (13μM)	no inhibition	------
Auranofin (0.007-0.7μM)	57.7 ± 16.6%	27%

$5x10^5$PMN/ml stimulated by 0.1μM chemotactic peptide or 1mg/ml opsonised zymosan in the presence of one of the six drugs shown (each in concentration found in plasma or synovial fluid invivo (Table 36)). The figures indicate the maximum inhibiti ons observed (means ± SD, n = 3). Krebs medium, pH 7.4, 37°C, 10μM luminol.

TABLE 45 Effect of D-Penicillamine on luminol chemiluminescence of PMN, resting or stimulated by chemotactic peptide

µM D-penicillamine	Background Chemilumnescence	1st Phase (60 sec)	2nd Phase (220 sec)	Probability that effect on 2 phases the same ($p<0.05$)
1.5µM (0.22µg/ml)	99.5 ± 5.5%	91.5 ± 3.5%	105 ± 4.3%*	NS
15µM (2.2 µg/ml)	105.3 ± 3.8%	58.6 ± 1.2%	106 ± 5.8%#	$p < 0.01$
150µM 22µg/ml)	130.5 ± 9.5%	33.5 ± 1.4%	103 ± 2.8%*	$p < 0.05$
1500µM (223 µg/ml)	132 ± 10%	26 ± 0.7%	109 ± 3.5%*	$p < 0.05$

$5x10^5$PMN/ml were stimulated by 0.1µM chemotactic peptide in the presence of 1.5 - 1500µM D-penicillamine. Krebs medium pH 7.4, 10µM luminol 37°C. Results expressed as percentage of chemiluminescence counts/second in absence of D-penicillamine interpolated from time course (*mean ± SEM, n = 2, #mean ± SEM, n = 3). (Dotted lines indicate range of concentrations found in vivo 10-20µg/ml = 67 - 134µM).

TABLE 46 Effect of Aurothiomalate on luminol dependent chemiluminescence of PMN resting or stimulated by chemotactic peptide

Concentration Drug	Background	1st Phase (60s)	2nd Phase (220s)	Probability that effect on two phases the same ($p<0.05$)
0.5µg/ml (1.3µM)	100 ± 23.6%	114 ± 21%	93 ± 12.4%	p = 0.10 (NS)
5.0µg/ml (13µM	119 ± 29.5%	133 ± 18%	103 ± 15.4%	p = 0.02
50.0µg/ml (130µM)	153 ± 36.8%	135 ± 29%	149 ± 23.0%	p = 0.24 (NS)
500.0µg/ml (1.3µM)	118 ± 22.1%	83 ± 13%	165 ± 12.1%	p = 0.0002

5x10^{5}PMN/ml stimulated by 0.1µM chemotactic peptide.
Results expressed as percentage of chemiluminescence expected in absence of aurothiomalate (but in presence of 10% (v/v) PBS in Krebs medium) derived by interpolation of time course).
Krebs pH 7.4, 37°C, 10µM luminol
Means ± SD, n = 4 or n = 3
Dotted lines indicate concentrations of drug found in vivo.

TABLE 47 Effect of Na Aurothiomalate on luminol dependent chemiluminescence of PMN resting or stimulted by opsonised zymosan

Conc. Aurothiomalate	Background CL counts/s	1st Phase (60s) CL counts/s	2nd Phase (420s) CL counts/s	
0ug/ml	1356 ± 259	9931 ± 3382	145,699 ± 4986	(n=2)
50ug/ml (130μM)	1541 ± (119%)	7244 (73%)	123,433 (85%)	(n=1)
500ug/ml (1.3mM)	1207 (89%)	6289 (63%)	91,222 (63%)	(n=1)

$5x10^5$PMN/ml stimulated by 1mg/ml opsonised zymosan.
Results expressed as chemiluminescence counts/s
Krebs medium, pH 7.4, 10% (v/v) PBS. 37°C, 10μM luminol.

resting chemiluminescence (mean 116%, range 89-190%, n = 17 [no drug = 100%]). Auranofin, however, exerted a dose dependent inhibitory effect on PMN resting chemiluminescence, such that 0.5μg/ml (0.74 μM), the mean plasma concentration found in vivo (Table 36) inhibited chemiluminescence by 38.7 ± 6.8% (mean ± SD, n = 3) while 50μg/ml (74μM) inhibited chemiluminescence by > 99% (Table 48).

Stimulated PMN chemiluminescence

Of the three anti rheumatic drugs, at concentrations found in vivo, only auranofin caused inhibition of the peak height of chemotactic peptide stimulated PMN chemiluminescence. This was dose dependent such that 0.74μM auranofin (the mean plasma concentration found in vivo) inhibited the peak height by 57.7 ± 16.6% (mean ± SD, n = 3) (Tables 44 and 48). Auranofin (0.74μM) also achieved a 27% inhibiton of the peak height of opsonised zymosan stimulated PMN chemiluminescence. The effect of D-penicillamine on opsonised zymosan stimulatd cells was not examined. The effect of aurothiomalate was not examined at concentrations of the drug found in vivo (Table 47).

