THE CHEMILUMINESCENCE OF OVINE NEUTROPHILS

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ABSTRACT

The development, structure and function of polymorphonuclear leucocytes (PMN) is reviewed and methods for determining neutrophil competence are discussed.

A technique, based on differential centrifugation and red blood cell lysis, is described for isolating neutrophils of 80 to 90% purity from ovine blood. A standardised, luminol-enhanced chemiluminescence (CL) assay was developed for ovine neutrophils using latex beads as the phagocytic stimulus and some conditions influencing the level of CL generated are described.

Normal sheep of similar age, housed under identical conditions and bled at approximately the same time on different days produced CL responses ranging from 386 to 3084 millivolts (mV). Animals sampled once daily over 5 days showed large fluctuations in CL values both between and within individuals. Furthermore, sheep bled at 4 hourly and 6 hourly intervals for 48 and 96 hrs respectively produced CL responses in a single individual with a range of 618 to 2946 mV. There was no evidence of periodicity in CL activity over the time periods examined.

Since peak CL responses showed such large variations between individuals, integrated CL values were also measured. Variations between and within individuals similar to those recorded by peak CL were seen in these results.

To examine the possible role of genetic differences in neutrophil function on the variability of CL, pairs of bovine monozygous twins were sampled. There was no correlation in CL response between genetically identical animals with the CL values from pairs of animals differing by as much as 2943 mV.

The effect of cortisol on PMN CL was assessed. Synthetic corticosteroid *in vivo* and *in vitro* did not increase the peak CL response from isolated neutrophils.

Profiles produced by recording CL against time were examined. Some cell isolates produced single peaked profiles while others gave a double peaked response. Single and double peaked profiles were recorded from the same donor at different times during a 24 hr period. Storage of the cells for prolonged periods sometimes resulted in an increase in the magnitude of the first peak possibly indicating an increase in the amount of more readily available myeloperoxidase (MPO). Prominent first peaks were still displayed after the cells were washed and resuspended in fresh media suggesting that the more readily available MPO was cell attached rather than truely extracellular.

Neutrophils from ceroid lipfuscinosis-affected sheep produced peak CL responses and CL profiles similar to those given by normal sheep. These results did not confirm the postulated myeloperoxidase deficiency of this condition.

It is concluded that ovine neutrophil CL is subject to large variations which cannot be controlled by standardising the cell isolation and CL analysis techniques. The assay is therefore unsuitable as a measure of neutrophil function where single samples are examined. Where there are consistant differences between individuals over a number of days, then CL may be of use when considered in conjunction with other tests of PMN function.

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CHAPTER 1

THE POLYMORPHONUCLEAR NEUTROPHIL GRANULOCYTE

1.1 Origin

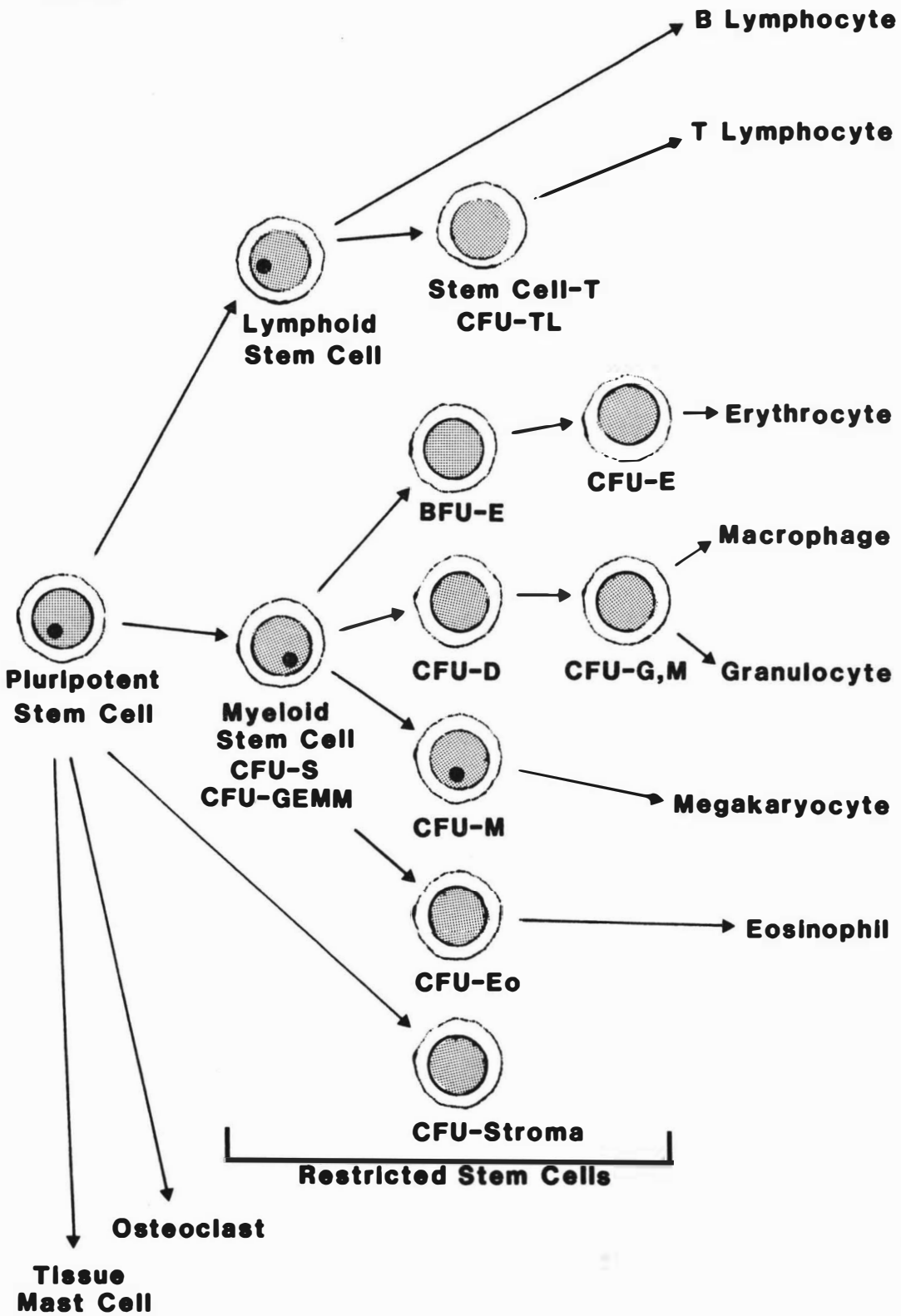
A common precursor for all blood cells has long been postulated but until Till and McCulloch (1961) developed the spleen colony-forming technique, definitive experimental work to test this hypothesis had not been possible. The introduction of *in vitro* soft agar cell culture by Bradley and Metcalf (1966) increased our understanding of haematopoietic tissue although the original assumption that both techniques identify pluripotent stem cells has been modified. It is now accepted that pluripotent or true stem cells, which are defined as cells that maintain their own numbers throughout the lifetime of the animal despite removal by differentiation, are the same as spleen colony-forming units (Lord, 1983). Culture colony-forming units from the original technique have a more limited differentiation potential and probably should not be referred to as pluripotent. Dexter *et al.* (1977) have descibed a modified culture method which is capable of supporting the growth of various types of bone marrow cells including stem cells. With all these techniques it is assumed that each colony originates from a single cell which can be defined by the morphology of the progeny cells. Evidence that a mixture of male and female cells does not produce mixed sex colonies has been cited to confirm this concept (Fauser and Messner, 1979).

The bone marrow pool is believed to contain both early pluripotent stem cells (Trentin et al., 1967; Nowell et al., 1970; Abramson et al., 1977; Messner et al., 1981; Lim et al., 1982) and later restricted stem cells (Becker et al., 1963; Adamson et al., 1976; Fauser and Messner, 1978; Fauser and Messner, 1979). The current understanding of haematopoietic cell development and the nomenclature applied to experimental colony forming units are summarised in Fig. 1.1.

The earliest progenitors are probably present in the mesoderm of the yolk sac and later migrate to the liver, spleen and bone marrow (Moore and Metcalf, 1970). The pattern of colonisation differs between species (Cline, 1976). In man, stem cells are observed in peripheral blood at the thirteenth week of gestation, in the liver at the twelfth to twentythird week, in bone marrow at the fifteenth to sixteenth week and in the spleen at the eighteenth to nineteenth week. The thymus has no haematopoietic activity in the midtrimester foetus (Hann et al., 1983). Granulopoiesis does not occur in significant amounts until after bone marrow haematopoiesis has been established (Thomas and Yoffey, 1962). Stem cells continue to migrate in post-natal life (Quesenbury and Levitt, 1979a). Restricted colonies can be cultured from adult peripheral blood (Fauser and Messner, 1978) and bone marrow which has been subjected to lethal irradiation will be repopulated if a limited area of the body is shielded (Maloney and Patt, 1972).

FIGURE 1.1. Haematopoietic cell development (CFU - colony-forming unit, BFU - blast-forming unit).

FIGURE 1.1.


B Lymphocyte
T Lymphocyte
Stem Cell-T
CFU-TL
Lymphoid
Stem Cell
Erythrocyte
CFU-E
BFU-E
Macrophage
CFU-D
CFU-G,M
Granulocyte
Pluripotent
Stem Cell
Myeloid
Stem Cell
CFU-S
CFU-GEMM
CFU-M
Megakaryocyte
Eosinophil
CFU-Eo
CFU-Stroma
Restricted Stem Cells
Osteoclast
Tissue
Mast Cell

While the morphology of the stem cell has not been fully established, Dicke et al. (1973) described it as ressembling a small lymphocyte. Van Bekkum et al. (1971) isolated cells from mouse bone marrow which appeared similar to small lymphocytes but did not contain Golgi apparatuses, centrioles, endoplasmic reticula or lysosomes, all of which are seen in lymphocytes. Rubinstein and Trobough (1973) isolated what they referred to as a presumptive stem cell; this cell had a round nucleus with numerous folds and a thin rim of cytoplasm containing many ribosomes and few mitochondria and accounted for approximately 0.3% of the bone marrow population.

The stimulus for commitment to a particular line of cellular development is unknown. In vitro, differentiation from stem cell to leucocyte is dependent on the presence of certain colony stimulating factors (CSF). These are a family of glycoproteins which appear to be antigenically related. Each CSF is specific for a particular type of colony. In man the main sources of CSF are macrophages, lymphocytes (Cline and Golde,1979) and placenta (Burgess et al., 1977). Kohsaki et al. (1983) produced granulopoietic stimulation in mice with an extract of human urinary CSF showing that there is a degree of cross-reactivity between species. In vivo, urinary CSF stimulates monocytes to produce specific granulocyte/macrophage colony stimulating factor (GM-CSF) (Motoyoshi et al., 1983). Van Zant and Goldwasser (1977) tested the simultaneous effects of erythropoietin, a hormone necessary for red blood cell development, and CSF on bone marrow cells. They

showed an overlap in specificity and concluded that the target cells of the two factors are the same or closely related. Foetal cells are more sensitive to appropriate stimuli than are adult cells because of intrinsic differences in the stem cells at different developmental stages (Linch et al., 1982).

Cellular development , in vitro, is dependent on cell-derived molecules which stimulate or inhibit proliferation and differentiation of progenitor cells (Cline and Golde, 1979). Such interactions probably keep cell numbers within controlled limits in vivo (Broxmeyer et al., 1980a). Von Schulthess and Mazer (1982) presented a model for the control of granulopoiesis based on data from patients with cyclic neutropaenia. They assumed that the controlled compartment is the bone marrow pool of mature polymorphonuclear neutrophil granulocytes (PMN). In situations where there is an increasing release of mature granulocytes to the blood, PMN production is stimulated. The model is dependent on a negative feedback signal from mature cells to the mitotic pool of early precursors.

Lactoferrin, which is present in the specific granules of PMN, has been identified as a negative feedback regulator of granulocyte and macrophage production (Broxmeyer et al., 1978b). Broxmeyer et al. (1980a) postulated that a subpopulation of mature cells contains an active form of this metal-binding glycoprotein; younger cells are suggested to have proteolytic enzymes capable of inactivating lactoferrin which are lost with maturity. Lactoferrin,

particularly in its iron-saturated form, inhibits the production of GM-CSF from macrophages. It is highly specific, having no effect on macrophage-produced B-lymphocyte CSF and lymphocyte-produced GM-CSF (Broxmeyer et al., 1980b). When the number of mature granulocytes in bone marrow is decreased less lactoferrin is secreted and macrophages release more GM-CSF, stimulating increased myelopoiesis.

A dual feedback control mechanism for granulopoiesis involving a balance between CSF and prostaglandin E, both of which are produced by macrophages, has been proposed (Kurland et al., 1978; Quesenbury and Levitt, 1979b). It is interesting to note that lactoferrin decreases the production of prostaglandin E from human blood monocytes (Broxmeyer et al., 1980b).

The stem cell is influenced by the microenvironment in which it is developing. Thus cells in the spleen tend to develop into erythroid elements whereas those in the bone marrow tend towards myeloid elements (Wolf and Trentin, 1968). Evidence suggests that bone marrow stromal cells arise from the pluripotent haematopoietic stem cell (Izaquirre et al., 1982) and that their arrival in the marrow cavity precedes that of the developing haematopoietic progenitors (Hann et al., 1983). It has been proposed that both osteoclasts (Marks and Walker, 1981) and tissue mast cells (Kitamura et al., 1981) also develop from the pluripotent stem cell.

The relevance *in vivo* of these diverse factors has yet to be defined but it appears that complex cellular interactions control haematopoietic differentiation and development (Erslev, 1971; Haskill *et al.*, 1972; Cline and Golde, 1979).

1.2 Development

The earliest defined neutrophil precursor is the *myeloblast* which is approximately twice the size of the mature cell (Davis and Gallin, 1981). It has a large, oval nucleus containing one or more prominent nucleoli and diffuse chromatin which shows some condensation near the nuclear membrane. The cytoplasm is scant and stains basophilic due to evenly distributed ribonucleoprotein particles. It also contains numerous mitochondria and a sparse endoplasmic reticulum with a Golgi region adjacent to the nucleus (Klebanoff and Clark, 1978). It has been suggested that a neutrophil blast cell can be distinguished from those of the monocyte and lymphocyte series (Klebanoff and Clark, 1978, Davis and Gallin, 1981) but the evidence that all blast cells originate from a common stem cell makes such a classification equivocal (Murphy, 1976). The myeloblast divides and eventually becomes the *promyelocyte* with cytoplasm containing mitochondria, a rough endoplasmic reticulum and a prominent Golgi apparatus. Proteins are synthesised on ribosomes associated with rough endoplasmic reticulum and transported to the Golgi complex where they are incorporated into granules (Klebanoff and Clark, 1978). Primary,

or azurophilic granules, so called because they are the first to appear and colour blue with Wright's stain, develop during this stage. At the end of the promyelocyte phase the cell is approximately two and a half times larger than a mature neutrophil, has an eccentrically-placed, bean-shaped nucleus and cytoplasm packed with granules (Davis and Gallin, 1981). Azurophilic granule production ceases prior to further cell division and the granules are distributed between ensuing daughter cells. The next developmental stage is the myelocyte in which specific, or secondary, granules occur. Cell size decreases during this stage with the nucleus becoming markedly indented and its chromatin condensed and marginated. There is an increase in both ribosomes and endoplasmic reticulum and glycogen particles appear throughout the cytoplasm. Specific granules also arise from the Golgi complex but the mechanism of their production differs from that of primary granules (Bainton and Farquhar, 1966). The transition from myeloblast to myelocyte takes four to six days (Davis and Gallin, 1981). Cell division then ceases but it is five to seven days before the neutrophil is fully mature. Several intermediate stages are recognised, beginning with the metamyelocyte which has a kidney bean-shaped nucleus, the band cell with a horseshoe-shaped nucleus and finally the mature PMN which has a fully segmented nucleus. Throughout the final maturation both cell and nucleus become smaller with increasingly condensed nuclear chromatin. There is a reduction in the number of cellular organelles but the number of granules remains static with a ratio of specific to azurophilic of approximately 2 to 3:1 (Klebanoff and Clark, 1978).

1.3 Structure

The mature PMN has a multilobed nucleus with condensed, marginated chromatin, and cytoplasm containing abundant granules and glycogen particles. The plasma membrane is trilaminar in structure, the major constituents being protein, cholesterol and phospholipid. There is also a small amount of carbohydrate including N-acetylneuraminic acid and some adenosine triphosphatase (ATPase) (Woodin and Wieneke, 1966).It has been suggested that ATPase is responsible for maintaining cationic gradients within the cell (Sha°afi et al., 1976) and is also involved in phagocytosis (Medzihradsky et al., 1976). Changes occur in the cell membrane during the final stages of cell maturation. The negative surface charge decreases giving an increase in adhesiveness and a high degree of surface deformabiity. Motility rates increase and there appears to be development or activation of a chemico-mechanical system to facilitate pseudopod formation (Lichtman and Weed, 1972).

Microfilaments, consisting of actin and associated contractile proteins, occur as a randomly orientated meshwork in the granule-free peripheral cytoplasmic areas (Stossel, 1978). Functional microfilaments are probably necessary for cell orientation and may provide a locomotory apparatus (Gallin et al., 1978). Boxer et al. (1974) studied a child with neutrophils containing poorly polymerizable actin. The cells exhibited fewer

microfilament-rich pseudopodia, migrated more slowly and ingested particles at a 15% slower rate than did normal PMN; secretion of granule contents was two and a half times greater than that of control cells.

Centrioles, which are located between the nuclear lobes, have microtubule-organising sites associated with them. Microtubules, consisting of aggregates of the globular protein tubulin, originate in the organising sites and radiate outward (Malech et al., 1977; Hoffstein, 1980). The tubulin subunits require specific conditions to promote the non-covalent bonding of tubule assembly (Weissmann et al., 1975; Snyder and McIntosh, 1976). These include appropriate levels of certain divalent cations and cyclic nucleotides and the presence of guanosine triphosphate. Intact microtubules are essential for normal pseudopod formation, orientation and maximal migration during chemotaxis (Malech et al., 1977). They help maintain the distribution of specific molecules on the surfaces of cells (Snyder and McIntosh, 1976) and may be required for the movement of granules (Zurier et al., 1973).

The granules are the most prominent cytoplasmic constituents of the mature neutrophil. They have an important role in the destruction of ingested microrganisms and their contents have been studied in considerable detail. Since Cohn and Hirsh (1960b) first suggested that enzymes are important granule constituents various cytochemical techniques have been employed to demonstrate the presence of a range of proteins within these structures, including

a number of hydrolytic enzymes similar to those found in lysosomes. Most investigations have utilised human or rabbit PMN and as granule contents differ between species (Rausch and Moore, 1975), care must be taken in extrapolating results from one species to another.

The primary granules have limiting membranes with a distinct trilaminar structure and often vary in size and shape (Klebanoff and Clark, 1978). Their major enzyme constituent is myeloperoxidase (MPO) which occurs in most species, notable exceptions being chickens and geese (Schultz et al., 1965; Ackerman, 1968). The cellular content and resulting enzymic activity varies between species (Schultz et al., 1965), but in human PMN myeloperoxidase constitutes greater than 5% of the total dry-weight of the cell (Schultz and Kaminker, 1962). Azurophilic granules in some species also contain acid phosphatase, arylsulphatase, beta-galactosidase, beta-glucuronidase, indoxyl elastase, 5'-nucleotidase (Bainton and Farquhar, 1968), neutral protease and lysozyme (Spitznagel et al., 1974). In addition the granules of the immature cell contain sulphated mucopolysaccharide and basic protein, the amount decreasing with maturity (Horn and Spicer, 1964). It has been suggested that azurophilic granules are primary lysosomes since they contain lysosomal enzymes (Bainton and Farquhar, 1968).

The specific granules are also bounded by trilaminar membranes. Their enzyme content differs from that of the primary granule;

they contain lysozyme and in addition lactoferrin (Spitznagel et al., 1974), collagenase (Robertson et al., 1972), vitamin B12-binding protein (Kane and Peters, 1975), folate binder (Colman, 1983) and alkaline phosphatase (Bainton and Farquhar, 1978). The location of alkaline phosphatase is controversial but some reports suggest that it may be a component of the specific granule membrane (Henson, 1971, Spitznagel et al., 1974).

Evidence has been presented recently for the existence of a tertiary granule specifically enriched in cytochrome b and ubiquinone (Crawford and Schneider, 1983; Mollinedo and Schneider, 1984).

1.4 Kinetics

PMN are thought to exist in three major, interconnected compartments defined as bone marrow, blood and tissue. Movement between the three compartments is not random but the factors controlling it are not fully understood.

The granulocyte production system within the bone marrow can be further divided into a stem cell pool, a mitotic pool in which division and maturation occur, a post-mitotic pool in which maturation alone takes place and a storage pool of mature cells. The granulocyte production rate in man has been estimated at 1.6×10^9 cells/kg body weight/day and the mean myelocyte to peripheral

blood transit time at 10 days (Robinson and Mangalik, 1975). Deubelbeiss et al. (1975) suggest a transit time of about 82 hours in the dog. The bone marrow storage pool contains a large reserve of mature neutrophils which can be mobilised into the blood if required (Craddock et al., 1956; Craddock et al., 1960). The human granulocyte spends a mean of 50 hours between maturation and release (Maloney and Patt, 1968). Deubelbeiss et al. (1975) calculated a canine bone marrow pool of 5.6×10^9 PMN/kg body weight and Craddock et al. (1960) estimated a bone marrow reserve of 140×10^{10} PMN in a 70 kg man. Thus the marrow pool is greater than the circulating mass of mature cells. Some studies suggest that there is selection within the storage pool based on the changes in cell deformability and stickiness which occur during maturation (Lichtman and Weed, 1972) because entry to the blood is selective with segmented cells entering in preference to band cells (Maloney and Patt, 1968).

The stimulus for the release of granulocytes from the bone marrow is unknown. The work of Gordon et al. (1960) suggested that a humoral factor stimulated the release of leucocytes in normal animals. Many regulatory factors have been postulated including a diffusible granulopoietic substance (Rothstein et al., 1971), CSF (Cline and Golde, 1979), leucocyte endogenous pyrogen (Kampschmidt et al., 1972) and fragments of the third component of complement (Davis and Gallin, 1981). Endotoxin and etiocholanolone, one of the naturally occurring metabolites of androgens, promote granulocyte release (Davis and Gallin, 1981), possibly via a

mediating substance which may be equivalent to the neutrophilia-inducing factor of Boggs et al. (1966). The substance(s) which initiate bone marrow release probably differ from those concerned with the stimulation of PMN production (Quesenberry et al., 1975). Broxmeyer et al. (1974) suggested that the same factor is responsible but that it has varying effects at different stages of cellular maturity.

The number of circulating PMN remains relatively constant in a healthy animal. Human circulating neutrophils can be separated into two subpopulations by their ability to form rosettes with immunoglobulin-coated erythrocytes (Klempner and Gallin, 1978, Broxmeyer et al., 1978a) and their responses to monoclonal antibodies (Clement et al., 1983). Broxmeyer et al. (1978a) noted that cells which form rosettes contain and release colony inhibiting activity (CIA) while those which do not, contain an inhibitor of CIA. Davis and Gallin (1981) suggest that the subclasses may represent different states of maturation of a single cell line.