At concentrations of drugs above those found in plasma and synovial fluid in vivo (Table 36), auranofin again inhibited peak height chemiluminescence for both stimuli in dose dependent fashion (Tables 48 and 49). D-penicillamine (1500μM) caused a slight enchancement (109 ± 3.5%, n=3) (Table 45). Aurothiomalate caused dose dependent inhibiiton of the peak height of opsonised zymosan stimulated PMN chemiluminescence (1.3μM aurothiomalate causing 37% inhibiton) but enhanced the peak height of chemotactic peptide stimulated PMN chemiluminescence (1.3mM aurothiomalate enhancing by 165 ± 12.1% (mean ± SD, n = 3) (Fig 91).

When the degree of inhibition or enchancement of both phases of the stimulated chemiluminescence traces was compared, the most

TABLE 48 Effect of Auranofin on luminol dependent chemiluminescence of PMN resting or stimulated by chemotactic peptide

Conc. Drug	Background	1st Phase (60s)	2nd Phase (220s)	Probability that effect on 2 phases the same ($p<0.05$)
0.005µg/ml (7.4nM)	108%	85%	95%	---
0.05µg/ml (74nM)	95 ± 24%	103.5 ± 43.1%	96 ± 15.6% (n=2)	p = 0.41 (NS)
0.25µg/ml (368µM)	65%	102%	103%	---
0.5µg/ml (740µM)	61.3 ± 6.8%	86 ± 20.8%	42.3 ± 16.6% (n=3)	p = 0.023
2.5µg/ml (3.68µM)	10.7%	1.9%	0.1%	---
5.0µg/ml (7.4µM)	7.1%	1%	0.1%	---
50.0µg/ml (74µM)	<1%	<1%	<1%	---

5.0×10^5 PMN/ml stimulated by 0.1µM chemotactic peptide
Results expressed as percentage of chemiluminescence expected in absence of auranofin (but in presence of 0.1% (v/v) DMSO derived by interpolation of time courses).
Means ± SD. Dotted lines indicate concentration of drug found in vivo.

TABLE 49 Effect of auranofin on luminol dependent chemiluminescence of PMN resting or stimulated by opsonised zymosan (1mg/ml)

Conc. Auranofin	Background CL counts/s	1st Phase (60s)CL counts/s	2nd Phase (220s) CL counts/s
0µg/ml	433 ± 51	8121 ± 1380	75,167 ± 5845
0.05µg/ml (74nM)	394	7217	70,887
0.5µg/ml (740uM)	223	7044	54,718
2.5µg/ml (3.68µM)	96 ± 56	1,636 ± 921	2,271 ± 1705 (n = 2)
5.0µg/ml (7.4µM)	7.5	13	- 10

$5x10^5$PMN/ml stimulated by 1mg/ml opsonised zymosan.
Chemiluminescence counts/s in presence and absence of auranofin
Krebs medium, pH 7.4, 37°C, 0.1% (v/v) DMSO. 10µm luminol.
Dotted lines indicate concentration of drug found in vivo.

significant differences occurred with the drug D-penicillamine. While exerting no effect on the peak height chemiluminescence of chemotactic peptide stimulated cells, this drug inhibited the first phase in dose dependent fashion (Table 45), both at concentrations found in vivo and above.

Auranofin inhibited both first and second phases of chemotactic peptide and opsonised zymosan stimulated chemiluminescence, the effect being more profound on the second phase (Tables 48 and 49, Figs 92 and 93). The above mentioned enhancement of chemotactic peptide stimulated PMN chemiluminescence achieved by aurothiomalate, at concentrations above those found in plasma and synovial fluid, preferentially affected the second phase (Table 46, Fig 91). No differences between the two phases were noted for the inhibitory effect of this drug on opsonised zymosan stimulated PMN chemiluminescence.

7.4 Discussion

In the experiments described in this chapter the effects of three non steroidal anti-inflammatory drugs and three anti-rheumatic drugs on the luminol chemiluminescence of PMN which were resting or stimulated by opsonised zymosan or chemotactic peptide were examined. Evidence was sought for decreased oxygen radical production, and specifically for oxygen radical scavenging, by respectively examining the effects of the drugs on stimulated chemiluminescence both globally (peak height chemiluminescence) and differentially (on the first and second phases).

The maximum amount of inhibition was achieved by the anti rheumatic drug auranofin (table 44). This drug, at concentrtions found in vivo inhibited the peak height of chemotactic peptide sitmulated PMN chemiluminescence by up to 58% and that of opsonised

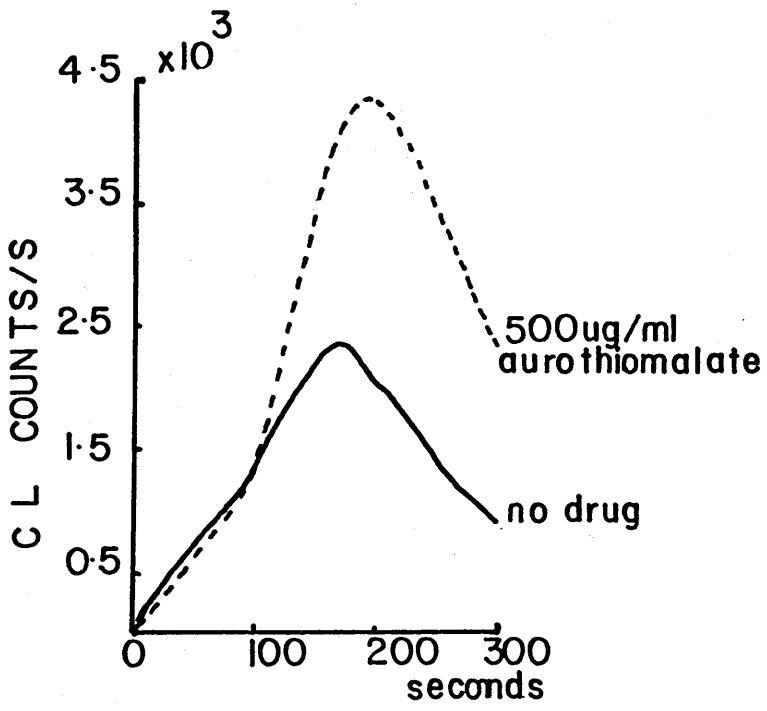


Figure 91 Effect of aurothiomalate on chemotactic peptide stimulated PMN chemiluminescence

Chemiluminescence traces produced by 5×10^5 PMN/ml stimulated by 0.1uM chemotactic peptide in the presence (dotted line) and absence (unbroken line) of 500ug/ml sodium aurothiomalate. Krebs medium, pH 7.4, 37°C, 10μM luminol.

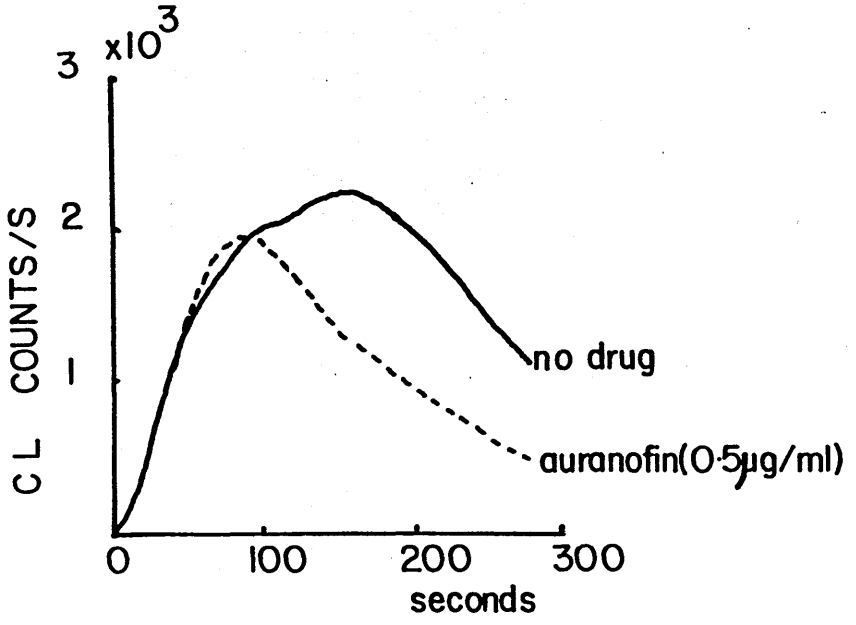


Figure 92 Effect of auranofin on chemotactic peptide stimulated PMN chemiluminescence

Chemiluminescence produced by 5×10^5 PMN/ml stimulated by 0.1µM chemotactic peptide in the presence (dotted line) and absence (unbroken line) of 0.5µg/ml auranofin (triethylphosphine gold). Krebs medium, pH 7.4, 37°C, 10µM luminol.