It has been demonstrated that the blood pool of neutrophils has two, approximately equal, components which are in dynamic equilibrium. The circulating pool consists of those cells sampled by venipuncture, while the marginating pool contains cells within the vascular space but caught in the slow moving flow along the endothelial surfaces of small veins (Athens et al., 1961). Neutrophils spend a relatively short period in the blood; the

blood half-life in man is about 6.6 hours (Athens et al., 1961) and in dogs this phase is probably even shorter (Craddock et al., 1956).

There are at least two routes by which neutrophils are lost from the blood. The first is a consequence of senescence and involves removal by the reticuloendothelial system, while the second is emigration to the tissues (Fliedner et al., 1964). During emigration the cells initially marginate, then adhere to the endothelial surface and finally migrate out of the vascular space. Gallin (1980) suggested that the mechanism of cell aggregation and margination was related to neutralisation of the negative surface charge of the neutrophil by a limited secretion of intracellular, cationic granule contents. Examination of neutrophil function in a patient with specific granule deficiency did not support this concept (Gallin et al., 1982). Others consider that local changes in blood flow shift the balance in favour of adhesion (Lackie and Smith, 1980) or that the change in adhesiveness rests entirely with the endothelial cell (Murphy, 1976). The eventual exit from the venules is by a process called diapedesis. Electron micrographs show the formation of pseudopodia and cell deformation permitting granulocytes to pass through gaps between endothelial cells (Marchesi and Florey, 1960; Marchesi, 1961).

Although increased numbers of cells shift into the marginating pool during local vascular injury and inflammation, vessel damage is not necessary to initiate migration. Cronkite et al. (1954) found that

granulocytes migrate to areas covered by a high density of commensal organisms setting up a defensive barrier against opportunistic infection. Thus large numbers of leucocytes are found in the oral cavity, presumably due to the chemotactic influence of the commensal flora (Tempel et al., 1970).

The suggested primary role of neutrophils within the body is the localisation and removal of microorganisms. The cells must move to the area where infection exists (chemotaxis), immobilise the pathogens (phagocytosis) and effectively deal with them so that they can no longer function (microbial killing).

1.5 Chemotaxis

Chemotaxis is a phenomenom in which the direction of locomotion of cells is determined by substances in their environment (Wilkinson and Allan, 1978). The process requires three interconnected events, namely, detection of the extracellular chemoattractant, orientation of the cell and locomotion.

Neutrophils in buffer solution exhibit spontaneous random movement. When a chemoattractant is added to the buffer, movement is stimulated but non-directional. Directed migration requires a gradient of chemoattractant across the surface of the cell. Extremely low concentrations of some substances can be detected.

The synthetic peptide N-formyl-methionyl-phenylalamine (FMLP) will initiate directed locomotion at concentrations of 1×10^{-11}M (Showell et al., 1976). Many different substances will stimulate a chemotactic response. Clinically, factors released from bacteria are obviously important in evoking the body's defence mechanisms (Harris, 1954; Keller and Sorkin, 1967), but many agents of host origin have also been defined. These include collagen (Chang and Houch, 1970), a factor from cultured fibroblasts (Sobel and Gallin, 1979), fibrinopeptide (Kay et al., 1974), lymphokines (de Franco et al., 1977) and the complement derived factors C3a and C5a. Experimentally, synthetic peptides have proved useful for investigating the interaction of chemotactic factors with the neutrophil surface (Showell et al., 1976).

Chemotaxis is thought to involve a spatial mechanism, comparing the concentration of a substance at two or more locations on the cell surface at any one time (Zigmond, 1974). In optimal gradients a 1% difference in concentration of a peptide between two points can be detected (Zigmond, 1977).

There are specific receptors for chemotactic factors on the neutrophil's surface, with a correlation between a substance's chemotactic effectiveness and ability to compete for binding sites (Williams et al., 1977). Schiffmann et al. (1978) have suggested a two site model; a binding site at which the signal is initiated and a hydrolytic site which frees the cell of the substance so that continued detection of the gradient can occur. They also found

specificity within binding sites. Some factors did not compete with others indicating different cellular sites of interaction for different types of chemotactic substances.

The mechanism for translation of receptor binding to a chemotactic response at the molecular level is unclear. Snyderman and Pike (1978) showed that binding of a chemoattractant is not sufficient to trigger a chemotactic response and suggested that the transmission of free energy from the ligand to the membrane is necessary. Seligmann et al. (1980a) found that neutrophils exposed to a chemotactic stimulus initially showed a response suggesting membrane depolarisation, followed by hyperpolarisation. The alteration in transmembrane potential activates ionic fluxes, particularly of calcium (Gallin and Rosenthal, 1974; Boucek and Snyderman, 1976; Gallin et al., 1978; Bareis et al., 1982), and leads to a rearrangement of cytoskeletal elements (Snyderman and Goetzl, 1981). Experimental data suggest that microtubule assembly is regulated, at least in part, by the level of cytoplasmic calcium (Gallin and Rosenthal, 1974, Snyder and McIntosh, 1976) and Gallin and Rosenthal (1974) have proposed that an assymetic assembly of microtubules is instrumental in imparting the net vector of motion during chemotaxis. In addition, an increase in cytoplasmic ionised calcium may be important for microfilament function (Gallin et al., 1978). Stimulation of anaerobic glucose metabolism appears to be essential for maximal chemotactic response (Goetzl and Austin, 1974).

In locomotion there is a linear alignment within the cell with the nucleus away from the stimulus. Microfilaments are present in increased numbers at both the leading and posterior ends of the cell. Centrioles adjacent to the nucleus have an associated radial array of microtubules extending towards, but not into, the filament-enriched poles (Malech et al., 1977; Snyderman and Goetzl, 1981). Explanations for the mechanism of locomotion are speculative at best. Stossell (1978) suggests that substrate-adherent cellular molecules, linked to a contractile meshwork, act like tank treads to propel the cell forward, the torque being provided by units of moving contractile proteins at the base of the cell which are attached to proteins fixed to the cell membrane.

Factors which suppress PMN chemotaxis have been described. Such factors are of two basic types, those which inactivate chemotactic factors (Van Epps and Williams, 1976) and those which act directly on the cell (Goetzl, 1975a; Goetzl, 1975b). It is speculated that humoral inhibitors regulate the inflammatory response in vivo (Van Epps and Williams, 1978). Goetzl and Austin (1972) descibed a preformed, soluble neutrophil immobilising factor (NIF) produced by PMN and mononuclear cells which causes an irreversible chemotactic inhibition but no impairment of cell viability or phagocytosis (Goetzl, 1973). Thus PMN can be localised in an area of inflammation while still retaining their ability to deal with phagocytosable substrates. Diffusion of NIF, which has minimal effect on macrophages, will stop the further influx of PMN, thus

favouring the transition to the mononuclear predominance of chronic inflammation.

1.6 Phagocytosis

Phagocytosis is the process by which a particle is taken within the cell while in effect remaining outside the plasma membrane. The process is initiated by contact between the PMN and the particle, the latter usually attaching firmly to the plasma membrane via specific receptors.

Microorganisms may become coated with IgG and associated complement, a process referred to as opsonisation. The coated bacterium binds directly to receptors present on the phagocyte membrane. PMN have at least two types of receptors, one specific for the Fc portion of IgG (Henson, 1969, Messner and Jelinek, 1970, Sajnani et al., 1974) and another for the C3b component of complement (Lay and Nussenzweig, 1968, Jasin,1972, Scriber and Fahrney, 1976). These receptors are present on 90% of neutrophils (Wong and Wilson, 1975) and have some mobility within the cytoplasmic membrane (Sajnani et al., 1974; Weinbaum et al., 1983). Their nature and ability to recognise opsonins is unclear but Fearon (1980) isolated the C3b receptor as a specific glycoprotein. Particle-bound C3b functions primarily in the adherence phase while IgG serves as a trigger for internalisation (Scriber and Fahrney, 1976; Ehlenberger and Nussenzweig, 1977;

Newman and Johnson, 1979; Roos et al., 1981). Where specific IgG is present, complement decreases the amount of antibody required and enhances the rate of phagocytosis (Scriber and Fahrney, 1976). Evidence suggests that many agents which evoke cell migration also cause complement receptor enhancement (Kay et al., 1981), increasing the degree of adhesion of the phagocyte to the opsonised particle.

Specific opsonisation cannot account for the ability of phagocytes to ingest uncoated bacteria and inert particles such as latex. Watt (1980) speculates that some bacteria have surfaces of an appropriate physicochemical nature to directly adhere to granulocytes while Hoffstein (1980) points to non-immune mechanisms related to hydrophobic interactions between cell and particle surfaces.

Following contact there is localised cellular contraction resulting in a depression containing the particle. Invagination continues and the particle is drawn into the cell interior. Eventually the margins of the depression fuse to complete the phagocytic vacuole (Hoffstein, 1980).

Internalising a bacterium does not guarantee that it will be killed. Once cell/particle contact is established specific mechanisms are activated which contribute to microbial destruction.

1.7 Degranulation

Cohn and Hirsch (1960a) allowed neutrophils to ingest bacteria and demonstated that within 30 minutes some, or all, of the granules were lost from the cytoplasm. Hirsch (1962) found that only granules adjacent to the phagocytic vacuole ruptured and that the vacuole grew larger with increasing granular breakdown. Zucker-Franklin and Hirsch (1964) produced a series of electron micrographs which showed the granule membrane fusing with that of the phagocytic vacuole. Thus granule enzymes can be brought into contact with a microorganism without contaminating the cytosol where they may cause cell damage or lysis. Trowell and Brewer (1976), using chicken heterophils, found individual granule lysis to be extremely rapid , often taking less than 0.25 of a second.

Degranulation occurs in a sequence, with the specific granules participating initially and azurophilic granules taking over later (Bainton, 1973). There is a drop in the vacuole pH from 6.5 to 4.0 during cell activation; this favours the specific granule enzyme optimum of neutral pH initially, and the azurophilic optimal acid range later. The granule membranes have distinct properties which may offer some explanation for the differential fusion rates (Bainton, 1973).

Degranulation begins 20 to 60 seconds after the initial stimulation provided by cell/particle contact (Weissmann et al.,1980) and thus may precede closure of the phagocytic vesicle. Several

investigators have noted some leakage of enzymes and reaction products to the surrounding environment (Zucker-Franklin and Hirsch, 1964; Weissmann et al., 1971; Bainton, 1973; Weissmann et al., 1980, Ohno et al., 1982). Cells exposed to a large antibody/antigen coated surface can adhere and be stimulated to release granule enzymes (Hawkins, 1972, Henson, 1971). This is pathologically important in immune complex nephritis and vasculitis and rheumatoid joint disease (Klebanoff and Clark, 1978).

1.8 Respiratory Burst

The resting PMN consumes little oxygen (Baehner et al. 1970; Weening et al., 1974) with most of its energy requirements being obtained through anaerobic glycolysis (Sbarra and Karnovsky, 1959). The fraction of glucose metabolised via the hexose monophosphate shunt (HMS) is of the order of 2 to 3% (Beck, 1958).

On exposure to appropriate stimuli the neutrophil undergoes a burst of metabolic activity. This respiratory burst does not depend on particle engulfment (Selvaraj and Sbarra, 1966; Goldstein et al., 1975a, Ohno et al., 1982) and can occur in response to soluble agents such as C5a (Goldstein et al, 1975b) and sodium fluoride (Selvaraj and Sbarra, 1966) and to aggregated, membrane-bound IgG (Johnston and Lehmeyer, 1976).

The mode of initiation of the burst is not understood. Takanaka and O'Brien (1975) stated that a reversible confirmational change in the plasma membrane is responsible for the activation of oxidase activities while Kaplan et al. (1972) suggested that events are initiated through hydrolysis and repair of the cell membrane. Within 10 seconds of exposure to a stimulus there is rapid membrane hyperpolarisation, then depolarisation followed by slow hyperpolarisation (Korchak and Weissmann, 1978). It is thought that these changes in membrane potential are involved in the primary triggering of the cellular response. Mottola and Romeo (1982) were able to show that calcium fluxes occur prior to neutrophil activation and appear to be responsible for initiating a number of cell processes. There is a transient increase in the concentration of free cytoplasmic calcium ions which is not due to an influx of extracellular cations (Roos et al., 1981) but probably results from release of bound calcium from the plasma membrane area in contact with the stimulus (Hoffstein, 1979). The removal of calcium from membranes can induce a decreased constraint on either enzyme or substate diffusion indicating that this molecule is involved in the regulation of enzyme activity (Horvath and Sovak, 1973). Neutrophils contain proteolytic activity which is inactive at normal cellular calcium levels and it is suggested that activation of NADPH-oxidase requires proteolytic processing of a latent form of the enzyme (Legendre, and Jones, 1983). English and McPherson (1978) isolated an inhibitor of NADPH-oxidase from resting PMN and concluded that inactivation of the inhibitor is involved in initiation of the respiratory burst. Phagocytes show

an enhanced response in the presence of certain chemoattractants (Allred and Hill, 1978; Kay et al., 1981) and it has been suggested that PMN activation is related to chemoattractant alteration of membrane potential (Seligman, and Gallin, 1980b).

With the respiratory burst there is an increase in glucose oxidation via the HMS (Sbarra and Karnovsky, 1959; Iyer et al., 1961) and a marked increase in oxygen uptake (Baldridge and Gerard, 1933; Baehner et al., 1970) which may be as high as 10 to 15 times the resting oxygen consumption (Weening et al., 1974). Iyer et al. (1961) demonstrated that at least part of the oxygen is converted to hydrogen peroxide (H_2O_2). Babior et al. (1973) showed that the superoxide radical (O_2^-) is also produced and Root and Metcalf (1977) were able to show that all the oxygen taken up is converted to superoxide and 80% of this superoxide is converted to hydrogen peroxide via a dismutation reaction;

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

Superoxide generation occurs in two stages, a rapid burst preceding endocytosis and a slower, progressive increase occurring with particle uptake (Baxter et al., 1983). Tauber and Curnutte (1978) suggest that the type of stimulus determines the level of response but Baxter et al. (1983) obtained contradictory results. With latex beads as a stimulus the extent of the burst is roughly proportional to the mass of particles taken up by the phagocyte (Krenis, 1961).

Nicotinamide adenine dinucleotide phosphate (NADP+) serves as an electron acceptor in the HMS. NADPH-oxidase catalyses the conversion of NADPH to NADP+ with NADPH acting as an electron donor. This is the superoxide-forming reaction (Babior, 1978);

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

$NADP^+$ may also be available from the glutathione peroxidase-glutathione reductase system (Reed, 1969) which protects the cell from damage by hydrogen peroxide;

$$\underset{\text{reduced glutathione}}{2GSH} + H_2O_2 \longrightarrow \underset{\text{oxidised glutathione}}{GSSG} + 2H_2O$$

$$GSSG + NADPH \longrightarrow 2GSH + NADP^+$$

The activity of the HMP is contolled by the rate at which NADP+ becomes available (Beck, 1958).

The identity of the primary oxidase responsible for initiation of the metabolic burst is an area of controversy. There is agreement that the enzyme catalyses the reduction of oxygen to superoxide by a reduced pyridine nucleotide but whether this is NADPH or NADH remains a point of debate. NADPH would seem the most obvious candidate (Rossi *et al.* 1971; Patriarca *et al.*, 1971; Rossi *et al.*, 1972; Dechatelet *et al.*, 1975; Babior, 1978; Seger *et al.*, 1983; Shurin *et al.*, 1983) but arguments have been put foreward for NADH (Karnovsky *et al.*, 1970; Baehner *et al.*, 1970;

Karnovsky, 1973; Curnette et al., 1975). Babior (1984) suggests that the enzyme is capable of using both nucleotides but has a greater affinity for NADPH which is probably the physiological electron donor. Karnovsky (1973) working with leucocytes from patients with chronic granulomatous disease (CGD), an inherited condition in which the PMN respiratory burst is absent (Holmes et al., 1967), constructed an overall scheme for the events which follow phagocytosis (Fig. 1.2).

For convenience the activity in question is usually considered to be due to a single enzyme although recent investigations suggest that an electron transport chain exists which incorporates a flavin cofactor and cytochrome b activity (Crawford and Schneider, 1983; Seger et al., 1983; Light et al., 1981; Gabig et al., 1982). A b-type cytochrome is found in neutrophils but its participation in the electron transport pathway between NADPH and oxygen has not been demonstrated. Evidence suggests a role outside this electron chain, for example, in the conversion of the oxidase from its resting to activated form (Babior, 1984). Crawford and Schneider (1983) demonstrated an enriched ubiquinone content in phagosomes from activated PMN and suggested that this compound, which acts as an intermediate electron acceptor in mitochondrial oxidative phosphorolation, is an important component of neutrophilic oxygen metabolism.

In man, most evidence has supported a plasma membrane location for the initiating enzyme(s) (Kaplan et al., 1972; Takanaka and

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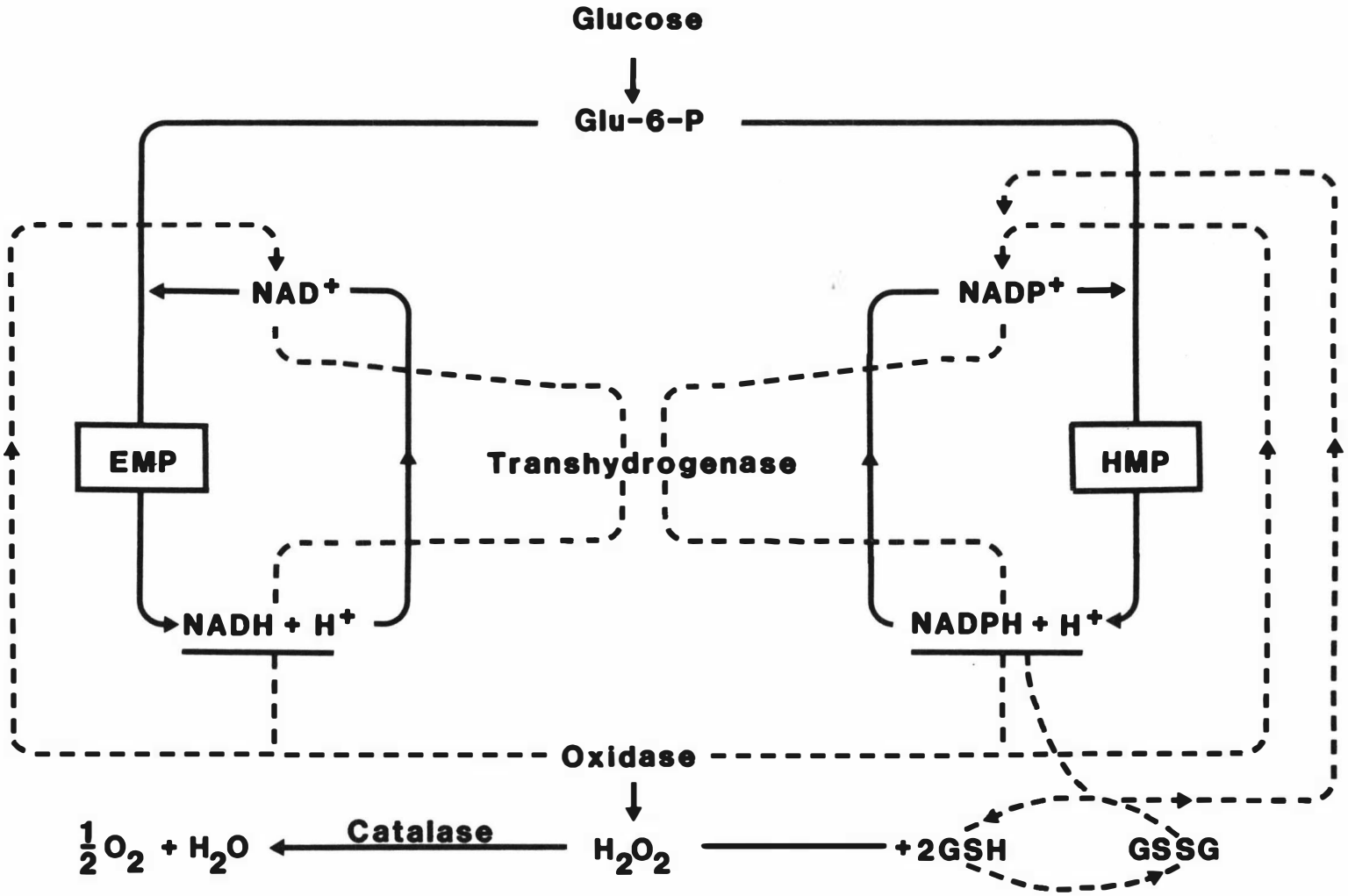
FIGURE 1.2. Suggested intracellular events following phagocytosis (from Karnovsky, M.L., (1973)). (EMP = Embden-Meyerhof-Parnas pathway, HMP = hexose monophosphate shunt).

FIGURE 1.2.


Glucose
Glu-6-P
NAD+
NADP+
EMP
Transhydrogenase
HMP
NADH + H+
NADPH + H+
Oxidase
½ O2 + H2O
Catalase
H2O2
+2GSH
GSSG

O'Brien, 1975; Goldstein et al., 1977; Dewald et al., 1979; Ohno et al., 1982; Seger et al., 1983) but subcellular fractionation suggests that at least part of the activity is not associated with the plasma membrane (Tauber and Goetzl, 1978; Crawford and Schneider, 1983). Badwey et al. (1979) noted species differences in location of enzyme activity.

1.9 Oxidative metabolism

Significant spontaneous dismutation of superoxide can occur in the acid environment of the phagocytic vacuole (Badwey and Karnovsky, 1980). A small amount of superoxide dismutase (SOD), a metalloenzyme which promotes the conversion of superoxide to hydrogen peroxide (McCord and Fridovich, 1969; McCord et al., 1971) and varying amounts of catalase, which decomposes hydrogen peroxide, are present in the cytosol of neutrophils (Dechatelet et al., 1974; Johnston et al., 1975). These enzymes probably help protect the cell against damage from the highly reactive products of the respiratory burst.

Superoxide and hydrogen peroxide may interact to produce hyroxyl radicals (OH•) and singlet oxygen ($^1O_2$). Haber and Weiss (1934) proposed that hydroxyl radicals can be formed from the reduction of hydrogen peroxide by superoxide;

$$O_2^- + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2$$

This was supported by studies using methional which reacts with hydroxyl radicals to form ethylene (Beauchamp and Fridovich, 1970; Tauber and Babior, 1977). As would be expected SOD decreases ethylene production substantially, but surprisingly catalase has no effect. Thus while superoxide is implicated in hydroxyl radical production, hydrogen peroxide is not (Tauber and Babior, 1977). Recently, the efficiency of the Haber-Weiss reaction and the use of methional to detect hydroxyl radicals have been questioned (Badwey and Karnovsky, 1980).

Singlet oxygen is formed when an absorbtion of energy shifts an electron from the ground state, where the spins of the two valence electrons are in the same direction, to an orbital of higher energy with an inversion of spin so that the two electrons then have opposing spins. The production of singlet oxygen by leucocytes has been proposed for a number of years (Allen et al., 1972; Johnston et al., 1975; Rosen and Klebanoff, 1976; Allen, 1977; Rosen and Klebanoff, 1977) but Kanofsky and Tauber (1983) have produced evidence suggesting that singlet oxygen is not a significant product of the respiratory burst. A number of reactions are capable of generating singlet oxygen, including the spontaneous dismutation of superoxide;

$$\underset{\text{perhydroxyl radical}}{HO_2\cdot} + O_2^- + H^+ \longrightarrow {}^1O_2 + H_2O_2$$

the modified Haber-Weiss reaction;

$$O_2^- + H_2O_2 \xrightarrow{\text{metal}} {}^1O_2 + H_2O_2$$

the interaction of hypochlorite with hydrogen peroxide (Piatt et al., 1977);

$$OCl^- + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O$$

and the interaction of superoxide with hydroxyl radicals;

$$O_2^- + OH^\cdot \longrightarrow {}^1O_2 + OH^-$$

Overall the interrelationships of the proposed oxygen derivatives of the metabolic burst are complex (Fig. 1.3, Fig. 1.4) and their occurrence in the PMN for the most part unconfirmed.

1.10 Microbial Killing

The mechanisms of microbial killing are ill-defined but probably involve an array of factors cooperating for effectiveness against the broad spectrum of pathogenic organisms.

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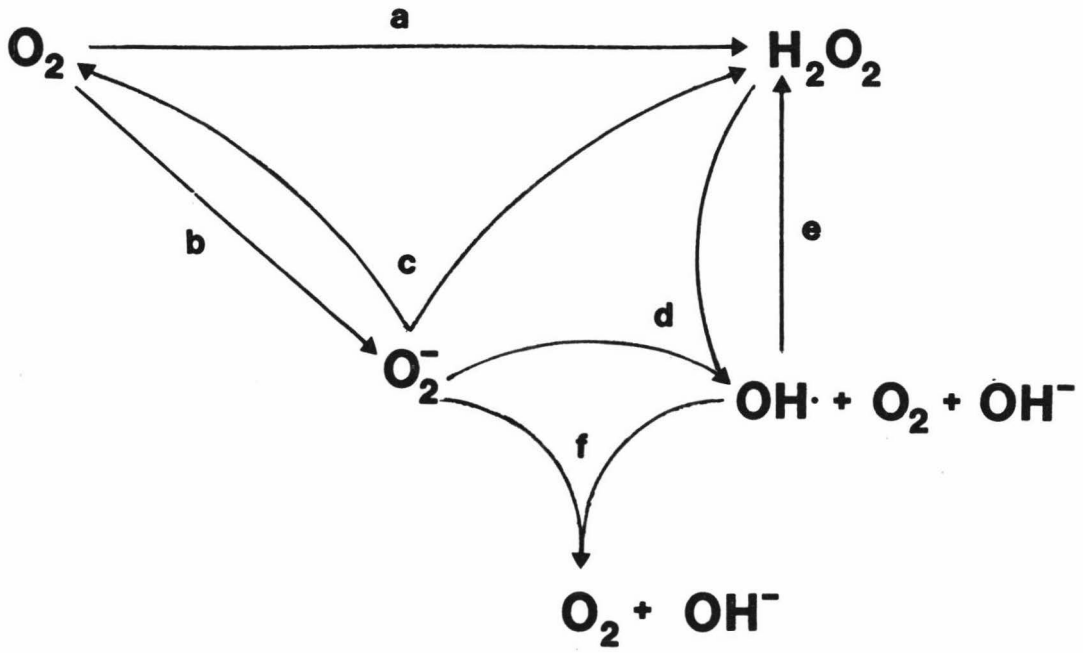
FIGURE 1.3. Suggested oxidative interactions involved in respiratory burst activity (from Klebanoff, S.J. and Clark, R.A., (1978)).

FIGURE 1.3.



a. $O_2 \longrightarrow H_2O_2$

b. $O_2 \longrightarrow O_2^-$

c. $O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$

d. $O_2^- + H_2O_2 \longrightarrow OH\cdot + O_2 + OH^-$

e. $OH\cdot + OH\cdot \longrightarrow H_2O_2$

f. $OH\cdot + O_2^- \longrightarrow O_2 + OH^-$

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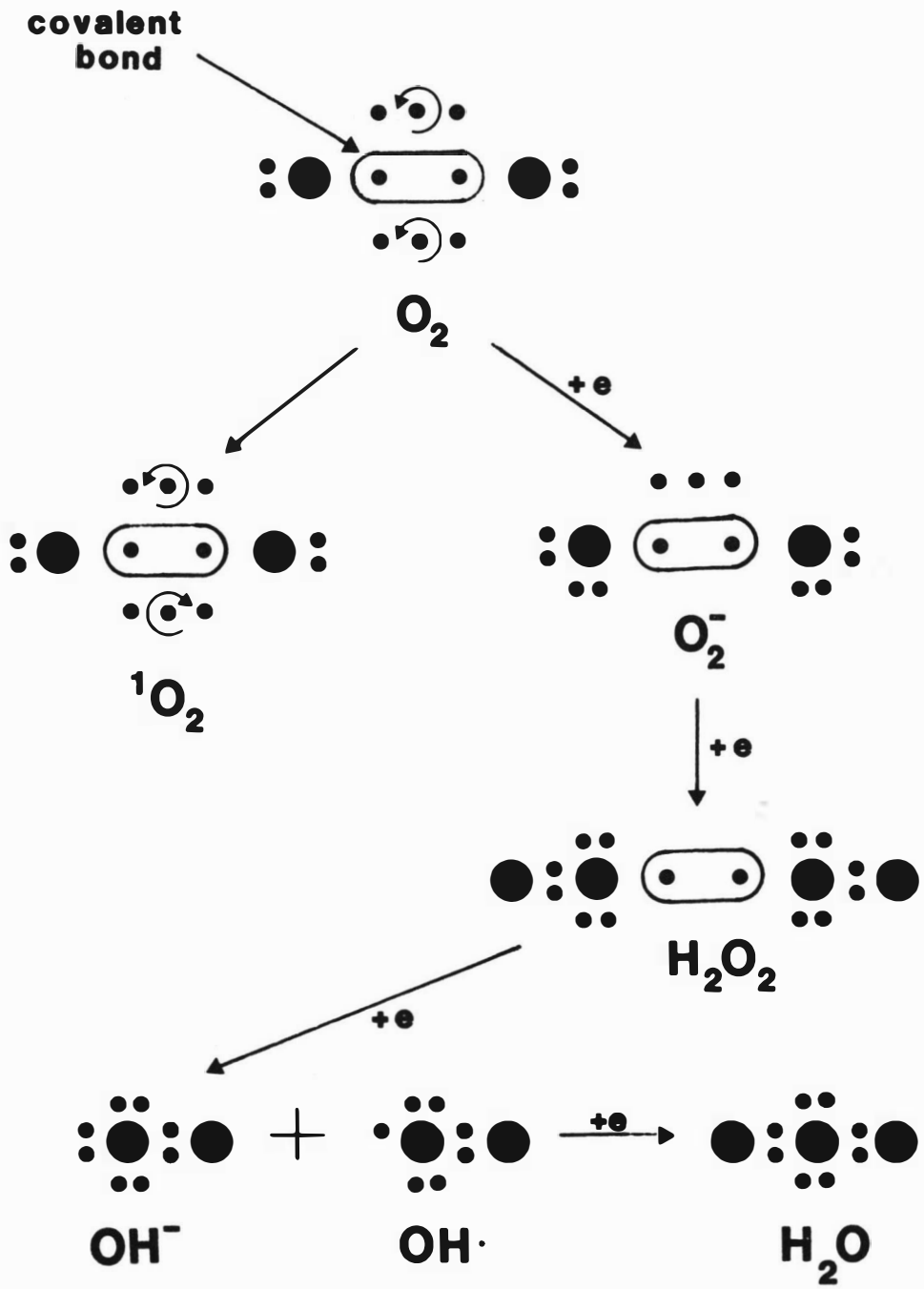
FIGURE 1.4. Possible interrelationship of the reactive oxygen species of the respiratory burst.

FIGURE 1.4.



a. Oxidative Bactericidal Mechanisms

Superoxide is not highly toxic to microorganisms (Johnston et al., 1975; Babior et al., 1975) and its importance seems to lie in the oxygen species it is capable of generating during the respiratory burst.

One well documented oxidative killing mechanism consists of MPO, hydrogen peroxide and a halide. Hydrogen peroxide is bactericidal to some extent (Babior, 1978) and it has also been proposed as a factor in a non-enzymic killing system with ascorbic acid and certain metals (Drath and Karnovsky, 1974). However, Winterbourn and Vissers (1983) found that cellular ascorbate was not released into phagosomes and concluded that there was no simple link between bacterial killing and ascorbate oxidation. The microbicidal capacity of hydrogen peroxide is greatly increased by the presence of the azurophilic granule haemoprotein, MPO, and a halide (Klebanoff, 1967). MPO catalyses the oxidation of halide ions (X^-) to hypohalite ions (Harrison and Schultz, 1976);

$$X^- + H_2O_2 \longrightarrow XO^- + H_2O$$

Chloride ions, being the most abundant halide in cells, are probably the physiological substrate (Babior, 1978). The MPO-hydrogen peroxide-chloride system is responsible for hydrolysis of proteins and decarboxylation of amino acids which may be bactericidal if it involves the essential amino acids of the

microbial cell wall (Selvaraj et al., 1974). It is proposed that the system works via the production of as yet undefined, reactive free radicals. In addition, it has been suggested that halogenation of the bacterial wall results in a loss of its integrity (Klebanoff, 1967; Simmonds and Karnovsky, 1973), but the correlation between halogenation and bacterial death is poor (McCall et al., 1971).

Hypochlorite is known to react with hydrogen peroxide to produce singlet oxygen (Rosen and Klebanoff, 1977) and the latter has been proposed as a bactericidal agent (Allen et al., 1972; Krinsky, 1974; Babior, 1978). In addition, production of singlet oxygen via the modified Haber-Weiss reaction has been implicated in bacterial killing (Rosen and Klebanoff, 1979), as has the hydroxyl radical (Johnston et al., 1975). Human neutrophils have the capacity to peroxidate ingested lipids, a reaction which probably contributes to microbial killing (Stossel et al., 1974; Sholet et al., 1974). Peroxidation of lipids has been attributed to the action of hydroxyl radicals (Fong et al., 1973). The role of this latter radical and singlet oxygen in the microbicidal effect of the PMN has yet to be definitely established but Repine et al. (1984) reported a significant decrease in neutrophil bactericidal activity in the presence of several hydroxyl radical scavengers.

b.Non-oxidative Bactericidal Mechanisms

Certain species of bacteria are killed under anaerobic conditions (Mandell, 1974) and in the absence of HMS stimulation (Dechatelet et al., 1971), indicating that non-oxidative bactericidal mechanisms exist.

Few bacteria are capable of growing in a markedly acidic environment. The increased glycolytic activity of phagocytosis results in the generation of large quantities of lactic acid and consequently an acidic pH within the phagocytic vacuole (Rous, 1925). Bainton (1973) found that the pH fell to 4.0 within 15 minutes of particle uptake, although Mandell (1970) suggested that the intravacuolar pH did not fall below 6.0-6.5 in human neutrophils.

Lysozyme kills those Gram-positive bacteria that do not have a covering to their peptidoglycan layer (Murphy, 1976). If another agent damages the cell wall allowing access, lysozyme can kill many Gram-positive and Gram-negative organisms (Ginsberg and Sela, 1976). Miller (1969) found that a combination of hydrogen peroxide and ascorbate renders Salmonella pullorum susceptible to lysozyme. Antibody and complement, which are capable of causing bacterial cell wall damage, may also assist lysozyme action. Leijh et al. (1981) demonstrated that both are necessary for optimal bacterial killing.

Lactoferrin, which is present in the specific granules, can be bacteriostatic or bactericidal (Arnold et al., 1977) by chelating the iron which is necessary for bacterial growth.

The azurophilic granules contain several cationic antibacterial proteins differing in structure and specificity (Hirsch, 1965; Zeya and Spitznagel, 1968; Odeberg and Olsson, 1975); nothing is known of the mechanism by which these achieve bacterial killing.

1.11 Neutrophil Function Tests

a. Nitroblue Tetrazolium Reduction

Nitroblue tetrazolium (NBT) is an artificial electron acceptor which is reduced to an insoluble, pigmented formazan during the metabolic burst (Shaffer et al., 1953). The tetrazolium salt is yellow in solution, whereas formazan is blue-black. The pigmented substance can be viewed microscopically or extracted and measured spectrophotometrically. Baehner (1975) proposed that the NBT-reductase is located at the plasma membrane and is activated on contact with the particle. Partial inhibition of granulocyte NBT reduction by SOD indicates that superoxide is responsible for at least a part of the reaction (Johnston et al., 1975) and Baehner et al. (1975) suggested that the reduction was due entirely to a reaction between the dye and superoxide. Since the reduction occurs as a consequence of the respiratory burst it has been

accepted as a simple test of neutrophil functional ability (Segal, 1974).

The NBT test has been used in the diagnosis of CGD (Johnston et al., 1975; Baehner et al., 1975; Seger et al., 1983), glucose-6-phosphate dehydrogenase deficiency (Cooper et al., 1972), specific granule deficiency (Gallin et al., 1982) and Chédiak-Higashi syndrome (Root et al., 1972).

b.Microbial Killing Tests

When a standard number of microoganisms is mixed with a known number of granulocytes and incubated for a specific time, the number of live bacteria remaining within phagocytes will give an estimate of the cellular killing ability. Miles and Misra (1938) standardised the method using colony counts resulting from plated dilutions of a PMN/bacterial suspension. The test, while cumbersome, gives an accurate measure of PMN function and has been used in experimental and clinical investigations (Root et al., 1972; Weinstein and Young, 1976; Paape and Wergin, 1977; Stevens and Young, 1977; Williams and Bunch, 1981; Gallin et al., 1982) Phillips et al. (1979) have developed a simpler micro-assay system. A variation of the test, based on the differential staining of Candida albicans with methylene blue, has also been described (Lehrer and Cline, 1969; Root et al., 1972; Wood and White, 1978).

c. Chemiluminescence

The generation of light within biological systems, called bio- or chemiluminescence (CL), occurs in a variety of unrelated organisms. The phenomenom requires high amounts of energy accumulated by a molecular species, producing electronically excited groups which emit photons of light on relaxation to ground state. Oxidation reactions are capable of satisfying the high energy requirement. This observation lead Allen et al. (1972) to suspect that PMN would exhibit CL during the respiratory burst. Confirmation of neutrophil light emission following particle stimulation has lead to the possibility of developing CL as a method of assaying the functional ability of PMN. Investigations have been carried out to define the chemical reactions involved in producing luminescence, but it is still an area of debate. It is known that both superoxide and hydrogen peroxide are produced during the metabolic burst and it has been postulated that the hydroxyl radical and singlet oxygen occur as intermediate products in some of the reactions. Any or all of these oxygen products may be involved in neutrophil CL.

Native CL is assessed by providing neutrophils with a phagocytosable substate and measuring the resultant light emission. Studies of the native system suggest that there are at least two mechanisms of CL production, one involving superoxide and the other a MPO-dependent reaction (Rosen and Klebanoff, 1976). Sodium azide, an inhibitor of MPO (Klebanoff and Pineus, 1971) and a

scavenger of singlet oxygen, (Hasty et al., 1972), markedly impairs granulocyte CL (Sagone et al., 1977). Neutrophils from individuals with a genetic MPO deficiency have decreased CL during the early post-phagocytic period but still produce significant levels of light emission. Superoxide production in MPO-deficient cells does not differ significantly from that of normal cells but decreases less rapidly with time, as does CL (Rosen and Klebanoff, 1976). Addition to the reaction mixture of SOD decreases light emission in both the presence and absence of MPO (Webb et al., 1974; Rosen and Klebanoff, 1976; Dechatelet et al., 1982), suggesting a primary effect on superoxide. It is thought that superoxide has no intrinsic CL production, but rather causes a secondary excitation of components of the phagocytosed particle or reaction mixture (Rosen and Klebanoff, 1976; Cheson et al., 1976; Klebanoff and Clark, 1978). Evidence that soluble stimuli fail to generate CL, in the absence of a secondary emitter such as a protein (Nelson et al., 1977; Westrick et al., 1980), tends to confirm this hypothesis.

Singlet oxygen is an unstable, electronically excited molecule which emits light on returning to ground state. Allen et al. (1972) proposed that PMN CL was mediated entirely by this molecule. It is thought, however, that singlet oxygen CL is restricted to the gas phase (Kearns, 1971) and in addition, the emission spectrum of singlet oxygen alone differs from that of the stimulated PMN (Andersen et al., 1977). Interaction of singlet oxygen and perhaps other radicals with regions of high electron density present in

components of the cell wall, for example unsaturated lipids, may result in the formation of dioxetanes or other unstable compounds. These may then disintergrate yielding excited carbonyl groups capable of emitting light on relaxation to ground state (Kearns, 1971; Allen, 1977; McPhail et al., 1979). Cheung et al. (1983) suggest that arachidonic acid liberated from membrane phospholipids is oxidised via the lipoxygenase pathway by some or all of the oxygen species of the respiratory burst with resultant generation of reactive intermediate anions.

Recently, the cyclic hydrazide luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) (Fig. 1.5a) has been employed as an agent for amplifying native CL (Allen and Loose, 1976; Stevens et al., 1978; Wilson et al., 1978; Anderson et al., 1980). Oxidation of luminol results in the formation of aminophthalate (Fig. 1.5b), an electronically excited anion capable of light emission (Roswell and White, 1978). It was initially assumed that the luminol-enhanced system mirrored the native system, but later reports indicate that the reactions involved differ (Stevens et al., 1978; Faden and Maciejewski, 1981; Dahlgren and Stendahl, 1983). Dechatelet et al. (1982) suggest that increasing the sensitivity of the system allows such small numbers of cells to be used that measurable CL is not generated via some of the expected pathways. Cormier and Prichard (1968) studied the peroxidation of luminol by horse-radish peroxidase and concluded that the substate of the light-producing reaction is the luminol radical which arises from the interaction of peroxidase/peroxide intermediates and luminol;

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FIGURE 1.5a. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione).

FIGURE 1.5b. Aminophthalate ion.

FIGURE 1.5a.

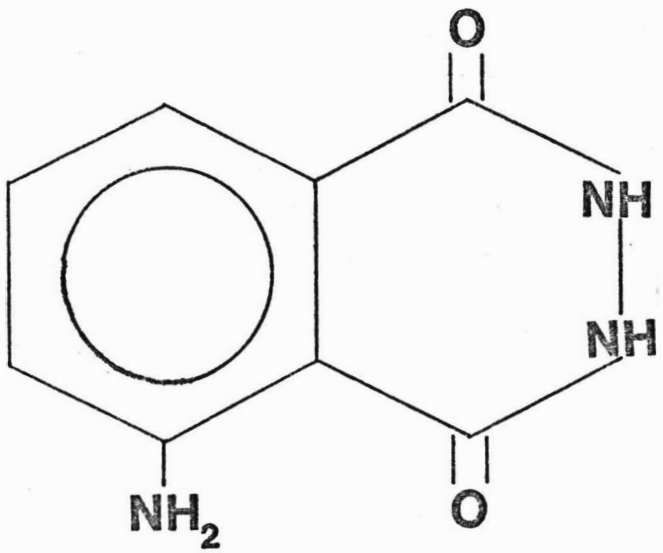
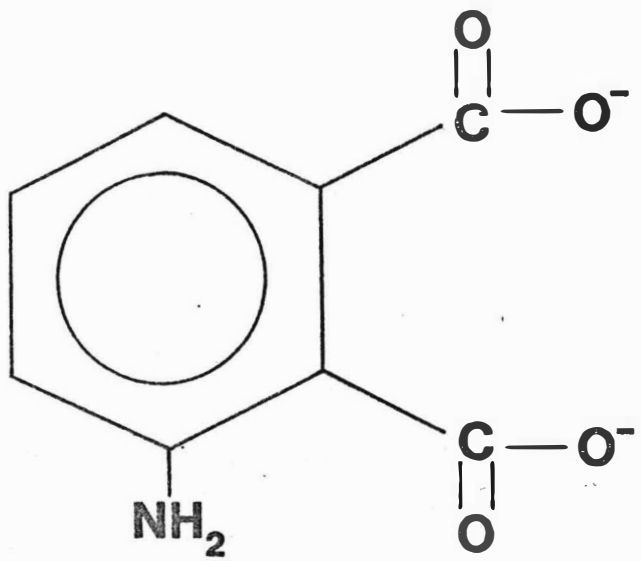


FIGURE 1.5b.



$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Complex I}$$

$$\text{Complex I} + \underset{\text{luminol}}{LH_2} \longrightarrow \text{Complex II} + \underset{\text{luminol radical}}{LH\cdot}$$

$$\text{Complex II} + LH_2 \longrightarrow \text{Peroxidase} + LH\cdot$$

$$2LH\cdot + H_2O_2 \longrightarrow \text{Light} + \text{Products}$$

Hodgson and Fridovich (1976), employing a superoxide-generating system, produced a scheme for the luminescence reaction of luminol which is dependent on superoxide;

$$\underset{\text{luminol monoanion}}{LH^-} + H_2O_2 \longrightarrow LH\cdot + OH\cdot + OH^-$$

$$LH^- + OH\cdot \longrightarrow LH\cdot + OH^-$$

$$LH\cdot + O_2^- \longrightarrow LOOH^- \longrightarrow N_2 + \underset{\substack{\text{aminophthalate} \\ \text{(excited)}}}{AP^*}$$

$$\text{--}\Lambda\Lambda\Lambda\text{-->} \text{ light} + AP$$

They further suggest that hydrogen peroxide is not essential because superoxide can also act as an oxidant, although it is less effective in this capacity than the hydroxyl radical;

$$LH^- + O_2^- + 2H^+ \longrightarrow LH\cdot + H_2O_2$$

In addition, Weiss (1983) notes that the perhydroxyl radical, a much stronger oxidant than superoxide, is formed by the protonation of superoxide and may be found in substantial concentration in the acid pH of the phagocytic vacuole. This scheme offers an explanation for the marked decrease in CL in the presence of SOD and the lesser effect of catalase (Faden and Maciejewski, 1981; Cheung et al., 1983). As superoxide can be produced by the action of any oxidant on luminol (Hodgson and Fridovich, 1975), the theory of hydroxyl radical participation based on the effect of SOD on the CL response (Fong et al., 1973) can be discounted. It should be noted that the experiments were conducted at pH 10.2. and it is possible that at the lower pH of the phagocyte, the reactions involved differ. Recently Repine et al. (1984) conducted a series of experiments with activated PMN exposed to various hydroxyl radical scavengers. The results of these experiments appear to confirm the participation of this metabolite in granulocyte CL.