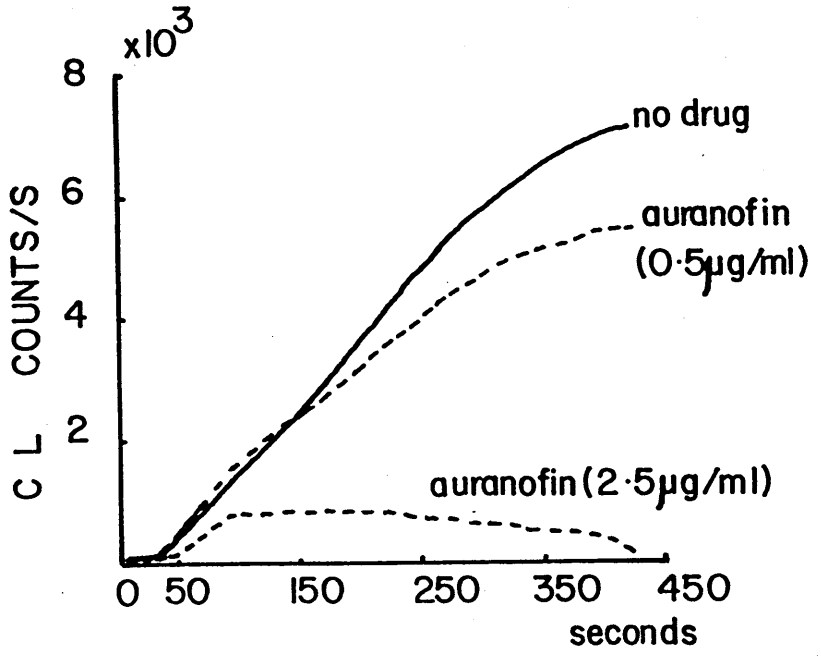


Figure 93 Effect of auranofin on opsonised zymosan stimulated PMN chemiluminescence

Chemiluminescence traces produced by 5×10^5 PMN/ml stimulated by 1mg/ml opsonised zymosan, in the presence (dotted lines) and absence (unbroken line) of 0.5-2.5µg/ml auranofin (triethylphosphine gold). Krebs medium, pH 7.4, 37°C, 10µM luminol.

zymosan stimulated chemiluminescence by up to 27%. Furthermore, auranofin exerted a differential effect on the chemiluminescence for both stimuli, preferentially inhibiting the second phase and thereby indicating that the decreased chemiluminescence resulted, at least in part, from oxygen radical scavenging or pathway modification.

The three non steroidal anti inflammatory drugs fenclofenac, ibuprofen and indomethacin each inhibited the peak height of chemotactic peptide stimulated PMN chemiluminescence by 30% (Table 44). For indomethacin and fenclofenac the inhibitory effect on the second phase was greater than that on the first ($p<0.05$) again suggesting that a proportion of the inhibition resulted from oxygen radical scavenging. It must be emphasised however that the sample numbers were small (n=3). For ibuprofen, such a differential effect only occurred at concentrations above those found in plasma and synovial fluid in vivo.

Although the anti-rheumatic drug D-penicillamine did not globally inhibit the chemiluminescence of PMN stimulated by chemotactic peptide (no decrease in peak height chemiluminescence), the strongest evidence for an oxygen radical scavenging action was none-the-less provided by this drug (Table 45) which preferentially inhibited the first phase.

At concentrations found in vivo the remaining anti rheumatic drug aurothiomalate had no effect on stimulated PMN chemiluminescence. Higher concentrations of the drug however preferentially enhanced the second phase of chemotactic peptide stimulated chemiluminescence to $165 \pm 12.1\%$ (mean $\pm$ SD, n=3). At such concentrations this drug therefore appeared capable of increasing oxygen radical production by PMN. This presumably occurred by modification of the oxygen radical generating pathway, or by enhanced receptor expression (Hurst et al 1986). Aurothiomalate did not enhance the chemiluminescence of

resting PMN however, and thus was not itself a cell stimulant.

The preferential inhibition of the second phase of chemotactic peptide and opsonised zymosan stimulated PMN chemiluminescence seen most obviously with auranofin (Figs 92 and 93) and less markedly with fenclofenac and indomethacin for chemotactic peptide stimulated PMN (Tables 38 and 42), being similar to the effect of azide (Fig 79, 6.3.2) was thus indicative of myeloperoxidase inactivation or $^1\Delta_g O_2$ scavenging (6.4). Likewise the preferential inhibitory effect of D-penicillamine on the first phase of chemotactic peptide stimulated PMN chemiluminescence (Table 45) being similar to the effect of superoxide dismutase (Fig 80 6.3.2) was indicative of scavenging of $O_2^{\cdot -}$, H_2O_2 or products of their reaction (6.4)

Additional evidence for an oxygen radical scavenging role for those drugs was provided concurrently by a number of groups (Table 50). Neither of the groups using luminol chemiluminescence had sought evidence of a differential effect of the drugs on the early or late phases of stimulated PMN chemiluminescence (Van Dyke et al 1979, Davis et al 1982). These authors thus had no mechanism for distinguishing oxygen radical scavenging from other causes of PMN chemiluminescence inhibiiton (altered pH, decreased light transmission, altered cell viability and function and impaired cell-stimulus interaction cf. Chapter 5).