A reaction system consisting of a MPO-deficient granule fraction, chloride, hydrogen peroxide and luminol emits no CL, but with the addition of a normal cell granule fraction there is an immediate intense burst of light. Harrison and Schultz (1976) descibed a hypochlorous acid-like intermediate formed during MPO-mediated reactions and Dechatelet et al. (1982) demonstrated that a hydrochlorous acid/luminol mixture produces measurable CL. Lovgren

and Cheng (1982) showed that a hydrogen peroxide/peroxidase system emitts light at physiological pH although the CL intensity is much lower than in more alkaline conditions. Cells from MPO-deficient patients exhibit minimal CL in spite of pronounced superoxide production, but when purified MPO is added to the reaction mixture CL output increases markedly (Dahlgren and Stendahl, 1983). These findings have been interpreted as evidence for complete reliance upon a MPO-mediated reaction within the enhanced system.

While the transfer of energy to luminol via an excited intermediate such as singlet oxygen is possible, Cheung et al. (1983) found that 2,5 dimethyl furan, a scavenger of singlet oxygen, decreased CL by only 15.4%. Thus the contribution of singlet oxygen to CL is probably minimal.

While the reactions responsible for light emission in neutrophils have yet to be defined, the emitted light is a product of the complex cellular metabolism occuring during the respiratory burst and as such reflects the metabolic activity of the cell.

This thesis descibes the use of ovine neutrophils in CL assays and explores some of the factors which influence the CL activity of such cells.

CHAPTER 2

THE DETERMINATION OF OPTIMAL CHEMILUMINESCENCE ASSAY CONDITIONS USING OVINE POLYMORPHONUCLEAR LEUCOCYTES

2.1 Introduction

The phagocytic competence of PMN is reflected by a range of excited oxygen metabolites produced by these cells on exposure to suitable stimuli (Klebanoff and Clark, 1978). The chemiluminescence (CL) produced by some or all of these metabolites can be detected using scintillation counters (Webb et al., 1974; Wilson et al., 1978; Stevens et al., 1978; Starkebaum et al., 1981) or luminometers (Easmon et al., 1980; Descamps-Latscha et al., 1982; Angle and Klesius, 1983). CL assays have been performed using phagocytes isolated from man (Allred and Hill, 1978; Easmon et al., 1980; Starkebaum et al., 1981; Dahlgren and Stendahl, 1982; Archibald et al., 1983), horses (Jacobson et al., 1982; Washburn et al., 1982), dogs (Andersen and Arimrault, 1979; Angle and Klesius, 1983), cattle (Weber et al., 1983), rabbits (Allen and Loose, 1976), rats (Welch et al., 1980) and striped bass (Stave et al., 1983). Measurable CL has also been demonstrated from human whole blood (Faden and Maciejewski, 1981; Descamps-latscha et al., 1982, Selvaraj et al., 1982). The assay has been used to detect cellular abnormalities in PMN function (Stjernholme et al., 1973; Johnston et al., 1975; Stevens et al., 1978; Wilson et al., 1978; Van Epps et al., 1978; Mills et al., 1979) and opsonic deficiencies

(Stevens and Young, 1977; Grebner et al., 1977).

This chapter descibes the assay conditions giving maximum CL from isolated ovine granulocytes phagocytosing latex particles and describes some factors which effect the amount of CL produced.

2.2 Materials and Methods

a. Animals

Clinically healthy, adult Southdown ewes of similar ages were used. They were housed together indoors and fed a diet of hay and water ad libitum.

b. Harvesting of PMN

Blood was withdrawn from the jugular vein into 10ml evacuated tubes containing 100µl of 15% potassium-EDTA (Terumo Corp., Tokyo, Japan.). Five ml aliquots of blood were layered over 5ml amounts of sodium metrizoate/Ficoll solution ("Lymphoprep", Nyegaard and Co., Oslo, Norway) and centrifuged at 850 x g for 30 min. The supernatants were discarded and each cell pellet was suspended in 6ml of NH_4Cl erythrocyte-lysing solution (Appendix) for 10 min with constant mixing before being centrifuged at 200 x g for 20 min. The cells were resuspended in 3ml of lysing solution for a further 10 min, centrifuged at 200 x g for 10 min and then washed twice in phosphate buffered saline (PBS, pH7·2, 0·01M). The cells were

pooled and suspended in PBS containing glucose at a final concentration of 2%, counted and the cell concentration adjusted as required. The entire isolation procedure was carried out at 4°C and all reagents were maintained at this temperature.

Cytocentrifuge (Cytospin, Shandon Southern Products Ltd, Runcorn, UK) preparations of the isolated cells showed 80%-90% PMN of characteristic morphology. Viability measured by trypan blue exclusion (Appendix) was 98-100%.

c. Luminol

Luminol (Sigma Chemical Company, St Louis, USA) was dissolved in dimethylsulphoxide at a concentration of 2mg/ml and stored at room temperature in the dark. This stock solution was diluted as required in PBS, the dilutions being held in dark bottles(Descamps-Latscha et al., 1982).

d. Latex Beads

Latex beads (Sigma Chemical Company, St Louis, USA) of 0·81 µm average diameter were purchased as a 10% suspension and diluted as required in PBS.

e. Haemoglobin

Since residual erythrocytes and/or haemoglobin (Hb) have been shown to quench the native CL from canine neutrophils (Andersen and Brenzel, 1978) the effect of Hb on the luminol-enhanced CL of sheep neutrophils was investigated. The supernatant obtained from erythrocyte lysis during the isolation procedure was used as a source of free Hb. The Hb concentration of this material was measured in a haemoglobinometer (950 Hb Meter, Corning Ltd, Essex, England.) and adjusted as required with PBS.

f. Chemiluminescence Analysis

CL was measured in an LKB 1251 luminometer (LKB-Wallac, Turku, Finland) at 27°C with continuous mixing. The constituents of the reaction mixture were equilibrated at 27°C for 30 min after which 500µl of cell suspension and 250µl of luminol solution were placed in a plastic cuvette. Immediately prior to counting 50µl of latex suspension were added to initiate phagocytosis. In the experiment which investigates the effect of storage on the cells, the isolated neutrophils were maintained at 4°C with gentle mixing. Aliquots were taken at specified intervals, diluted to give the required cell concentration and equilibrated at 27°C for 30 min following which the phagocytic stimulus was added and the CL measured. The results given are the mean peak millivolt (mV) values of three aliquots from each sample unless otherwise stated. Controls consisted of luminol and cells only.

2.3 Results

a. Effect of Cell Concentration

The relationship between CL activity and the cell concentration was examined using cells from a single animal on 3 separate occasions (Fig. 2.1). A concentration of 5 x 10^6 PMN/ml gave the maximum response of those tested. Since this number of cells was readily obtainable from 10ml of blood, this cell concentration was used in all subsequent experiments.

b. Effect of Latex Concentration

Using 5 x 10^6 PMN/ml maximum CL was recorded with a 1:5 dilution of the commercial latex suspension (Fig. 2.2). This dilution was used in all subsequent experiments.

c. Effect of Luminol Concentration

A 1:10 dilution of the stock luminol solution gave maximum CL (Fig. 2.3). This dilution was used in all subsequent experiments.

d. Controls

CL values for controls never exceeded 0.6mV.

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FIGURE 2.1. The relationship between chemiluminescence and cell concentration.

FIGURE 2.1.

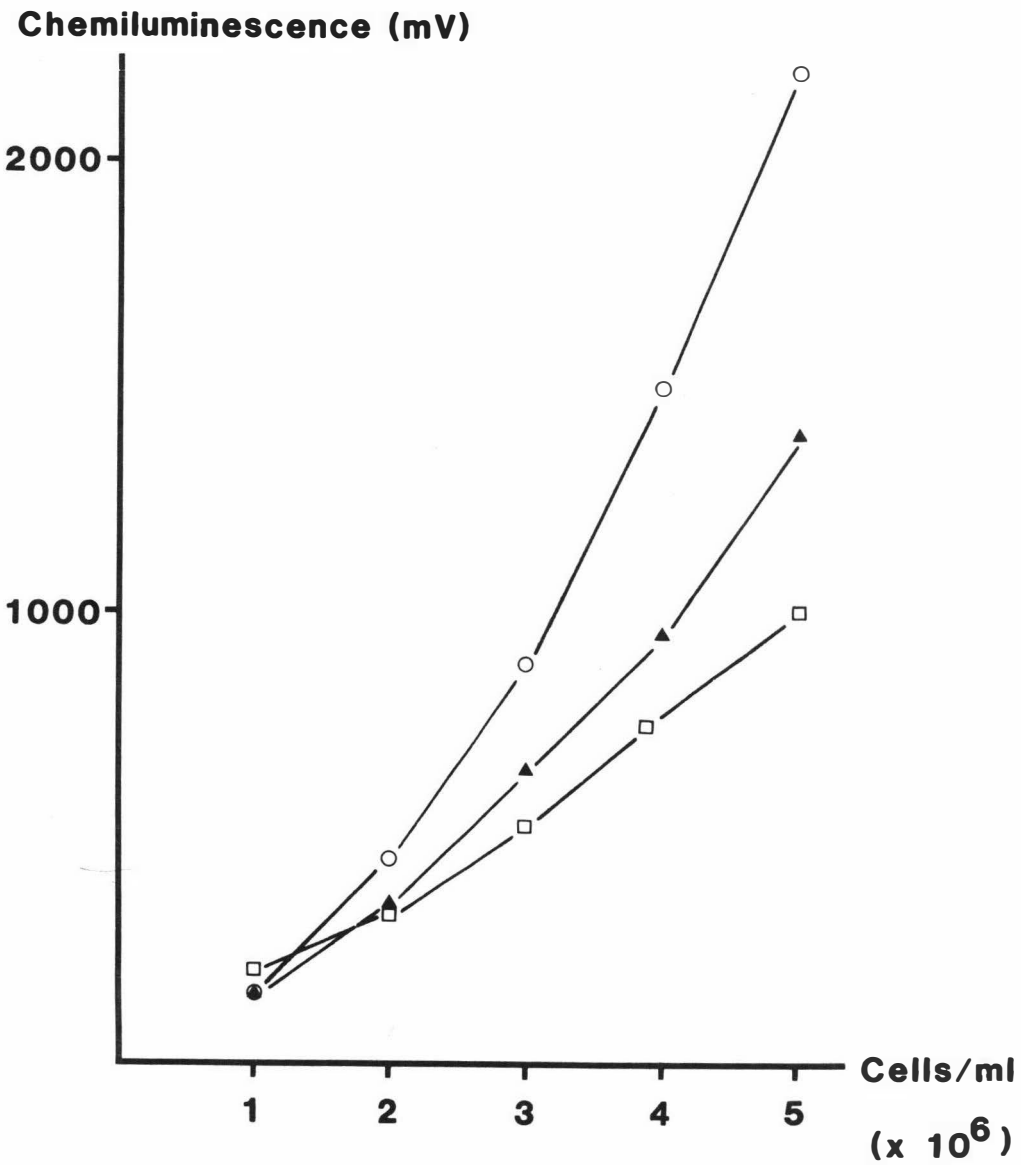

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FIGURE 2.2. The effect of latex concentration on chemiluminescence.

FIGURE 2.3. The effect of luminol concentration on chemiluminescence.

FIGURE 2.2.

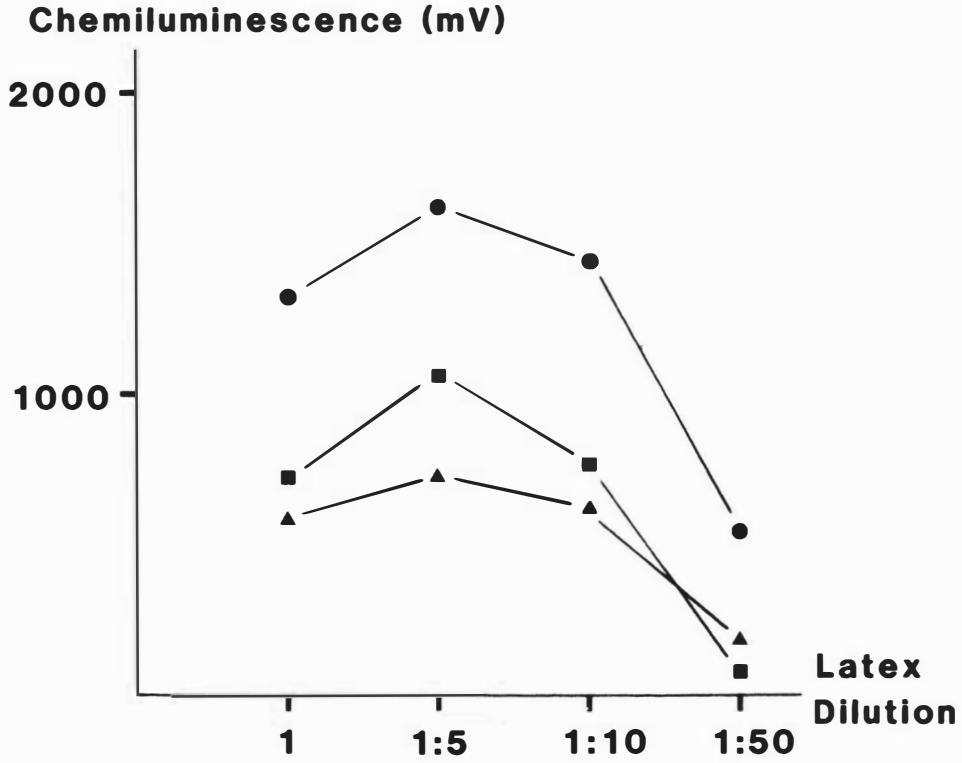
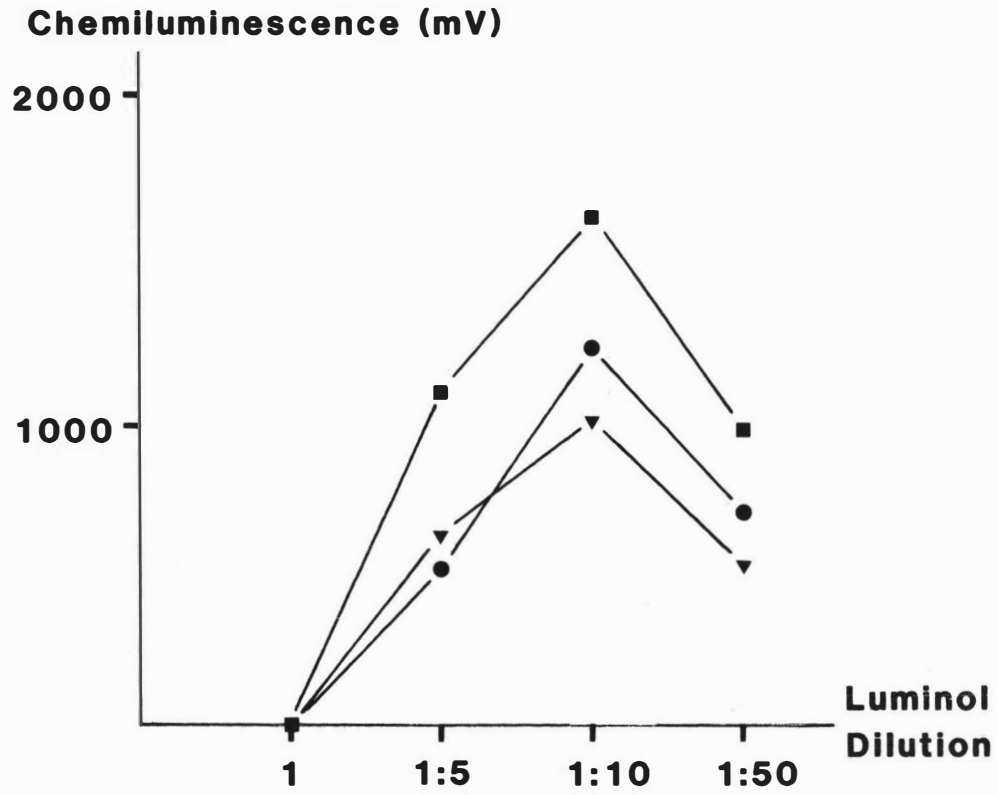


FIGURE 2.3.



e. Replication

The results of 4 experiments to determine the replication within samples are listed in Table 2.1. The replication was such that the mean result from 3 aliquots was accepted as an accurate measurement of a sample's CL.

f. Effect of Haemoglobin on CL

The addition of Hb to a concentration of 0.05 mg/ml in the final reaction mixture decreased the CL by 16%. With 1.5 mg/ml of Hb the decrease was 78% (Fig. 2.4).

g. Effect of Storage of the Isolated Cells at 4°C

The amount of CL produced by ovine PMN was modified by storage at 4°C for various periods. The CL increased for the first 4 to 8 hr following isolation and was still relatively high at 16 hr (Fig. 2.5). The cells of sheep (a) were also assayed at 24 hr and gave a CL of 800 mV. The cell viability was 93% at 8 hr and 86% at 24 hr.

2.4 Discussion

Ovine granlocytes exposed to suitable stimuli produce CL responses as seen in other species. The conditions of the CL assay using ovine neutrophils were standardised to reduce variations due to outside influences while giving maximum light output.

TABLE 2.1. Replication of chemiluminescence values within samples.

Experiment	Number of Replicates	Range (mV)	Mean ± S.D.
1	10	1875 - 2151	1981 ± 101
2	6	1442 - 1591	1525 ± 54
3	7	1588 - 1674	1623 ± 30
4	6	1484 - 1598	1547 ± 47

FIGURE 2.4. The effect of haemoglobin on luminol-enhanced chemiluminescence from isolated neutrophils (mean $\pm$ standard deviation).

FIGURE 2.4.

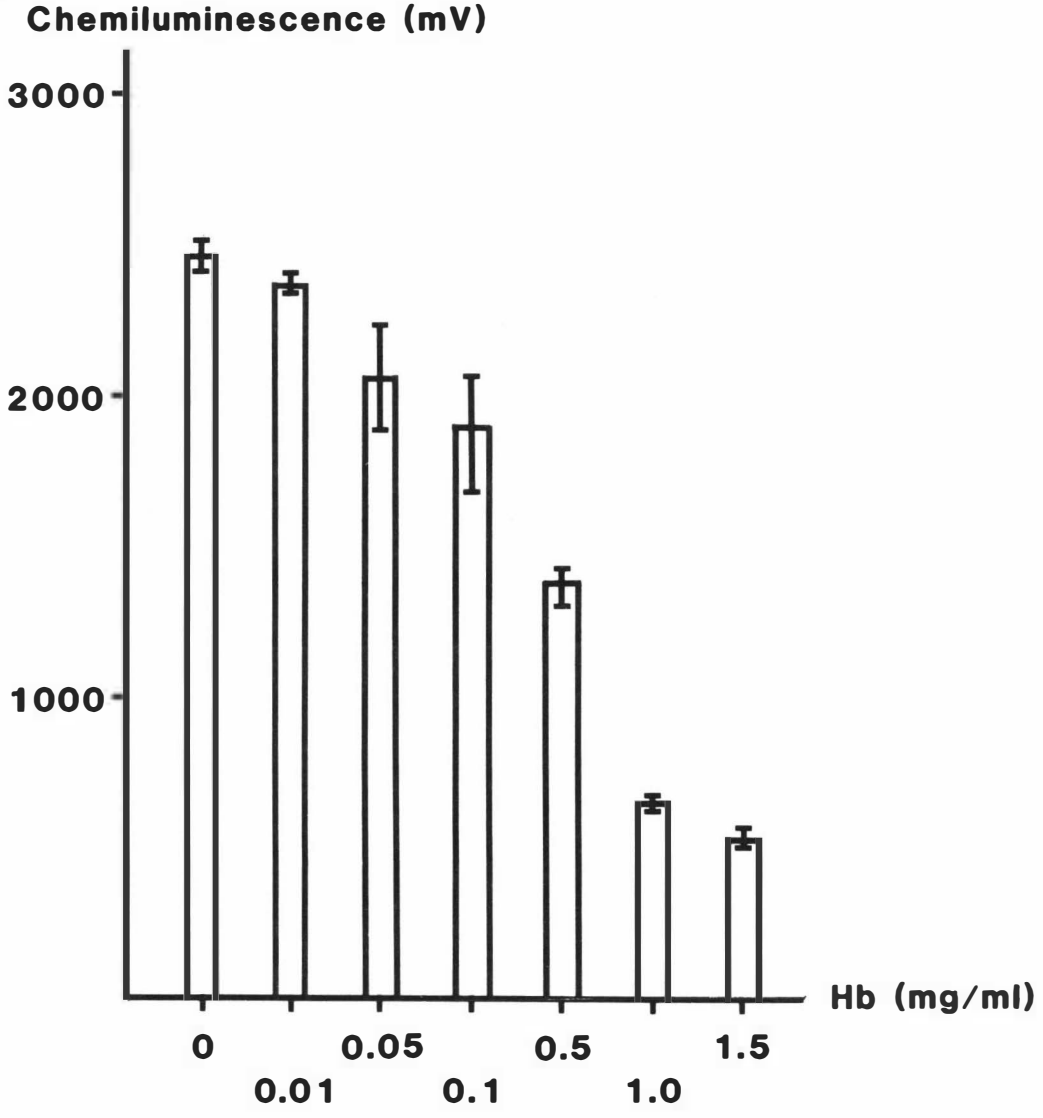
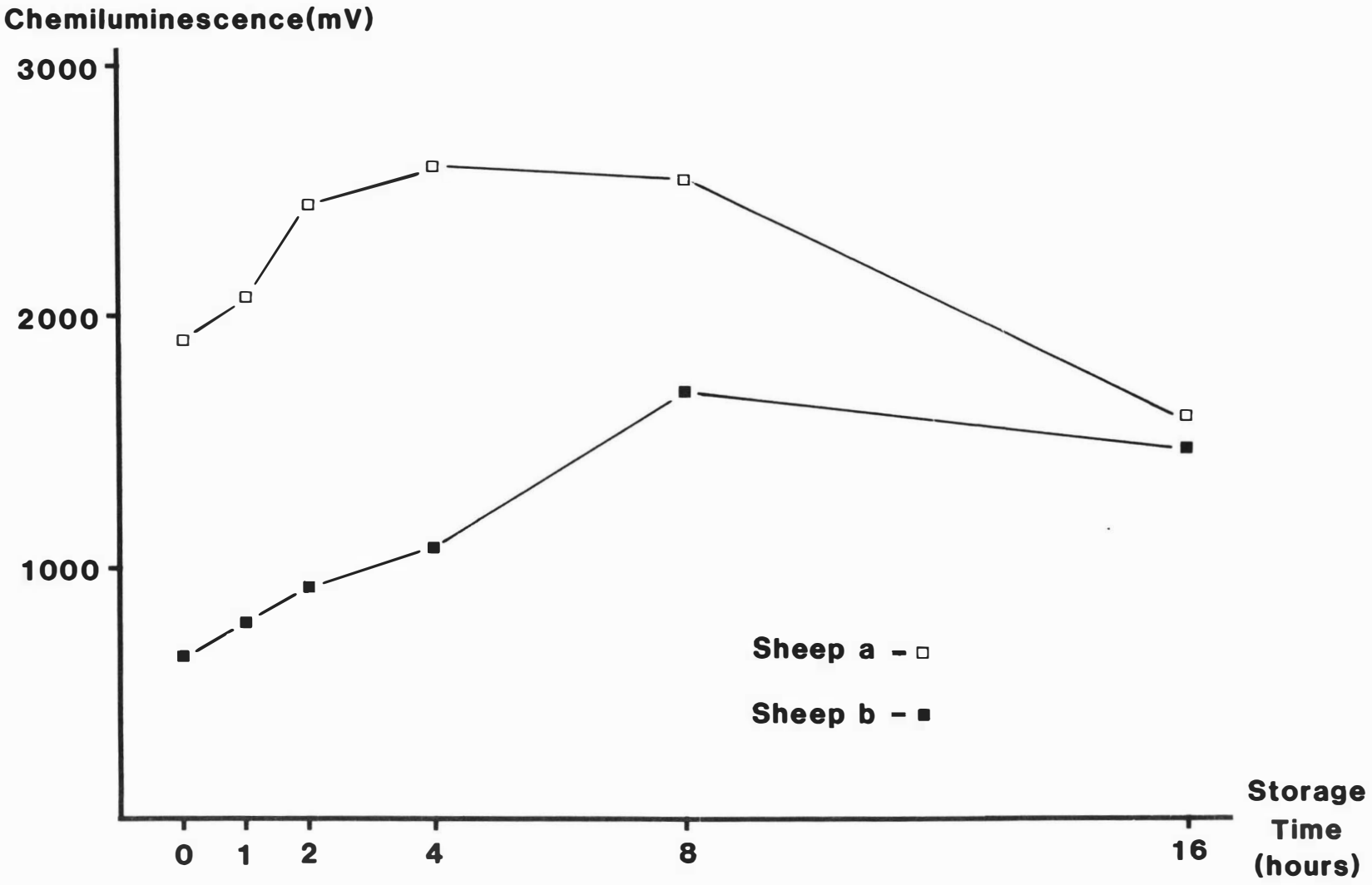

Chemiluminescence (mV)
3000
2000
1000
Hb (mg/ml)
0
0.01
0.05
0.1
0.5
1.0
1.5

FIGURE 2.5. Mean peak chemiluminescence from isolated neutrophils stored at $4^{o}C$.

FIGURE 2.5.


Chemiluminescence(mV)
3000
2000
1000
Sheep a - □
Sheep b - ■
0
1
2
4
8
16
Storage Time (hours)

Normal ovine blood has a PMN/mononuclear cell ratio of approximately 1:3 (Schalm et al., 1975) and a neglegible erythrocyte sedimentation rate (Bunce, 1954), factors which preclude the use of many of the isolation procedures employed with non-ruminant blood. The isolation method developed relies on sodium metrizoate/Ficoll centrifugation to eliminate most of the mononuclear cells and hypotonic lysing to remove red blood cells. The lysing was done twice to ensure the removal of all erythrocytes, bearing in mind the light quenching effect of any residual red cells (Andersen and Brenzel, 1978). Preliminary experiments with sodium citrate or heparin as anticoagulants produced isolates containing aggregates of cells and a high proportion of damaged leucocytes. The unsuitability of heparin for use in isolating bovine leucocytes has been reported (Carlson and Kaneko, 1973). Initially all isolates were examined by cytocentrifugation for characteristic morphology and differential cell count but as the technique became standardised and the proportion of PMN in the cell isolates was consistently 80-90%, these examinations were performed only on those preparations giving abnormal results, for example, isolates which gave very low CL values or took longer than usual to peak. There was no apparent correlation between cell morphology and such abnormal CL values.

Latex particles were chosen as the phagocytosable substrate because their uptake is independent of the influence of serum factors and specific receptor activity and thus provides a basic measurement of

phagocytic ability (Selvaraj et al., 1982). Various cell:particle ratios were tested to establish the optimal conditions of light generation, that is the point where the reaction is not limited by the availability of the phagocytic stimulus. Beyond this concentration CL is probably quenched by the turbidity of the particle suspension (Andersen and Brenzel, 1978).

CL increased with increasing PMN concentrations but in the experiments described the relationship between the two parameters was not strictly linear. Higher cell concentrations must increase the probability of cell:particle contact and, in addition, there may be a crowding effect where the cells are perhaps activated by the close proximity of other PMN.

The erythrocyte lysis supernatant produced marked quenching of CL. The effect of NH_4Cl lysing solution alone was not assessed but as Andersen and Brenzel (1978) reported quenching of canine neutrophil CL by residual red blood cells and Hb, the decrease in CL seen with ovine cells was assumed to be due to the presence of Hb. Although luminol-enhanced assays using human whole blood have been reported (Faden and Maciejewski, 1981; Descamps-Latscha et al., 1982; Selvaraj et al., 1982), preliminary experiments with ovine whole blood did not produce measurable CL. Isolated human PMN were assayed on several occasions using the same standardised system developed for the ovine PMN and produced CL values 2 to 3 times greater than those of the ovine cells. (Results not presented.) Differences in the PMN CL responses obtained from different species

have been reported previously (Hatch et al., 1978). Erythrocyte quenching combined with a lower CL level probably results in values below the sensitivity of the luminometer for sheep whole blood.

Replication within a single sample was acceptable providing that factors such as microbial contamination of reagents and residues from dirty glasswear were eliminated. Such contamination, which occurred on some occasions, effected both the replication and the time taken to reach the peak value. The cell suspension must be stored with gentle mixing. Samples left unmixed for short periods gave variable results, possibly because of uneven distribution of the cells within the suspension.

CL values increased for the first 4 to 8 hr after PMN isolation suggesting that some type of cell activation occurs during storage. Any delay during the isolation or CL analysis of cells may significantly effect the results. It was interesting to note that even after 16 hr storage at 4°C, neutrophils retain much of their viability and oxidative capacity. This is contrary to the findings of Washburn et al. (1982) who reported a decrease in the CL response from equine PMN of approximately 97% 7 to 8 hr after sample collection. In their experiments cell isolation and storage were carried out at room temperature. Angle and Klesius (1983) found that CL from canine neutrophils stored at room temperature increased for the first 2.5 hr following cell isolation and was still higher than the initial response 5 hr post-isolation. Purified human granulocytes are reported to have 99% viabilty after

24 hr when maintained in a suitable medium at 4°C (Contreras et al., 1980). Human PMN suspended in Hank's balanced salt solution showed a 40% decrease in viability and 30% decrease in CL response after 4 hr storage. When the cells were suspended in tissue culture medium supplemented with foetal bovine serum the CL remained stable for 8 hr at storage temperatures of 4 to 37°C (Easmon et al., 1980).

In conclusion, the CL activity of ovine PMN is influenced by cell isolation and storage procedures. With standardisation of the CL assay it was hoped to exclude the effects of as many extrinsic factors as possible allowing the assay to be used as a reliable method for estimating neutrophil functional ability.

CHAPTER 3

VARIATION IN NEUTROPHIL CHEMILUMINECENCE ACTIVITY

3.1 Introduction

Before CL can be accepted as a reliable indicator of neutrophil competence the range of CL values recorded for normal individuals must be established. Stevens *et al.* (1978), using a scintillation counter, recorded CL values ranging from approximately 360 x 10^3 to 680 x 10^3 counts per minute for PMN isolated from 7 human donors while Angle and Klesius (1983) showed day-to-day variations of a similar magnitude with PMN from a single canine donor assayed by luminometry. Further analyses of the variability between subjects and sampling times have not been published.

This chapter describes the variation in CL activity of PMN taken from different sheep on different days and from sheep bled every 4 or 6 hr for 48 and 96 hr respectively. The considerable variation in CL values recorded in these sheep suggested that the reaction(s) generating CL may be influenced by an extracellular element, for example, a factor present at a variable concentration in normal serum. One such factor, plasma cortisol, was examined for its effect on CL both *in vivo* and *in vitro*.

When it became apparent that there were large differences in the results from genetically dissimilar donors, PMN from pairs of

bovine monozygous twins were examined for their CL activity.

3.2 Materials and Methods

a. Animals

The sheep were those descibed in Chapter 2; they were used in 4 experiments. In the first of these, 3 sheep were each bled at approximately the same times on 5 consecutive days. This experiment showed marked day-to-day variations in CL activity. This variation was further explored in a second experiment by following the CL values of PMN taken from 2 sheep every 4 hr for 48 hr. Fourteen days later, in the third experiment, the same 2 sheep were bled every 6 hr for 96 hr to see if there was any periodicity in CL activity. In the fourth experiment 2 animals were housed in individual crates in a light-proof room. Lighting was controlled by an automatic time switch giving a 12 hr (6am-6pm) light and 12 hr (6pm-6am) darkness sequence. Blood samples were collected every 6 hr for 96 hr.

The cattle used were 4 pairs of identical twin heifers. These were members of an experimental dairy herd kept on pasture and supplemented with hay.

b. Blood Collection

Sheep blood was usually collected as described in Chapter 2 but in the case of the sheep housed under controlled lighting, samples were collected from indwelling jugular cannulae. These were inserted 3 days prior to the first bleeding (Appendix) and flushed with heparinised, isotonic saline several times daily in an attempt to maintain their patency. During the darkness periods samples were taken under low intensity red lighting.

The cattle were bled from the ventral coccygeal vein; one pair was bled each morning on 4 successive days.

c. Harvesting of PMN

PMN were harvested from sheep and cattle blood by the procedure described in Chapter 2.

d. Chemiluminescence Analyses

The conditions for generating CL using either ovine or bovine PMN, were as described previously. Peak CL values were recorded as the mean value of 3 aliquots of the reaction mixture. Because of the variability seen in the peak CL responses, integrated CL values were also examined. For these analyses each aliquot of cells was equilibrated at 27°C for 30 minutes, the phagocytic stimulus added and the CL output integrated at one second intervals for 30

minutes. The results given are the mean integrated millivolt values from 3 aliquots.

e. Blood Leucocyte Counts and Plasma Cortisol Determinations

Total and differential white blood cell counts and determinations of plasma cortisol levels were made on each sample of blood from the sheep housed under artificial lighting. A smear was prepared immediately after blood withdrawal and stained with Wright's stain for the differential cell count. Total leucocyte counts were performed manually using commercial dilution reservoirs ("Unopette" Becton-Dickenson, New Jersey, USA.) and a haemocytometer.

eor plasma cortisol determinations blood samples were centrifuged immediately following collection and the plasma stored at $^{-}20^{o}C$ until required. Plasma cortisol levels were measured by radioimmunoassay (Appendix).

f. The Effect of Corticosteroid in vivo on CL

To examine the effect of corticosteroid in vivo dexamethasone sodium phosphate (Dexadreson, Intervet, NZ) was administered intravenously to 2 sheep at a dose of 2mg/kg body weight. A control animal received a placebo injection of sterile isotonic saline at 1ml/kg body weight. The animals were sampled immediately prior to the injection (0 hr) and again 1 and 4 hr later.

g. The Effect of Corticosteroid in vitro on CL

The isolated PMN from a single donor were divided into 6 aliquots. Dexame cell suspensions at concentrations of 500 µg, 50 µg, 5 µg, 0.5 µg, 0.05 µg and 0 µg per 2250 µl of cell suspension. The highest concentration of corticosteroid used was calculated to be approximately 10 times the *in vivo* concentration achieved by the intravenous administration at 2mg/kg. The PMN were incubated for 1 hr at 27°C and the peak CL measured.

3.3 Results

a. Day-to-Day Variation in Peak CL Values

The mean peak CL values of 3 sheep on 5 successive days are recorded in Fig. 3.1. Aliquots from each animal were assayed in rotation to minimise any effects from cell storage. There were marked variations both between individuals on the same day and between samples from the same individual taken on successive days. The ranges of CL values from the 3 individuals over the 5 day period were 386 to 3084, 404 to 1903 and 702 to 1606.

b. Day-to-Day Variation in Integrated CL Values

The mean integrated CL values of 3 sheep over 4 days are listed in Table 3.1. There were large variations in the values from individual samples with ranges for the 3 donors of 346,966 to

621,700, 589,366 to 1,456,000 and 460,666 to 765,700.

c. Variability in Peak CL from Samples Taken at 4 and 6 Hourly Intervals

The mean peak CL values recorded from 2 sheep bled 4 hourly and 6 hourly for 48 hr and 96 hr respectively, are shown in Figs. 3.2 and 3.3. Over the 48 hr period CL values for the 2 animals showed ranges of 723 to 2328 and 849 to 2460; for the 96 hr observation period these values were 618 to 2946 and 1318 to 2678. There was no obvious periodicity in CL activity in either experiment.

d. CL, Leucocyte Counts and Plasma Cortisol Levels

The mean peak CL, total and differential leucocyte counts and plasma cortisol levels from a single individual sampled by indwelling jugular cannula at 6 hourly intervals are showm in Fig. 3.4. The total leucocyte count fell from a peak of 10.4 x 10^6 cells/ml at the second bleeding to 3.6 x 10^6 cells/ml at the seventh bleeding. The absolute PMN counts declined from 4.26 x 10^6 cells/ml (41%) at the first bleeding to 0.98 x 10^6 cells/ml (17%) in the final sample. CL values ranged from 1043 to 3182. The plasma cortisol levels ranged from 2ng to 15ng per ml with 2 peaks occurring approximately 48 hr apart. The results do not demonstrate readily apparent relationships between any of the parameters measured.

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FIGURE 3.1. The mean peak chemiluminescence values from 3 sheep over 5 successive days.

FIGURE 3.1.

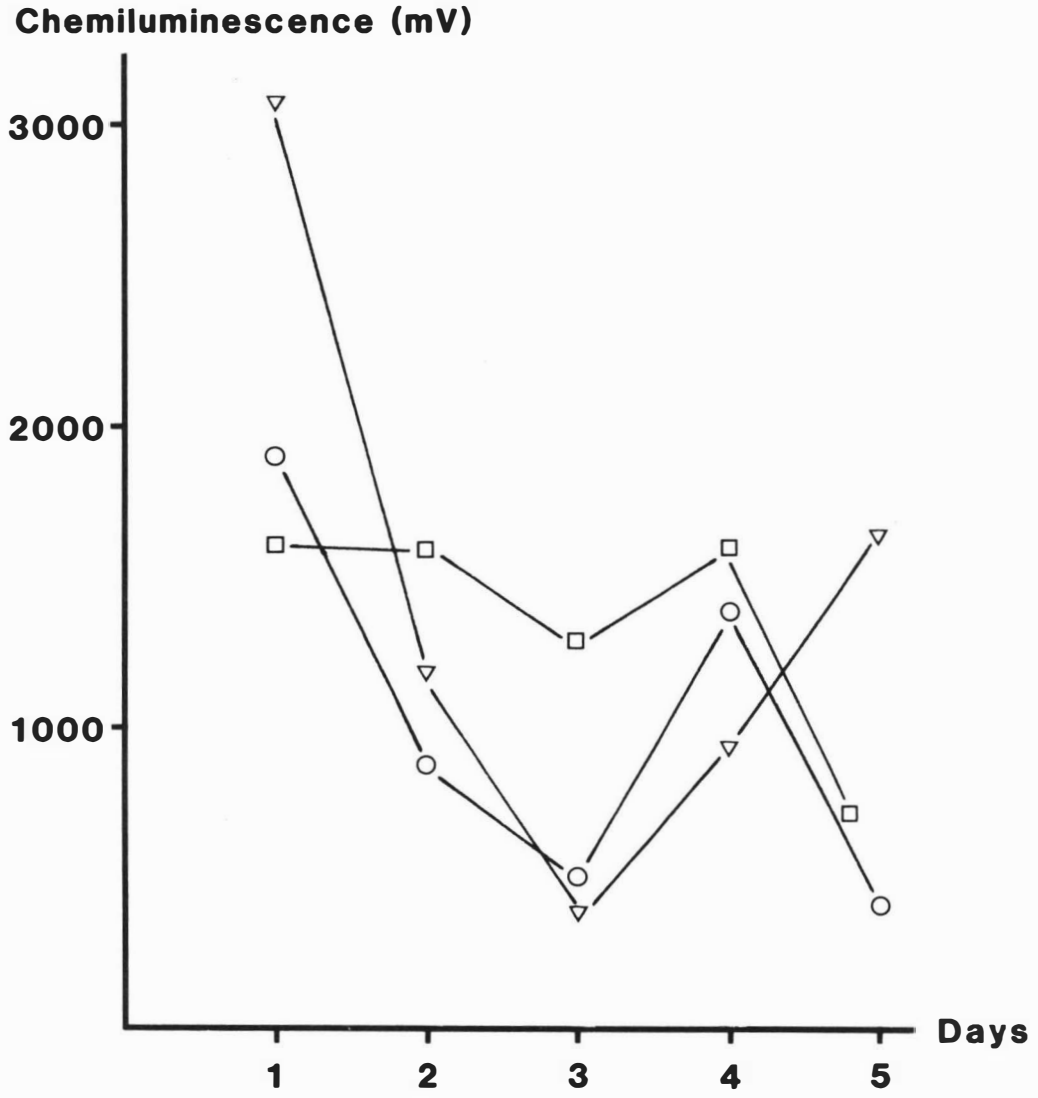

TABLE 3.1. Mean integrated chemiluminescence values from 3 sheep recorded on 4 successive days

Day	Chemiluminescence (mV)		
	Sheep a	Sheep b	Sheep c
1	354,266	589,366	471,033
2	621,700	1,061,500	509,333
3	599,033	1,456,000	765,700
4	346,966	693,033	460,666

TABLE 3.2. The effect of corticosteroid *in vivo* (2mg/kg body weight) on mean peak chemiluminescence values.

Hours post-injection	Chemiluminescence (mV)		
	Sheep a	Sheep b	Sheep c (control)
0	528	621	761
1	1687	927	2223
4	1864	2842	2020

e. Effect of Corticosteroid in vivo

Table 3.2 lists the peak CL values of 2 sheep before and after intravenous administration of dexamethasone at 2mg/kg body weight. The CL responses of sheep (a) and sheep (b) increased from 528 to 1864 and from 621 to 2842 respectively. The CL response from the control sheep also increased from 761 to 2223.

f. Effect of Corticosteroid in vitro

Table 3.3 lists the peak CL responses of PMN incubated with 10-fold dilutions of corticosteroid. At the highest concentration of corticosteroid the CL response was depressed but cells treated with lower concentrations produced CL values similar to untreated cells.

g. Variations in Neutrophil CL Between Animals of Identical Genetic Composition

The mean peak CL from 4 pairs of bovine monozygous twins are recorded in Table 3.4. There were large differences in the PMN CL response from animals of identical genetic composition.

3.4 Discussion

The results of preliminary experiments with ovine PMN indicated that the CL response was subject to large fluctuations. If such variations do not depend on factors within the assay technique then

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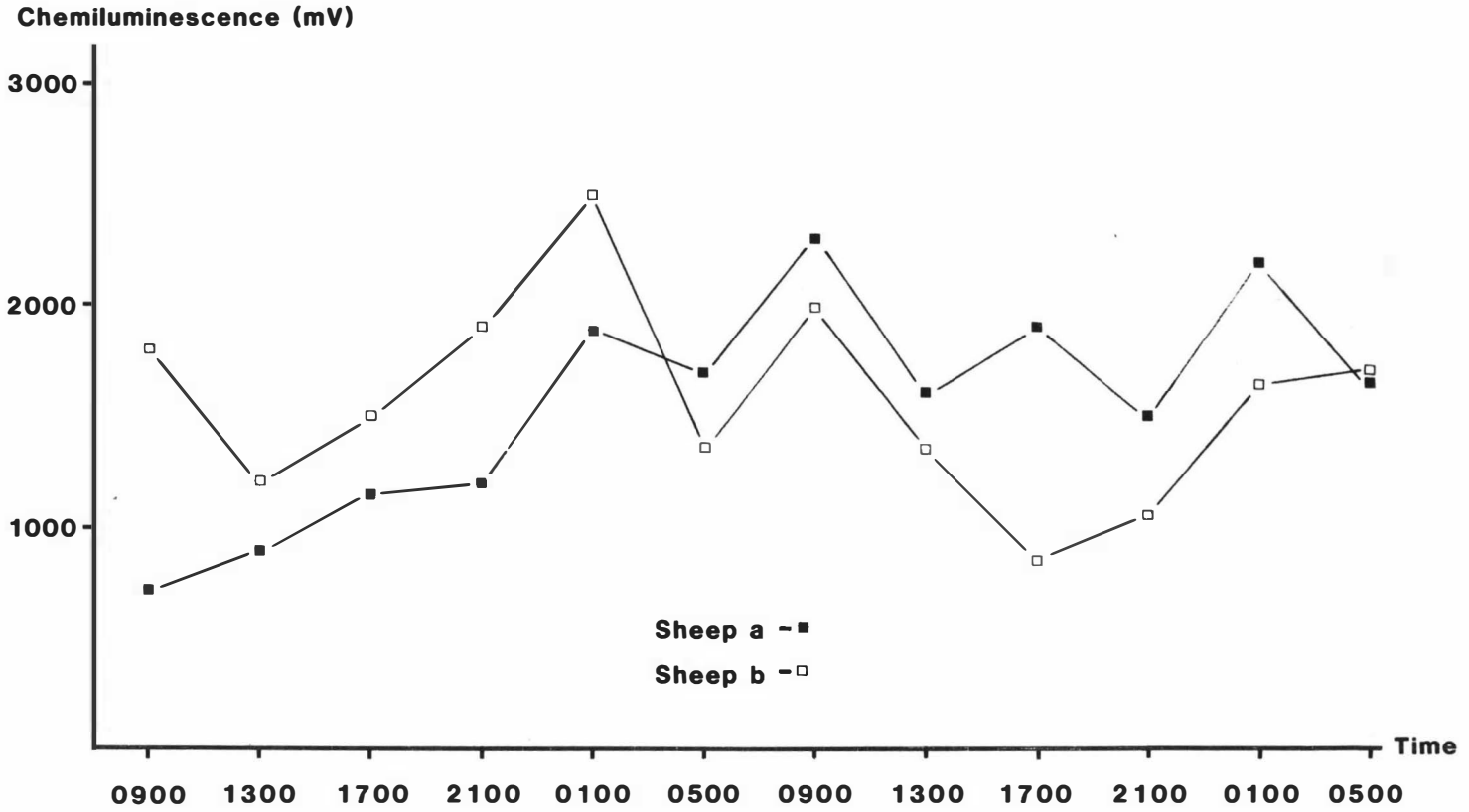
FIGURE 3.2. The mean peak chemiluminescence from 2 sheep bled at 4 hourly intervals for 48 hr.