In the experiments of this chapter the effects of pH and light transmission could be discounted (7.2.). While the effects of the drugs on cell viability were not formally tested (5.3.2) only auranofin had any consistent inhibitory effect on resting PMN luminol chemiluminescence. Whilst impaired cell viability cannot be ruled out as a cause of this inhibition it is more likely that the effect of auranofin mirrored that of albumin and azide which inhibited second

Table 50 Evidence for oxygen radical scavenging by non steroidal anti-inflammatory and anti rheumatic drugs.

Drug	System in which inhibition demonstrated	Authors	Year
Ibuprofen	PMN cytochrome c reduction	Simchowitz et al	1979
Ibuprofen Indomethacin	PMN luminol chemiluminescence	Van Dyke et al	1979
Indomethacin	PMN nitroblue tetrazolium reduction	Herzer and Lemmel	1980
Indomethacin	Synovial fluid degradation by enzymically generated oxygen radicals	Puig-Parellada and Planas	1978
D-penicillamine	Hyaluronic acid degradation by enzymically generated oxygen radicals	Betts and Cleland	1982
D-penicillamine	Inactivation of α-1-proteinase inhibitor by enzymically generated oxygen radicals	Matheson	1982
Auranofin	PMN luminol chemiluminescence	Davis et al	1982
Aurothiomalate	PMN chemiluminescence	Harth et al	1983

phase and resting PMN chemiluminescence (5.3.3 and 6.3.2). Albumin was shown not to cause a decrease in cell viability (5.3.2).

With regard to any effect on the drugs on cell stimulus interaction, Simchowitz et al (1979) had previously shown that 100μM indomethacin and 1mM ibuprofen had no significant effect on the cellular binding of tritrated chemotactic peptide (n-formyl-met-leu-phe). Subsequently Davis et al (1982, 1983) showed that neither auranofin nor aurothiomalate interfered with cell stimulus binding for chemotactic peptide or for candida albicans.

While, in the experiments of this chapter, the most convincing evidence of oxygen radical scavenging was provided for the antirheumatic drugs auranofin and D-penicillamine, the differing effects of these drugs, and of aurothiomalate would appear to mitigate against common involvement of the drugs 'thiol' groups in modification of the oxygen radical generating pathways or in a common method of oxygen radical scavenging on their mode of action in vivo. In fact only D-penicillamine was presented to the cells in vitro as a free thiol (Fig 90). This drug, alone, exhibited the same preferential inhibitory effect on the first phase of stimulated PMN chemiluminescence as reduced glutathione (Fig 80, 6.3.2). By contrast the sulphur moieties in auranofin and aurothiomalate were not available as free thiols (Fig 90). Although in neither molecule was the sulphur fully oxidised, and thus in both it remained a likely site for redox reactions and hence oxygen radical scavenging.

Davis et al (1982) found no inhibitory effect of whole aurothiomalate on stimulated PMN luminol chemiluminescence while Harth et al (1983) found a dose dependent inhibition of opsonised zymosan stimulated PMN chemiluminescence in the presence of 2-25μg/ml aurothiomalate. The enhancing effects of the higher concentrations of

aurothiomalate on the second phase of chemotactic peptide stimulated PMN luminol chemiluminescence demonstrated in this chapter had thus not previously been observed. Recently Hurst et al (1986) found that monocytes isolated from rheumatoid arthritis patients who had responded to therapy with aurothiomalate on D-penicillamine subsequently showed enhanced receptor mediated superoxide generation in vitro. The drugs themselves were said to have no such effect on monocytes in vitro. These authors proposed that the beneficial actions of the drugs in vivo might reflect a helpful effect of oxygen radical production on the immune system.

In the experiments of this chapter, however, an enhancing effect of aurothiomalate only occurred at concentrations of the drug which exceeded the concentration in plasma and synovial fluid. Furthermore the concentrations used were in excess of those found in cellular microlocations and were shown by others to impair cell viability (Penneys et al 1976, Davis et al 1982).

In vivo, aurothiomalate has been shown to dissociate to gold and thiomalate (Rudge et al 1983, Jellum and Munthe 1980). Matheson (1982) showed that the thiol group, rather than gold, was responsible for aurothiomalate inhibition of α-1-proteinase inhibitor inactivation by an acellular enzymically generated oxygen radical system. In the past, non thiol containing gold chloride and colloidal gold preparations containing equivalent amounts of elemental gold have been found ineffective in the treatment of rheumatoid arthritis (Drury et al 1984). In order to determine the potential of the 'thiol' component of aurothiomalate as an oxygen radical scavenger it would thus be appropriate to examine the effect of thiomalate on PMN luminol chemiluminescence.