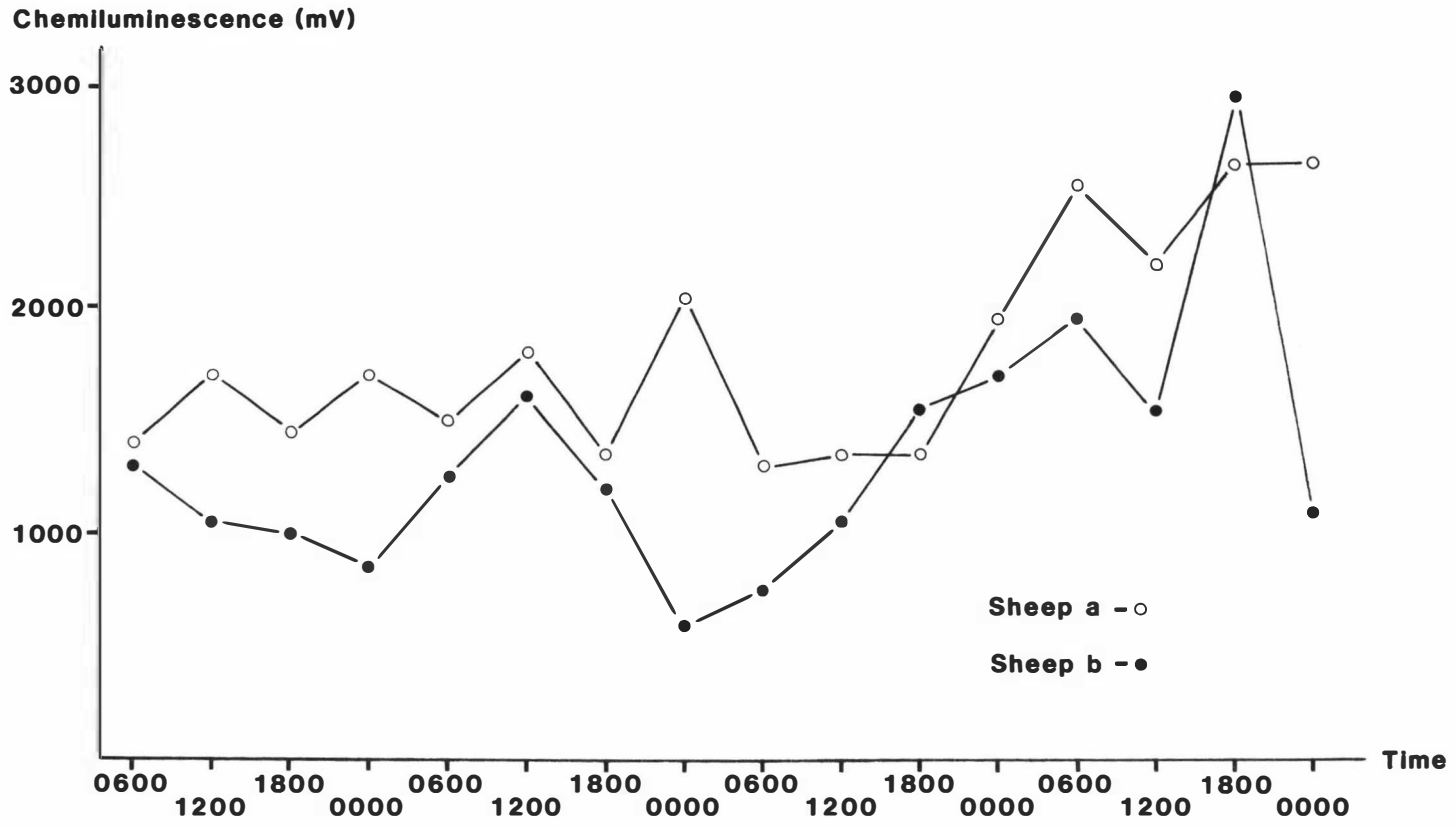
FIGURE 3.2.


Chemiluminescence (mV)
3000
2000
1000
Sheep a
Sheep b
Time
0900
1300
1700
2100
0100
0500
0900
1300
1700
2100
0100
0500

Facing Page 71

FIGURE 3.3. The mean peak chemiluminescence from 2 sheep bled at 6 hourly intervals for 96 hr.

FIGURE 3.3.


Chemiluminescence (mV)
3000
2000
1000
Sheep a
Sheep b
Time
0600
1200
1800
0000
0600
1200
1800
0000
0600
1200
1800
0000
0600
1200
1800
0000

its reliability as a measure of neutrophil function is open to question. The experiments descibed in this chapter were designed to minimise physiological variations by using animals of the same breed, sex and of similar age housed under identical conditions. A standardised assay was used to eliminate, where possible, variations arising from within the assay technique.

Previous reports have descibed the range of peak values obtained from human subjects sampled once. Van Epps et al. (1978) using a scintillation counter to measure CL, obtained a range of approximately 8-44 x 10^3 CPM. Mills et al. (1979) descibed a range of 100->400 x 10^{-3} CPM from adult donors. The variation in CL response from a single canine donor sampled over 3 days has been reported (Angle and Klesius, 1983) but day-to-day recordings from other species have not been descibed. The results presented in this chapter demonstrate large variations in the peak and integrated CL responses from ovine PMN taken from different animals on different days and from the same animals at regular 4 or 6 hourly intervals. There were no morphological differences between the cells of high and low yielding isolates on microscopic examination and the cell viability was never less than 97%. There was no evidence of a CL circadian rhythm within the time periods used.

With the sheep housed in controlled lighting, problems with the cannulae limited the samples to a single animal which may not be representitive. Both the total leucocyte and PMN count flunctuated

FIGURE 3.4. Neutrophil chemiluminescence, leucocyte counts and plasma cortisol from a single donor bled via an indwelling cannula at 6 hourly intervals.

FIGURE 3.4.

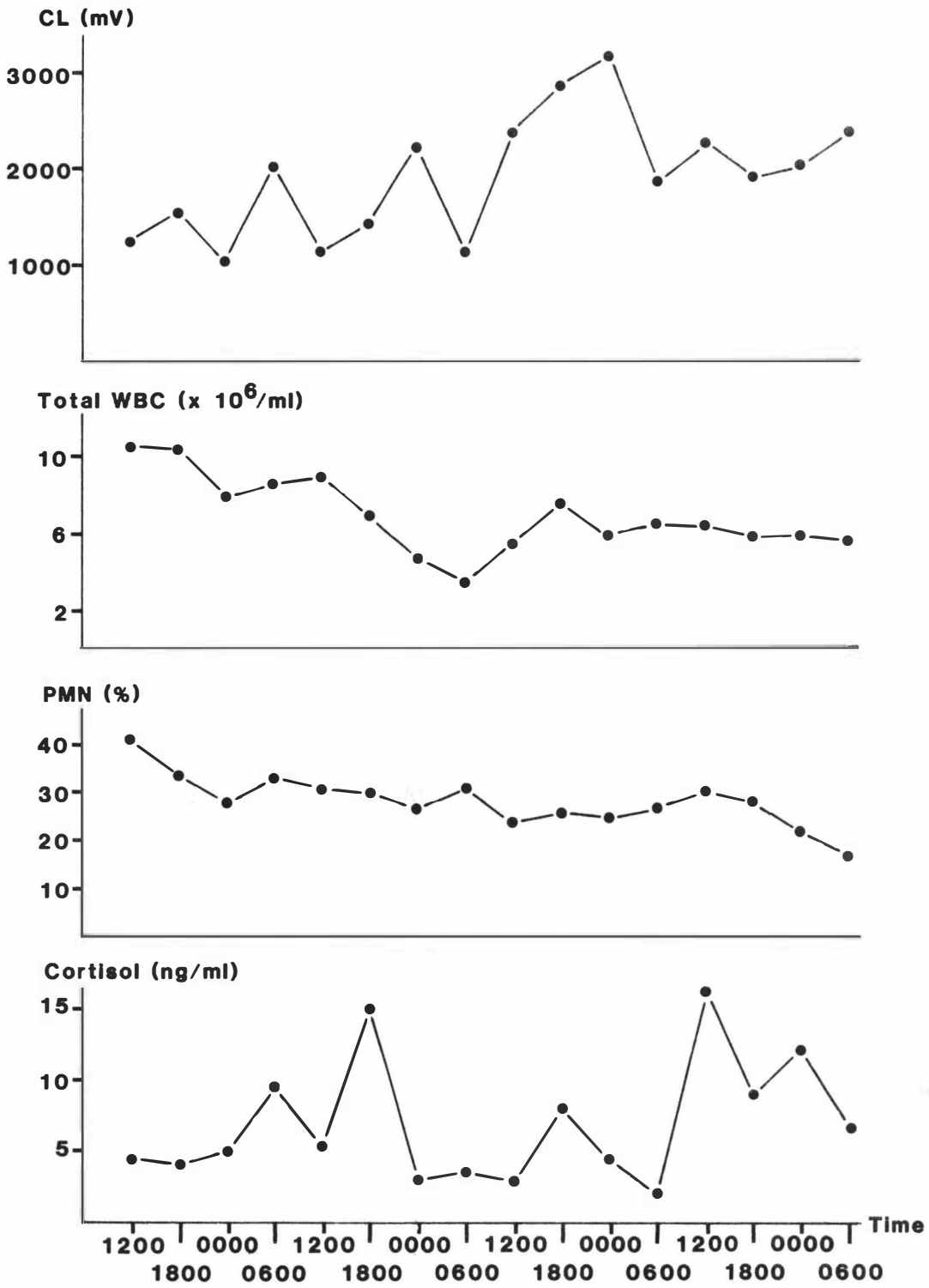

CL (mV)
3000
2000
1000
Total WBC (x 10^6/ml)
10
6
2
PMN (%)
40
30
20
10
Cortisol (ng/ml)
15
10
5
Time
1200 0000 1200 0000 1200 0000 1200 0000
1800 0600 1800 0600 1800 0600 1800 0600

TABLE 3.3. The effect of corticosteroid *in vitro* on mean peak chemiluminescence values.

Corticosteroid*	Chemiluminescence (mV)
0	1232
500	455
50	1055
5	1117
0.5	1126
0.05	1291

* ug/2250 ul of reaction mixture

TABLE 3.4. Mean peak chemiluminescence responses of pairs of bovine monozygous twins.

	Chemiluminescence (mV)	
	Twin a	Twin b
Fresian Heifers	1905	1244
Jersey Heifers	1739	1409
Jersey X Heifers	1570	1867
Jersey X Heifers	4687	1744

during the experiment with their values showing a tendency to decrease over time. The CL response was variable and there was no apparent correlation between this and the other parameters measured. Ovine plasma cortisol has been variously reported to have no circadian rhythm (Bassett, 1974), a single early morning peak (McNatty et al., 1972) and 2 daily peaks (Holley et al., 1975). It has been suggested that sheep require 6 to 28 days to adapt to cage restraint (McNatty et al., 1972; Holley et al., 1975), a factor which may have contributed to the erratic plasma cortisol results reported here. Dexamethasone is a synthetic corticosteroid which is reported to have 25 times the glucocorticoid activity of naturally occurring cortisol with virtually no mineralocorticoid activity (Ganong, 1979). Its actions are similar to those of natural cortisol. Exposure of neutrophils to this synthetic corticosteroid in vivo and in vitro did not increase the CL response. The results suggest that the variations seen in normal CL are not due directly to fluctuations in serum cortisol.

An attempt was made to further examine the effects of serum factors by incubating isolated PMN with sera taken from high and low CL-yielding samples (results not shown). The results of these experiments were equivocal in that while there was some suggestion that incubation with high sera increased the CL response, incubation with low sera gave variable results. These problems may have been due in part to the combined effects of cell damage from repeated washing and CL-quenching from residual serum.

The paired bovine twins showed differences in their PMN CL suggesting that the variability seen with the sheep PMN cannot be attributed to genetic differences in neutrophil metabolism alone.

CL is finding increasing use as a measure of neutrophil function in situations where individual results are compared (Stevens and Young, 1977; Wilson et al., 1978; Welsh et al., 1980; Selvaraj et al., 1982; Galgiani et al., 1983). For example, Stevens and Young (1977) recorded the CL from human PMN exposed to different isolates of E. coli. The results were presented as the means of duplicate recordings made with single PMN isolates from 2 donors and they concluded that CL correlated well with other methods of assessing PMN function.

The results presented here demonstrate that there is a marked variability in CL response which makes the validity of direct comparisons between individuals questionable unless consistent differences are recorded over a number of days. The assay may be useful where major defects in PMN function exist. However, more subtle differences could go unnoticed or be misinterpreted on the basis of a single blood sample.

CHAPTER 4

PROFILES OF LUMINOL-ENHANCED CHEMILUMINESENCE RESPONSES

4.1 Introduction

In Chapters 2 and 3 CL was reported as either a peak or integrated millivolt value. Another way of recording CL is to plot CL values against reaction time. In this way profiles of CL function are obtained which show any changes in CL associated with the sequence of phagocytosis. The luminol-enhanced CL responses seen using opsonised zymosan (Easmon et al., 1980; Faden and Maciejewski, 1981; Dechatelet et al., 1982; Weber et al., 1983; Cheung et al., 1983), opsonised bacteria (Stevens et al., 1978; Welch et al., 1980; Anderson et al., 1980;) or latex (Selaraj et al., 1982) have usually been descibed as single peaks of varying magnitude and duration. However, a two-peaked response has been noted with opsonised bacteria (Lofgren et al., 1980). The soluble stimuli phorbol myristate acetate (PMA), N-formyl- methionyl-phenylalamine and C5a produce two-peaked responses (Westrick et al., 1980; Dahlgren and Stendahl, 1982; Dahlgren and Stendahl, 1983; Bender and Van Epps, 1983) although Dechatelet et al. (1982) described a single peak with PMA. It has been suggested that the first peak is due to extracellularly released MPO (Dahlgren and Stendahl, 1982; Dahlgren and Stendahl, 1983; Bender and Van Epps, 1983) and the second is related to cellular superoxide production (Dahlgren and Stendahl, 1982) or to cell-associated MPO (Dahlgren

and Stendahl, 1983; Bender and Van Epps, 1983).

The experiments in this chapter were designed to further investigate the variability of CL by examining the profiles produced by normal ovine neutrophils phagocytosing latex beads.

4.2 Materials and Methods

a. Animals

The sheep were those described in Chapter 2.

b. Neutrophil Isolation

PMN were harvested by the method described in Chapter 2.

c. CL Analysis

The reaction mixtures and CL analysis were as previously described. Profiles of the CL responses were recorded on a chart recorder attached to the luminometer, each aliquot being measured over 30 min.

d. Post-equilibration Washing

The effect of the distribution of peroxidase on the shape of the CL profile was investigated by washing the isolated cells to remove

any extracellular MPO. After the routine 30 min temperature equilibration period the PMN were centrifuged for 15 min at 450 x g and the resulting supernatant reserved. The cells were then washed twice in PBS and divided into 2 aliquots; one was resuspended in the original supernatant and the other in fresh PBS with glucose. CL was measured before the washing procedure and again immediately after resuspension of the cells.

4.3 Results

a. Profiles of the CL Response in Normal Sheep

The CL response profiles of 4 sheep sampled at approximately the same time on different days are shown in Fig. 4.1. Two of the isolates showed a response which was essentially single-peaked and which declined rapidly. The other 2 samples each showed a biphasic response which declined less rapidly over time.

b. Profile of the CL Response in Samples Taken at 4 hourly Intervals

Two sheep were bled at 4 hourly intervals for 24 hr. The shape of the CL profile varied between samples (Fig. 4.2a and Fig. 4.2b). Single and double peaked traces were obtained with isolates from the same animal at different times during the 24 hr period. The peak CL values from the animals are given in Table 4.1.

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FIGURE 4.1. Profiles of chemiluminescence responses of neutrophils isolated from 4 different sheep (y axis = chemiluminescence (mV), x axis = time).

FIGURE 4.1.

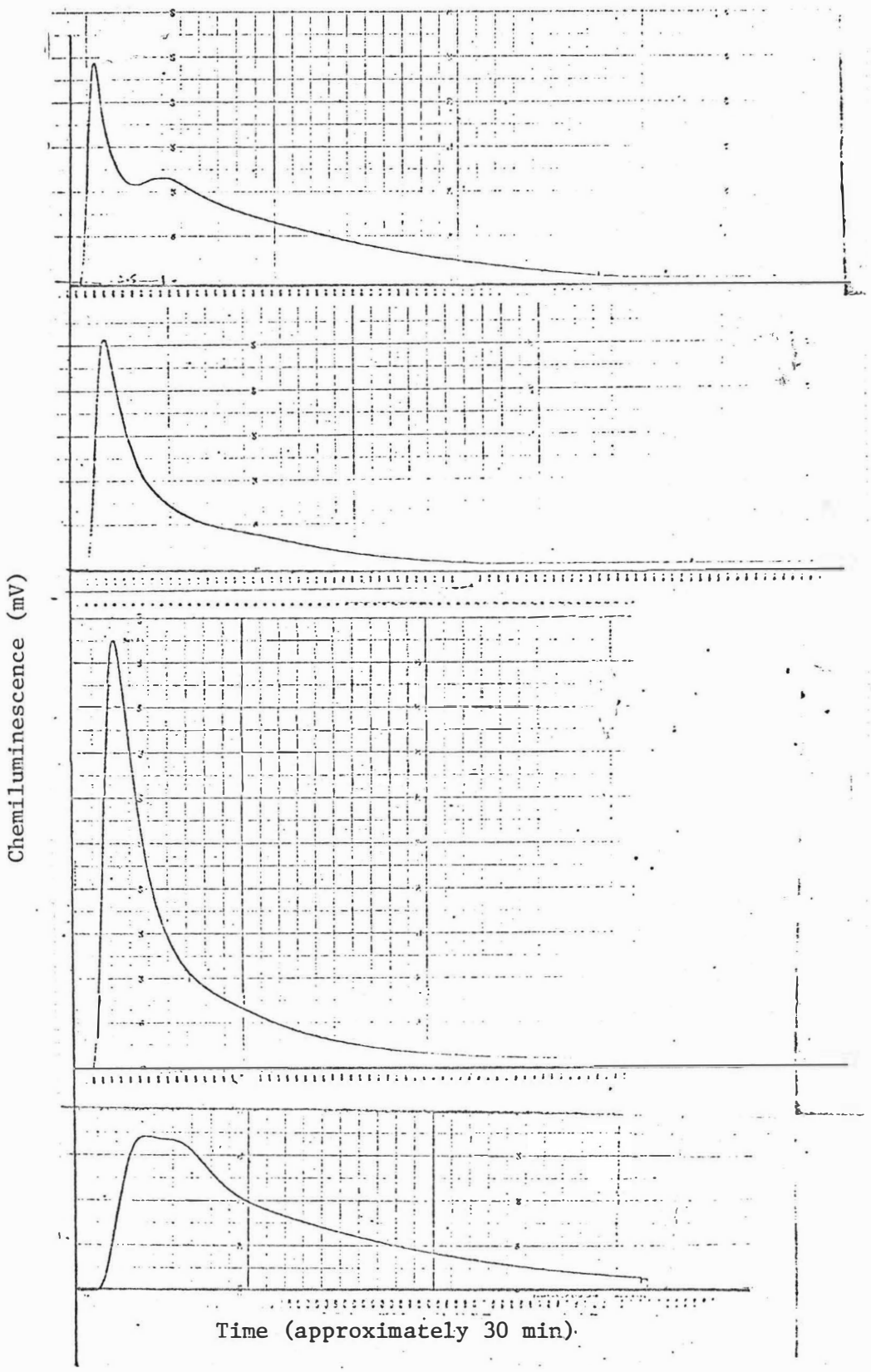

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FIGURE 4.2a. Sheep a, chemiluminescence profiles recorded at 4 hourly intervals.

FIGURE 4.2a

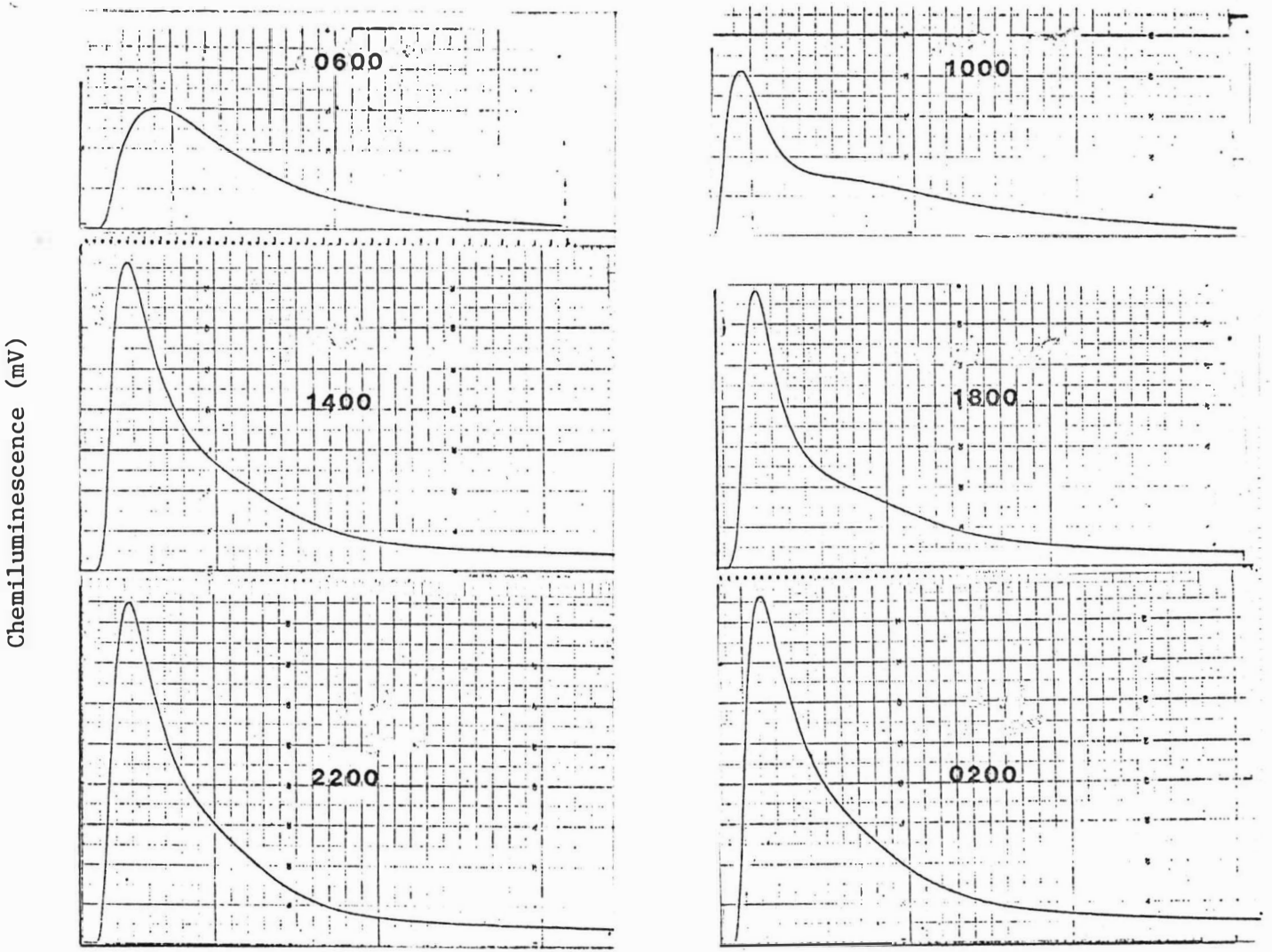
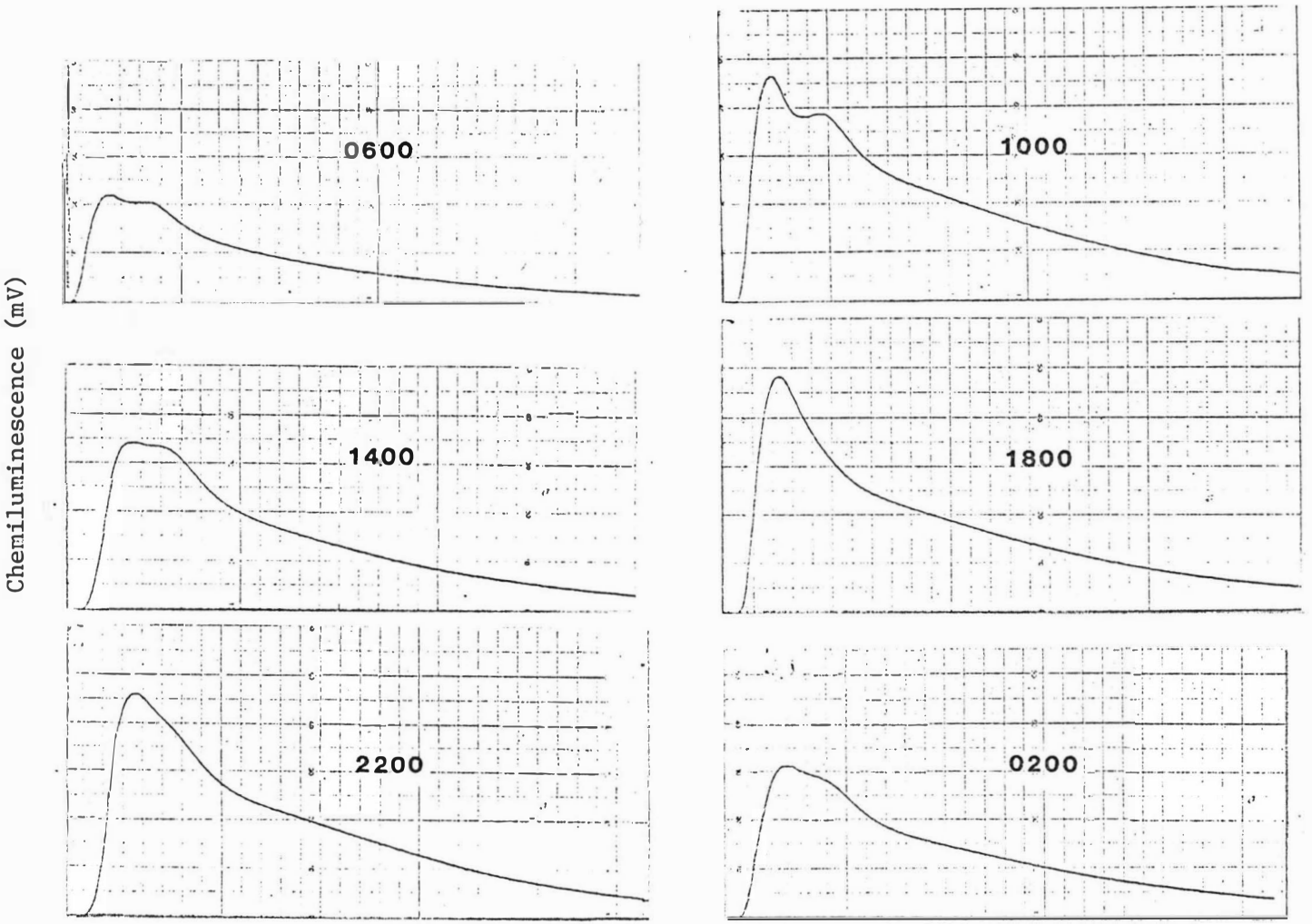

0600
1000
1400
1800
2200
0200
Chemiluminescence (mV)
Time (approximately 20 min)

Figure 4.2b. Sheep b, chemiluminescence profiles recorded at 4 hourly intervals.

FIGURE 4.2b.


0600
1000
1400
1800
2200
0200
Chemiluminescence (mV)
Time (approximately 20 min)

c. Effect of Incubation at 27°C on the CL Profile

The isolated PMN from three animals were incubated at 27°C with constant mixing. Following the standard 30 min temperature equilibration period, a control profile (0 min, Fig. 4.3a and Fig. 4.3b) was recorded. The assay was repeated 60 min and 90 min later. With sheep (a) the first peak increased while the second peak decreased with incubation. For sheep (b) there was little change in the CL profile with incubation while sheep (c) showed an increase in peak one and a decrease in peak two activity. The CL profile for sheep (a) at 0 min was unusual in that the second peak exceeded the first peak in CL activity.

d. Effect of Post-equilibration Washing on the CL Profile

CL profiles were recorded from the PMN of two sheep before and after cell washing (Fig. 4.4). The initial peaks of the profiles recorded before washing (controls) were smaller than those of the later profiles. The CL responses of the washed cells resuspended in either the supernatant or fresh PBS/glucose had more prominent first peaks than did the controls.

4.4 Discussion

The profile of the CL response for human neutrophils engaged in phagocytosis is usually recorded as a single peak but two-peaked responses have been reported, usually following exposure to

TABLE 4.1. Peak chemiluminescence values (mV) from the 2 sheep from which chemiluminescence profiles were recorded at 4 hourly intervals over 24 hr.

	Sheep	
Hours	a	b
0600	1082	797
1000	1475	1678
1400	2744	1231
1800	2453	1744
2200	3848	1663
0200	3101	1127

chemoattractants (Harvath et al., 1978; Lofgren et al., 1980; Dahlgren and Stendahl, 1982).

Previous authors have reported various time sequences for the peak response, which may be explained in part by the differing assay temperatures and instrumentation used. CL measured in a scintillation counter at room temperature peaked anywhere between 10 min (Westrick, 1980) and 80 min (Stevens et al., 1978; Welch, 1980). Where a luminometer was used the CL was reported to peak after 10 to 15 min (Archibald et al., 1983; Cheung et al., 1983) at room temperature and 5 min at 37°C (Easmon et al., 1980). Washburn et al. (1982) using equine granulocytes and a luminometer at 39°C obtained a peak at 1 to 4 min. The two-peaked response to soluble stimuli measured in a scintillation counter has been descibed as 2 and 8 min at 22°C (Dahlgren and Stendahl, 1982) and 1 and 4 min at room temperature (Bender and Van Epps, 1983). There is no published report of a dual peaked response measured with a luminometer. Using the standardised, luminol-dependent CL assay, all the isolates tested displayed one peak 1 to 2 min after addition of the phagocytic stimulus. Some isolates also showed a second peak 4 to 5 min later. When PMN were assayed at 4 hourly intervals isolates from a single animal produced different shaped profiles at different times during a 24 hr period.

The presence of 2 peaks may have been an indication of improved cell preparation and preservation, that is less cell damage with subsequent enzyme release. As cell harvesting was always performed

FIGURE 4.3a. Effect of incubation at 27°C on the chemiluminescence profiles from isolated neutrophils (normal sheep).

FIGURE 4.3a.

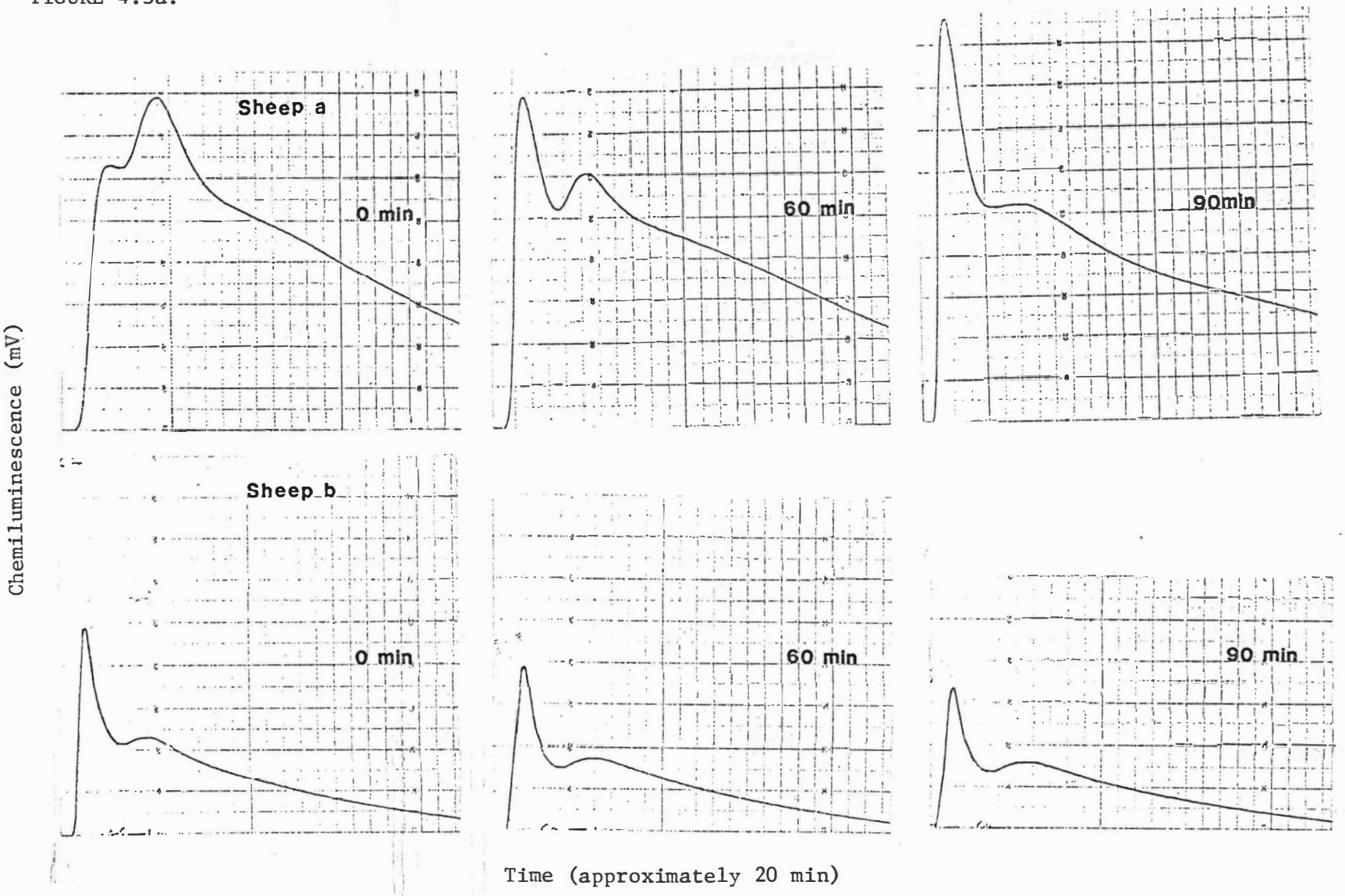

Sheep a
0 min
60 min
90min
Sheep b
0 min
60 min
90 min
Chemiluminescence (mV)
Time (approximately 20 min)

FIGURE 4.3b. Effect of incubation at 27°C on the chemiluminescence profiles from isolated neutrophils (normal sheep).

FIGURE 4.3b.

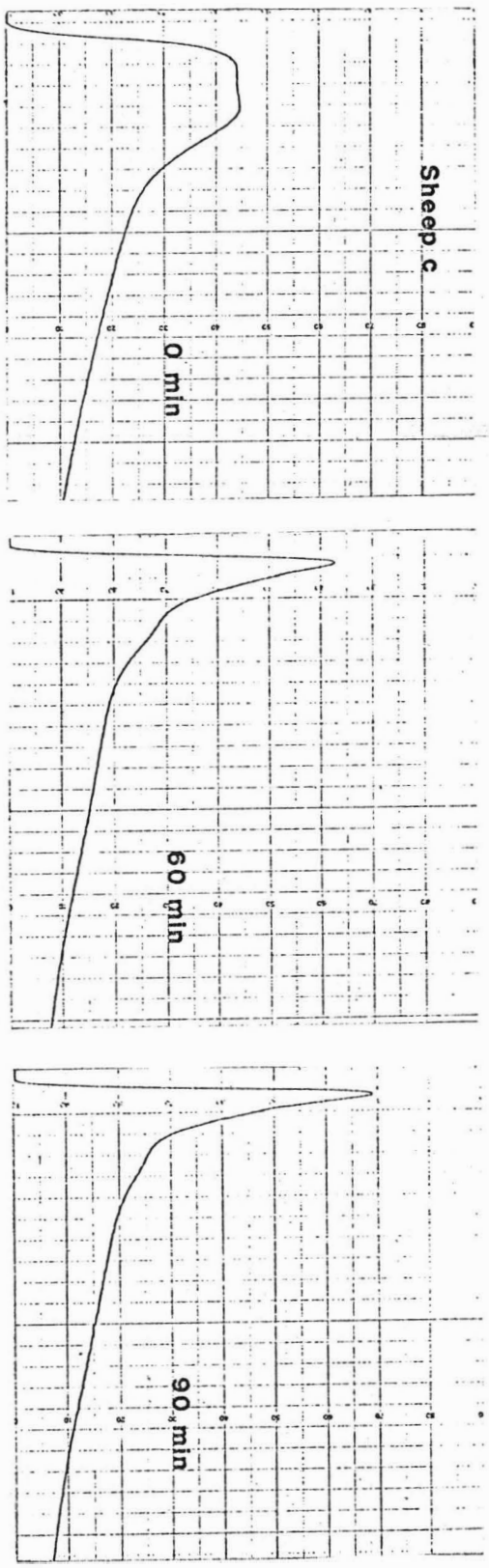
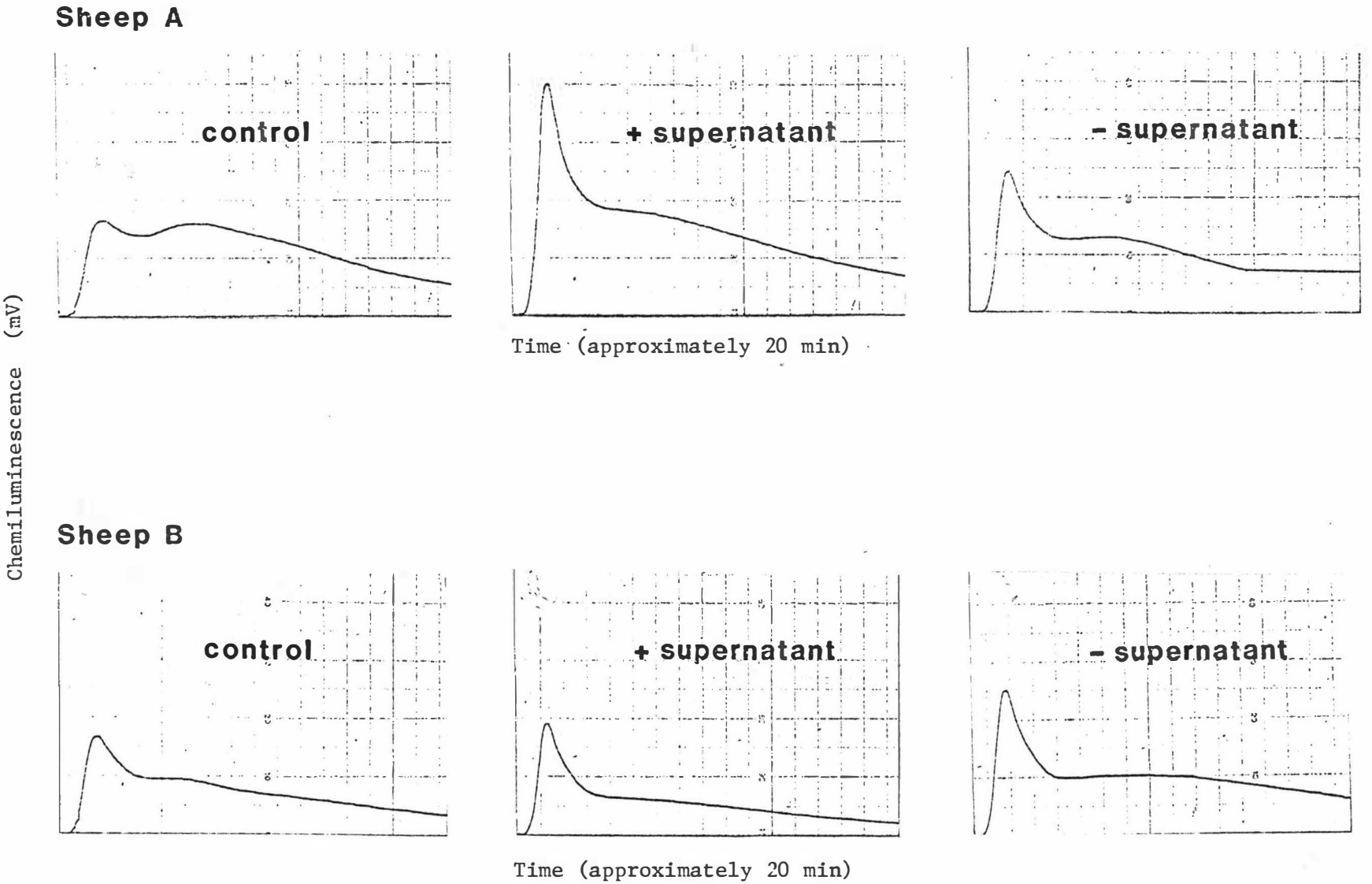

Chemiluminescence (mV)
Sheep C
0 min
60 min
90 min
Time (approximately 20 min)

FIGURE 4.4. Effect of post-equilibration washing on the chemiluminescence profile.

FIGURE 4.4.


Sheep A
control
+ supernatant
- supernatant
Time (approximately 20 min)
Chemiluminescence (mV)
Sheep B
control
+ supernatant
- supernatant
Time (approximately 20 min)

at the same temperature and the method was standardised to the point where the time taken to complete the isolation seldom varied by more than ± 5 min, it seems unlikely that there would be large differences in the proportion of damaged cells between isolates. Exposure to certain substances initiates the secretion of PMN granule enzymes (Wright, 1982). There may be an as yet unknown factor(s) which effects granule movement present in the serum at certain times influencing the availability of MPO. It should be noted that the agents descibed to date have a much greater effect on secondary granules than primary granules and in many cases do not stimulate any primary granule exocytosis (Showell et al., 1976; Bentwood and Henson, 1980; Wright, 1982).

The cell suspensions were incubated at 27°C for prolonged periods to investigate the effect of cell damage from aging and constant mixing on the shape of the curve. If this cell damage increased the availability of MPO then the resulting profiles would be expected to have increased primary and decreased secondary peaks. This occurred with sheep (a) and sheep (c) but in sheep (b) there was a small decrease in both peaks with incubation. A possible explanation for the fall in both peaks in the latter animal is decreased cell viability. The cell numbers isolated from this animal were sufficient only for the CL assay and consequently a trypan blue exclusion test could not be performed.

The first and second peaks are supposedly due to "extracellular" and intracellular MPO, respectively (Dahlgren and Stendahl, 1983;

Bender and Van Epps, 1983). Cell-washing to remove any extracellular elements followed by resuspension in fresh media did not abolish the initial peak. It is concluded that if the presence of individual peaks is a reflection of MPO availability then the initial reaction is due to more readily available, possibly surface-attached, MPO rather than truely extracellular, that is, fluid phase enzyme.

In the experiment in which CL profiles were recorded at 4 hourly intervals the first and final profiles of sheep (b) (Fig. 4.2b) are similar. These profiles were recorded at an interval of 20 hr and are the only suggestion seen of any periodicity in CL activity. The possibility of such periodicity could be investigated over an extended observation period.

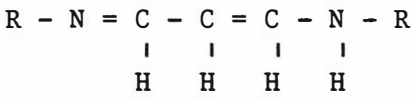
In conclusion, the CL profiles produced by normal ovine PMN show variations in shape which are possibly due to the availabilty of MPO. The use of standardised cell isolation and CL procedures do not ensure that all isolates react identically and therefore the use of CL profiles is no more valid than the use of peak or integrated CL values to assess neutrophil competence.

CHAPTER 5

CL in Ceroid Lipofuscinosis-Affected Sheep

5.1 Introduction

Generalised ceroid lipofuscinosis (CLF) is an inherited disease in which there is an accumulation of autofluorescent lipopigments within various types of cells. The basic metabolic defect(s) responsible for the disease is unknown but it has been speculated that it is caused by a disorder of peroxidation (Zeman, 1974). This suggestion is based on the structure of the storage pigments which are thought to be cross-linked polymers originating from the condensation of malonaldehyde, an unsaturated carbonyl, with the amino groups of nucleic acids, amino sugars and proteins and having the common structural feature:



Where abnormally high quantities of H_2O_2 or other oxidants are present, lipid peroxidation is increased leading to greater malonaldehyde production. Thus the pigments are said to arise from oxidative damage to the cells due to a defect in the defence

mechanism against oxidative damage by H_2O_2 (Awasthi et al., 1977), in particular a deficiency in peroxidase activity. Armstrong et al., (1974a, 1974b) demonstrated a deficiency in leukocyte peroxidase levels in human patients but other reports suggest that the deficiency is due to an increase in the proportion of insoluble, possibly membrane-bound, MPO which is more resistant to extraction than is soluble MPO (Pilz et al., 1976; Farrell and Sumi, 1977).

The absence of luminol-enhanced neutrophil CL in MPO deficiency has been reported (Stevens et al. 1978; Dahlgren and Stendahl, 1983) indicating that light emission in this system is totally dependent on a MPO-calalysed reaction. This observation suggested that CL might be a useful assay to investigate the proposed peroxidase deficiency of CLF. Dahlgren and Stendahl (1983) proposed that the profile of the CL response is determined by the presence of both extra- and intracellular MPO. If cells from CLF-affected individuals contain more insoluble than soluble MPO, their response profiles might be expected to display a smaller initial peak and a greater second peak than those of cells from normal individuals.

This chapter describes the peak CL responses and CL profiles of CLF-affected animals and compares their responses with those of normal sheep.

5.2 Materials and Methods

a. Animals

CLF-affected animals were obtained from a flock of South Hampshire sheep maintained to breed homozygous diseased individuals (Jolly et al., 1980). Diagnosis had been established by histopathology of brain biopsies prior to the onset of clinical signs. Throughout the experiment they were housed together in a covered pen and fed hay and water ad libidum. Normal Southdown sheep of a similar age housed under the same conditions were used as controls.

b. Neutrophil Isolation

PMN were harvested by the procedure descibed in Chapter 2.

c. CL Analysis

The reaction mixtures and CL analysis were as descibed in Chapters 2 and 4.

5.3 Results

a. Peak CL Values

Seven CLF-affected sheep were sampled at approximately the same time on different days and their peak PMN CL measured. The range

of the results for the 7 donors was 1393 to 3743 with a mean of 2638 (Table 5.1). The range of peak CL for normal, Southdown sheep established previously (see Chapters 2 and 3) was approximately 500 to 3900 with a mean value of approximately 2100.

b. Profiles of the CL Responses in CLF-affected Sheep

PMN from 4 CLF-affected lambs and 4 normal lambs were assayed to determine their CL profiles (Fig. 5.1 and Fig. 5.2). The CL profiles of the affected sheep were similar to those of the control animals.

c. Effect of Incubation at 27°C on the CL Profiles of CLF-affected Sheep

Isolated PMN were incubated at 27°C with constant mixing. The CL profiles were measured after the standard 30 min equilibration period (0 min, Figs. 5.3a and 5.3b) and again 60 and 90 min later. There was no distinct second peak at 0 min in any of the 3 animals examined, although sheep (b) showed some indication of one developing. With sheep (a) and sheep (b) the height of the CL peak was increased at 60 min but declined more rapidly than that of the initial recording. In sheep (b) and (c) second peaks had emerged at 90 min.