In summary the experiments of this chapter have shown that four

drugs commonly used in the treatment of rheumatoid arthritis, at concentrations found in plasma and synovial fluid in vivo, are capable of decreasing PMN oxygen radical production and/or scavenging oxygen radicals. No common effect for the three antirheumatic drugs (D-penicillamine, auranofin and aurothiomalate) was observed. Only the effect of D-penicillamine was directly attributable to its sulphydryl content. The antirheumatic drug auranofin caused the greatest inhibition of peak height chemiluminescence. Its preferential inhibition of the second phase was mirrored by the anti-inflammatory drugs fenclofenac and indomethacin.

7.5 Summary

Three non-steroidal anti-inflammatory drugs and three 'thiol containing' antirheumatic drugs were examined for their ability to influence the production of oxygen radicals by resting and stimulated PMN. Selective modification of the first or second phases of stimulated PMN chemiluminescence was taken, in particular, as evidence of oxygen radical scavenging by the drugs.

(a) At concentrations found in vivo following oral or parenteral administration the three non steroidal anti inflammatory drugs (fenclofenac, indomethacin and ibuprofen) inhibited peak height of chemotactic peptide stimulated chemiluminescence by 30%. The inhibition preferentially affected the second phase thereby confirming that at least part of the inhibition resulted from oxygen radical scavenging.

(b) The antirheumatic drug auranofin, at concentrations found in vivo, inhibited the peak height of stimulated PMN chemiluminescence by up to 58%. The

second phase was preferentially inhibited, confirming oxygen radical scavenging. Resting chemiluminescence was also inhibited.

(c) D-penicillamine caused no inhibition of peak height chemotactic peptide stimulated PMN chemiluminescence but caused selective inhibition of the first phase (by up to 66%) in a similar fashion to the inhibition caused by reduced glutathione. Strong evidence for oxygen radical scavenging by the reduced thiol group on D-penicillamine was thereby provided.

(d) The remaining antirheumatic drug, aurothiomalate, had no effect at concentrations found in vivo but at higher concentration enhanced the second phase of chemotactic stimulated PMN chemiluminescence by up to 165%.

No common effect for the three sulphur containing anti rheumatic drugs was therfore observed. Only one of these drugs (D-penicillamine) was presented to the cells in vitro as a free thiol, however.

CHAPTER 8

Conclusions, and Future Prospects

8.1 Conclusions

8.2 Future Prospects

8.1 Conclusions

The overall aims of this thesis (1.6) were to provide direct evidence of oxygen radical production by PMN in rheumatoid arthritis and to identify any major physiological and pharmacological scavengers. While at the end of this work direct evidence for oxygen radical production by PMN in rheumatoid arthritis is still lacking, the major and novel conclusion of the work has been the identification of albumin as a potent extracellular oxygen radical scavenger (Chapter 5). In addition the antirheumatic drugs D-penicillamine and auranofin and the non steroidal anti inflammatory drugs fenclofenac and indomethacin were shown to exert oxygen radical scavenging effects in vitro (Chapter 7).

Before the work of this thesis began, the putative role for oxygen radicals in the pathogenesis of rheumatoid arthritis hinged upon the availability of these highly reactive moities for tissue damage (1.5). It followed that any extracellular presence of oxygen radicals in the disease would be a function not only of their release by stimulated phagocytes but of their scavenging by physiological or pharmacological agents.

Demonstration of the oxygen radical scavenging ability of albumin highlighted both the potential importance of this extracellular molecule as a protector against oxygen radical mediated tissue damage, and the vulnerability of it and possibly other protein molecules to simulataneous covalent structural modification by the radicals (such as thiol oxidation) (Chapter 6). Thus albumin, and possibly other protein containing residues capable of scavenging oxygen radicals, may both compensate for the extracellular paucity of superoxide dismutase and catalase (1.2.3) and undergo structural changes of consequence to their function and integrity. Simultaneous with the work of this

thesis evidence has been provided by other authors for the structural modification of proteolytic enzyme inhibitors (Matheson 1982) and IgG (Wickens et al 1983) exposed to oxygen radicals in vitro.

The emphasis on the potential role of oxygen radicals in the pathogenesis of rheumatoid arthritis is therefore changing from one of mere participation in the non specific extracellular destruction of the inflammatory response, to one of structural modification of key protein molecules as a consequence of which the disease may be exacerbated or perpetuated. Demonstration of the in vitro oxygen radical scavenging capability particularly of the anti rheumatic drugs D-penicillamine and auranofin, further suggests that at least part of the beneficial effect of these drugs in vivo in rheumatoid arthritis may occur via this means.