TABLE 5.1. Peak chemiluminescence responses from 7 ceroid lipofuscinosis-affected sheep.

Sheep	Chemiluminescence (mV)
a	1996
b	1393
c	3182
d	2360
e	3483
f	2380
g	3743

5.4 Discussion

The assays used to investigate the proposed peroxidase deficiency in CLF have been unsatisfactory, primarily because the results could not be duplicated with assays incorporating hydrogen donors other than p-phenylendiamine (PPD) (Patel *et al.*, 1974, Armstrong *et al.*, 1974a, Armstrong *et al.*, 1974b; Clausen and Jensen, 1975; Jensen *et al.*, 1977), although Awasthi *et al.* (1977) claimed to have demonstrated a peroxidase deficiency with guaniacol and o-dianisidine as the hydrogen donors. Tsan *et al.* (1978) found that horse-radish peroxidase oxidised PPD in the absence of H_2O_2 giving a product with the same absorbtion spectrum as the peroxidative product and concluded that PPD was not a suitable hydrogen donor for the study of peroxidase.

Experiments with cells from human subjects with an absolute MPO deficiency suggest that in a luminol-enhanced CL assay, the response is totally dependent on a MPO-catalysed reaction (Stevens *et al.*, 1978; Dahlgren and Stendahl, 1983). The apparently normal response of neutrophils from the CLF-affected sheep indicate that these cells contain normal levels of peroxidase but it is also possible that MPO is not a rate-limiting component of the light-producing reaction and that a partial MPO deficiency might not be detected on the basis of the CL response.

Some investigators have noted difficulties in solubilizing leucocyte peroxidase (Katsushima, 1963; Himmelhoch *et al.*, 1969)

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FIGURE 5.5. Chemiluminescence profiles of ceroid lipofuscinosis-affected lambs.

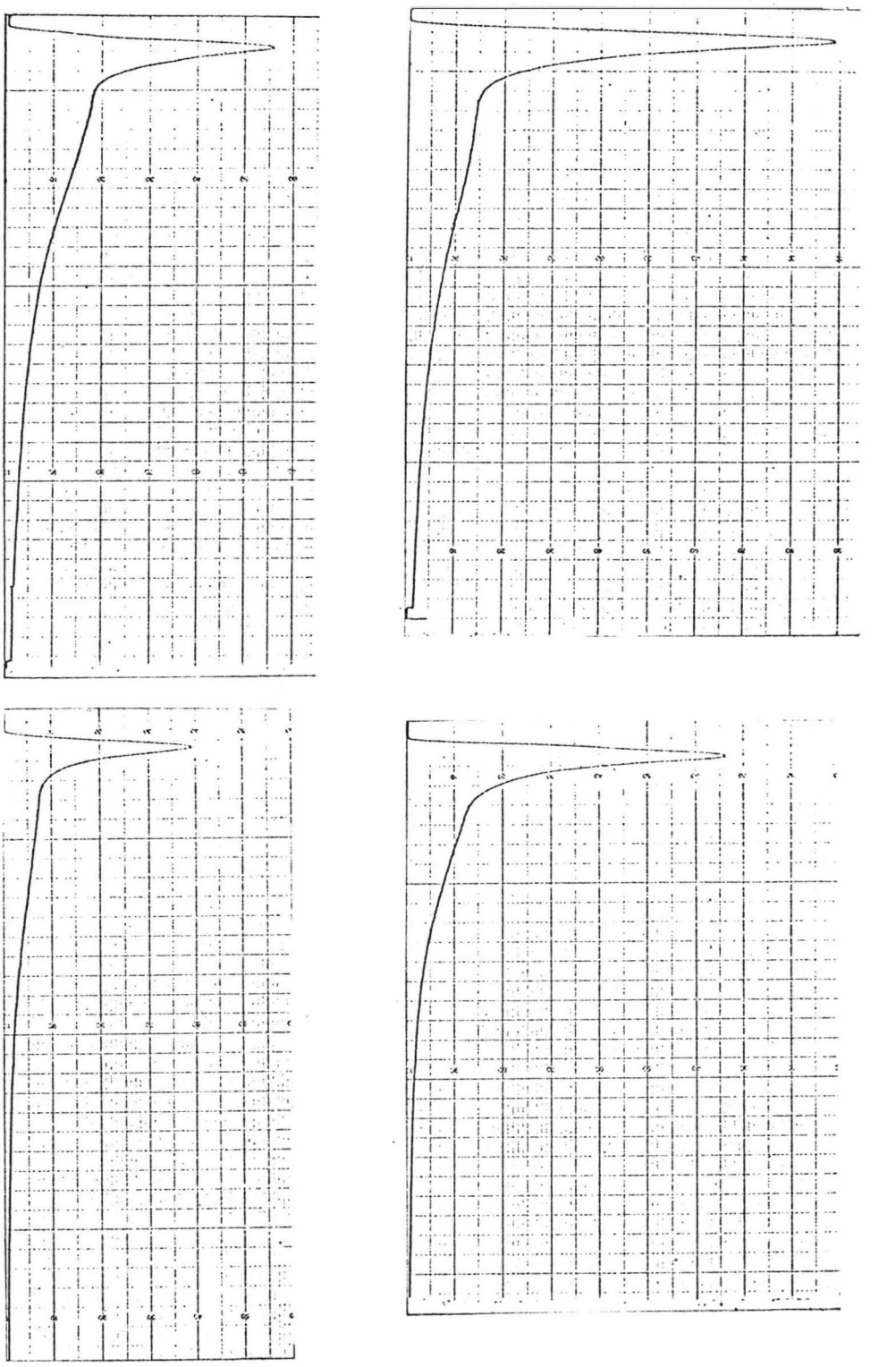

FIGURE 5.1.

Facing Page 98

FIGURE 5.2. Chemiluminescence profiles of normal, Southdown lambs.

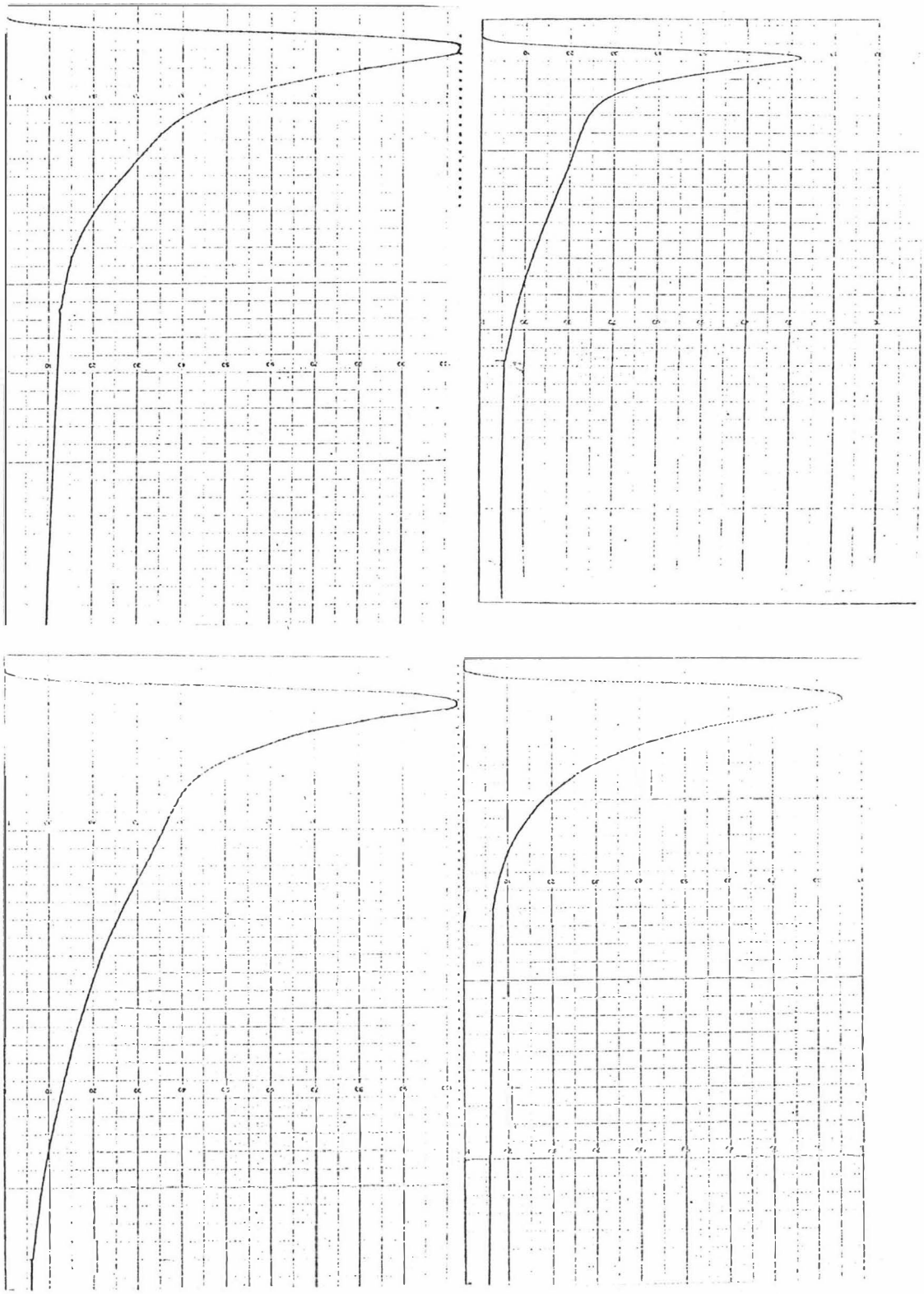

FIGURE 5.2.

suggesting that the differences in total activity recorded by various authors might stem in part from inefficient extraction methods. Using a system developed to extract maximum PMN peroxidase, Pilz et al. (1976) concluded that leucocytes contain both soluble and insoluble, probably membrane-bound, peroxidase fractions with the insoluble fraction having 10 to 15 times greater activity than the soluble fraction. They found that the soluble but not the membrane-bound fraction was decreased in CLF patients. Later investigators also commented on solubilization difficulties and recorded levels of activity within the normal range for CLF patients when care was taken during enzyme extraction or a whole cell homogenate was used as the enzyme source (Anzil et al., 1974; Farrell and Sumi, 1977; Pilz et al., 1978; Den Tandt and Martin, 1978). These findings have lead to the suggestion that the total peroxidase is not decreased in affected subjects but that there is a relative increase in the insoluble fraction, possibly due to an abnormally stored product (Farrell and Sumi, 1977; Bozdech et al., 1980).

The CL profiles obtained from CLF-affected lambs did not differ noticeably from the profiles of normal lambs. If the ratio of insoluble (membrane bound) to soluble MPO was increased in the neutrophils of CLF-affected sheep then it might be predicted that extended storage of the cells could not lead to externalisation of the MPO. If this was so then the first peaks of the CL profiles of CLF-affected animals would not increase by the same magnitude shown by those of normal sheep. The results presented here show that the

FIGURE 5.3a. Effect of incubation at 27°C on the chemiluminescence profiles of ceroid lipofuscinosis-affected animals.

FIGURE 5.3a.

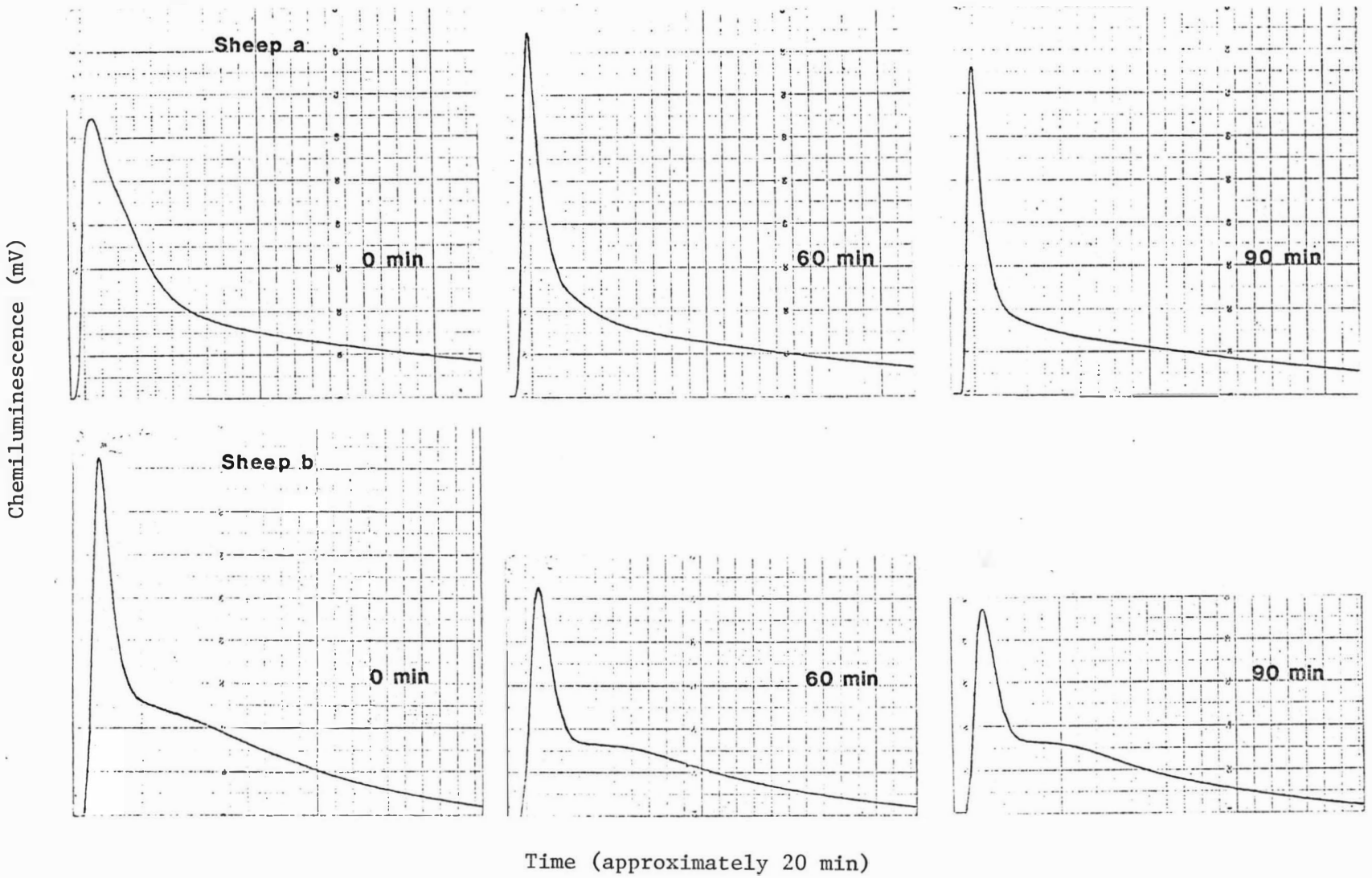
Sheep a
0 min
60 min
90 min
Sheep b
0 min
60 min
90 min
Chemiluminescence (mV)
Time (approximately 20 min)

FIGURE 5.3b. Effect of incubation at 27^{o}C on the chemiluminescence profile of a ceroid lipofuscinosis-affected animal.

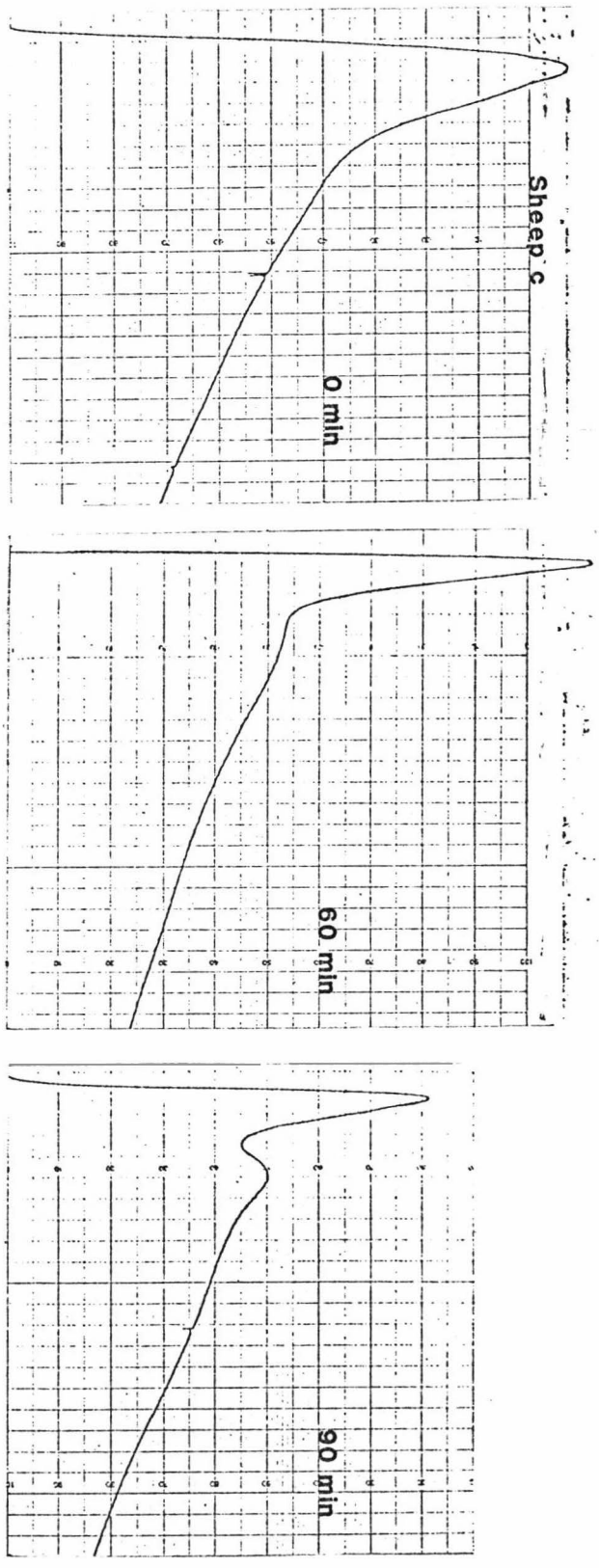
Chemiluminescence (mV)
Sheep c
0 min
60 min
90 min
Time (approximately 20 min)

FIGURE 5.3b.

CL profiles of PMN from CLF-affected sheep and normal sheep (Chapter 4) are similar following extended incubation periods. A possible explanation for this which does not exclude changes in MPO solubility in affected sheep, is that accelerated death and lysis of the PMN occurs during the incubation resulting in exposure of insoluble peroxidase to the fluid phase. However, cytocentrifuge preparations of CLF-affected PMN examined during the initial isolation procedure did not show any obvious differences between these cells and those from normal sheep.

The results presented here suggest that the peroxidase activity of the PMN from CLF-affected and normal sheep are similar and do not indicate any change in the proportions of soluble and insoluble MPO.

CHAPTER 6

A Concluding Statement

This investigation has examined the luminol-enhanced CL produced by isolated ovine PMN phagocytosing latex beads. The CL was considered in three ways; as peak and integrated values and as profiles of the CL output against time. There were variations in the CL response between animals and from day-to-day within the same animal with all of these methods and large ranges of "normal" values were encountered. It is concluded that CL alone is an inappropriate assay for defining the competence of ovine neutrophils.

Many of the reactions which are suggested to occur in the neutrophil during phagocytosis have the potential to produce CL but the actual reactions responsible for the light emission have yet to be defined. Secondary excitation of components of the phagocytosed particle, of the reaction mixture or of the cell membrane might contribute to the CL response. In addition the amount of MPO available and the light-producing oxidation reactions may differ from time to time within the PMN from a particular donor. Furthermore, the role of humoral factors in influencing CL has not been thoroughly explored.

Latex beads were the only phagocytic stimulus examined in detail. An investigation of the CL responses induced by complement and

antibody-opsonised zymosan and bacteria, which react with specific membrane-associated receptors, and by soluble stimuli seems a logical extension of this study.

The CL output from ovine PMN was low when compared with a limited number of human and canine samples assayed under the same conditions. A comparative study of the ranges of CL responses from different species under standardised conditions may indicate those in which the assay is a more reliable investigative tool.

At present the assay may be of use where the results are obtained from a single cell isolate, that is, for comparing the in vitro effects of specfic treatments on separate aliquots of cells. In particular this technique may be useful for evaluating the effects of certain theraputics on neutrophil function.

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APPENDIX

a. Erythrocyte-Lysing Solution

NH_4Cl	8.32g
$NaHCO_3$	0.84g
EDTA	43.2mg

Make up to one litre with distilled water and adjust to pH 7.2 (Andersen and Amirault, 1979).

b. Trypan blue staining for cell viability

Two hundred µl of PMN suspension were mixed with 1.8ml of trypan blue stain (1gm trypan blue in 100ml of 0.9% NaCl in water). The cells which took up the stain were assumed to be dead and were expressed as a percentage from a total count of 200 cells.

c. Indwelling jugular cannulae

The cannulae consisted of silicon rubber ("Silastic", Dow Corning Corporation, USA) with silicon cement beads ("Silastic Epimer", Dow Corning Corporation, USA) attached at approximately 10 and 15 cm from the venous end.

Anaesthesia was induced with intravenous alphaxalone/alphadolone ("Saffan", Glaxo NZ Ltd) and maintained with a mixture of 2% halothane ("Fluothane", Imperial Chemicals Ltd) in oxygen via an

endotracheal tube. A midline incision was made in the ventral neck and both jugulars isolated by blunt dissection. The veins were occluded cranially and a small incision made caudal to the occlusion within a preplaced pursestring suture. Each cannula was inserted, tied in place beyond the second bead and the vessel checked for leakage. Both cannulae were exteriorised through a small opening in the dorsal neck and immobilised under an elasticised netting vest.

d. Cortisol assay

One hundred µl of serum were added to 2ml of dichloromethane and vortexed for 30 sec. Following overnight storage at -20°C the liquid fraction was poured off and dried down under air. One ml of ethanol was added to each tube and duplicate 100µl aliquots dried under air. Two hundred µl of antiserum/tracer solution (see below) were mixed with the residue and incubated at room temperature for 12 to 16 hours after which time 500µl of dextran/charcoal (see below) suspension were added. The mixture was incubated for 10 min and centrifuged at 1900 x g at 4°C for 20 min. A 500µl aliquot from each tube and 5ml of scintillation fluid were mixed in a scintillation vial and counted for 2 min.

i. Antiserum/Tracer solution

12ml borate buffer

250µl tracer stock ([1,2,6,7-^{3}H]-cortisol, Amersham International Ltd, Amersham, UK)

300µl 2-5% Bovine gamma globulin

240µl 10% bovine serum albumin

100µl antiserum (Cortisol antiserum F3-314, Endocrine Scientific Products, California, USA)

ii. Dextran/Charcoal suspension

0.5% dextran and 0.25% charcoal stirred in distilled water for 2 hr prior to use.