Despite the above, however, evidence for the involvement of oxygen radicals in the pathogenesis of rheumatoid arthritis is, and is likely to remain, circumstantial. The short half lives (ranging from milli to micro seconds) of $O_2^{\cdot -}$, $OH^{\cdot}$ and $^1\Delta_g O_2$ mitigate against their direct detection in serum and synovial fluid. In Chapter 4 of this thesis an attempt was made in vitro to directly measure their rate of production by PMN exposed to rheumatoid synovial fluid and serum known to contain stimuli of phagocytosis and chemotaxis. The failure of the majority of these samples (once denuded of all live cells) to stimulate PMN luminol chemiluminescence probably resulted from the albumin content of the fluids. Albumin in physiological concentration (4g% (w/v)) was subsequently shown to inhibit PMN luminol chemiluminescence by >80% and to account for all of the inhibition of PMN luminol chemiluminescence caused by dialysed serum (Chapter 5).

Having shown that the inhibition of PMN luminol chemiluminescence by albumin resulted from oxygen radical scavenging (and not from

impaired cell viability, altered pH or light transmission, luminol excited state quenching etc (Chapter 5)), the question of precisely which oxygen radical or reactive oxygen metabolite is scavenged by albumin remains unanswered and underlines the chief difficulty in interpretation of luminol chemiluminescence. What luminol lacks in specificity of reaction however, it gains in sensitivity and in facility for continuous global monitoring of the production of oxidising species by PMN. Use of a more specific oxygen radical detector such as cytochrome c would not have allowed the identification of albumin's oxygen radical scavenging capability in the first place (Chapter 5).

The preferential inhibition of albumin on the second phase of chemotactic peptide stimulated PMN chemiluminescence was shared by the myeloperoxidase inhibitor azide (Chapters 5 and 6). The discrepancy between PMN luminol chemiluminescence and oxygen consumption (Chapter 3) further indicated that in the second phase luminol reacted with oxygen metabolites whose existence was not temporally dependent on cellular oxygen consumption. On both accounts it is therefore likely that albumin scavenges a reactive oxygen metabolite produc ed by the interaction of myeloperoxidase - H_2O_2 - Cl^- (Chapter 6).

The consequences to the albumin molecule of its extracellular scavenging of oxygen radicals was explored in Chapter 6. While potential moieties for oxygen radical scavenging on the molecule included amine, tyrosine and histidine residues, the effect of exposure to stimulated PMN on albumin's lone free thiol group was particularly examined. Stimulated PMN were shown to oxidise thiol groups not only on albumin, but in whole serum and on glutathione by up to 60%. This oxidation was inhibitable by azide but not by superoxide dismutase (Chapter 6).

The preferential inhibition of the first phase of chemotactic peptide stimulated PMN chemiluminescence achieved by the low molecular weight thiol glutathione, suggested either that a different moiety on albumin and glutathione caused their inhibition of PMN chemiluminescence or that a different radical was scavenged by each (Chapter 6). Preferential inhibition of the first phase of stimulated PMN luminol chemiluminescence was shared also by the free thiol containing anti-rheumatic drug D-penicillamine (Chapter 7). Preferential inhibition of the second phase was caused by the anti-rheumatic drug auranofin, and to a lesser extent by the anti-inflammatory drugs fenclofenac and indomethacin.

Albumin is the principle source of free thiol in serum. Serum thiol levels were known to be depressed in active rheumatoid disease and to normalise with a clinical response to therapy (Haataja (1975)). In the work of this thesis, the parallel effects of albumin and the antirheumatic drugs on PMN luminol chemiluminescence suggested that one action for these drugs in vivo may be protection of extracellualr proteins from oxygen radical attack, and particularly from thiol oxidation. In rheumatoid arthritis the likely site for such attack is the inflammed joint in which phagocytes and their stimuli accumulate in large numbers.

In Chapter 6 a preliminary attempt was made to correlate the thiol concentrations of rheumatoid and control synovial fluids with their cell counts, albumin concentrations and with drug therapy. It was predicted that the lowest thiol concentrations would occur in patients with rheumatoid arthritis not receiving antirheumatic drugs. In fact the lowest concentrations were found in the control post menisectomy/osteoarthritis group. The contribution of non albumin protein (raised in the rheumatoid patients) to the total thiol levels

of each group was, however, undetermined.

Although predominantly composed of disulphide bonds, immunoglobulin G (1gG) has been shown to possess 0.2 moles/mole free thiol groups. Oxidation of these thiol groups by stimulated PMN could cause aggregation of 1gG, exposure of new antigenic sites and hence a mechanism for the production of rheumatoid factor and the perpetuation of rheumatoid arthritis. Covalent modification of proteolytic enzymes or of protein components of the complement cascade by oxygen radicals produc ed by stimulated PMN may similarly augment the immune response.

One hundred years later, Metchnikoff's proposal that stimulated phagocytes may liberate substances which are detrimental to their hosts is proving correct. For oxygen radicals and rheumatoid arthritis, there is now a requirement for investigation of the occurrence and consequences of oxygen radical mediated covalent modification of extracellular protein molecules, a better understanding of which may lead not only to more specific markers of rheumatoid disease activity but, hopefully, to more effective drug therapy.

8.2 Future Prospects

The experiments described in this thesis, in identifying the oxygen radical scavenging capability of albumin, have drawn attention to the potential of oxygen radicals to covalently modify the structure of extracellular proteins and have suggested that one of the beneficial effects of antirheumatic drugs in vivo may be the prevention of such modification.

Logical extensions to the work are the following:-

(a) More detailed examination of the consequences both structural and functional, which may result in a

variety of proteins following their exposure in vitro to cellular or acellular oxygen radical generating systems.

(b) Examination of rheumatoid serum and synovial fluid for the presence of the defined oxidation products of given oxygen radical-protein interactions.

(c) Investigation of the efficacy of antirheumatic and anti-inflammatory drugs in preventing oxygen radical mediated covalent modification of proteins in vitro and in vivo.

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Appendix I - List of publications and communications to learned societies and scientific meetings arising directly from this work

1. Written publications

(1) Holt Mary E, Ryall M. E. T., & Campbell A. K. (1984)
Albumin inhibits human polymorphonuclear leukocyte luminol dependent chemiluminescence : Evidence for oxygen radical scavenging.
British Journal of Experimental Pathology, 65: 231-241

(2) Holt Mary E. & Campbell A. K. (1984).
Human polymorphonuclear leukocyte luminol chemiluminescence identifies serum albumin as an oxygen radical scavenger. In, Analytical Applications of Bioluminescence and Chemiluminescence, ed Kricka L. J., Stanley P. E., Thorpe G. H. G., Whitehead T. P., pp 311-314. New York, Academic Press.

(3) Campbell A. K., Holt Mary E., & Patel A. (1985)
Chemiluminescence in Medical Biochemistry. In
Recent Advances in Clinical Biochemistry Vol II, pp 1-30,
ed Alberti KGMM and Price C. P. , Edinburgh, Churchill Livingstone.

2. Communications

(1) Poster Holt Mary E., Hallett M. B. & Campbell A. K. (1982)
Chemiluminescence as a monitor of oxygen radical production by polymorphonuclear leukocytes in rheumatoid arthritis. Presented to the National Meeting of the Association of Clinical Biochemists, Cambridge,

Semptember 28th - October 1st.

(2) Oral Holt Mary E. & Campbell A. K. (1983). Synovial fluid chemiluminescence Presented to the South Wales, West Country and Wessex Rheumatology Club Meeting, Talygarn, June.

(3) Oral Holt Mary E. & Campbell A. K. (1983). Albumin scavenges oxygen radicals produced by human polymorphonuclear leukocytes. Presented to the Heberden Society, Edinburgh, July 8th - 9th.

(4) Oral Holt Mary E. & Campbell A. K. (1984). Human polymorphonuclear leukocyte luminol chemiluminescence identifies serum albumin as an oxygen radical scavenger. Presented to 3rd International Symposium in Analytical Applications of Bioluminescence and Chemiluminescence, Birmingham, April 17th - 19th.

Appendix II - List of Abbreviations used in text of Thesis

ABEI	-	amino butyl ethyl iso luminol
ATP	-	adenosine tri phosphate
DMSO	-	dimethyl sulphoxide
DTNB	-	dithiobis 2 nitrobenzoic acid
$^1\Delta_g O_2$	-	singlet oxygen - delta form
EDTA	-	ethylene diamino tetra acetate
HEPES	-	N_2 hydroxy-ethylpiperazine n´2 ethanol sulphonic acid
H_2O_2	-	hydrogen peroxide
1gG	-	immunoglobulin G
M.E.H.	-	Mary Elizabeth Holt
OA	-	osteoarthritis
$O_2^{\cdot -}$	-	superoxide anion
$OH^{\cdot}$	-	hydroxyl radical
PMN	-	polymorphonuclear leukocyte
POPOP	-	p-Bis (2-(5-phenyloxazoyl)-benzine
PPO	-	2,5-Diphenyloxazole
RA	-	rheumatoid arthritis
S.D.	-	standard deviation
S.E.M.	-	standard error of mean
SF	-	synovial fluid
SH	-	sulphydryl or thiol group