DETERMINANTS OF PHAGOCYTOSIS:

A STUDY OF THE VARIABLE FACTORS INVOLVED IN THE INTERACTION BETWEEN RED BLOOD CELLS AND MACRO- PHAGES.

CONTENTS.

SUMMARY.

The experimental study of the phagocytic process presents considerable difficulty. These difficulties arise principally because phagocytosis occurs only with living phagocytic cells whose phagocytoci ability is related to the environmental conditions in which they occur. Measurements of phagocytic ability can be made on living animals, but the data obtained from IN VIVO experiments are not applicable to individual phagocytic cells and cannot therefore be used to investigate the process of phagocytosis at the cellular level. The phagocytic process can be investigated using IN VITRO techniques, and can be quantitated in tersm of the reactivity of the individual phagocytic cells comprising the culture. In using such techniques strict control must be exercised over the conditions of culture.

Phagocytosis is an active process involving three elements, the phagocytic cell, the particle to be phagocytosed, and the medium in which phagocytosis takes place. Each of these elements is subject to variation. The variability can be controlled by employing a system which fulfills the following criteria:

1. The phagocytic cell employed must be shown to be functionally active under the conditions of the experiment.

2. The test particle must be of a constant composition, and should be capable of controlled modification, according to the needs of the experiment, without affecting the system which phagocytosis of the particle is to be measured.

3. The medium in which phagocytosis occurs must be rigidly defined in terms of its composition and biological activity.

4. That the time of contact between the phagocytic cell and the test/

test particle must be constant, and preferrably short.

5. That the phagocytic cells employed are of similar age, and are maintained under the same conditions as the phagocytic cells comprising the control series.

6. That the experimental model allows the independent study of the phagocyte, the particle and the medium, and the quantitative assessment of the effect of each of these variables on the phagocytic act.

In the following experiments, the system employed monolayer cultures of mouse macrophages obtained by peritoneal lavage. These macrophages were maintained in media containing sera, and in media containing no serum protein. The test particles employed throughout were erythrocytes from the mouse, and from a variety of other species, including man. All cultures were carefully controlled, strict attention being paid to the proof of the functional capacities of the macrophage cultures under the experimental conditions. In this system the independent contribution of each of the three variables to the phagocytic process could be assessed.

The particle.

The test particle employed in these experiments was the human red cell. Previous reports indicate that the human red cell is rendered susceptible to phagocytosis by incubation in physiological saline for 18 to 24 hours. (Stuart & Cumming 1967). In the system employed fresh human red cells were not susceptible to phagocytosis. These experimental findings were confirmed, and an investigation made into the "injury"which rendered red cells susceptible to phagocytosis. Injury did not occur if red cells were incubated/

incubated for similar periods of time in media containing serum, but they rapidly became injured in defatted serum. Supplying the red cells with glucose, co-enzymes, and vitamins during the incubation period did not prevent injury occurring. Inhibition of cellular respiration with metabolic inhibitors, and stimulation of cellular respiration with methylene blue did not prevent injury occurring in red cells incubated in saline. It was concluded that injury was related to some membrane deficiency in the red cell caused by incubation in media lacking the phosphatides necessary for the maintainence of cell membrane integrity. Analysis of the supernatant from suspensions of injured red cells showed that the injured cells had lost both protein and lipids from their membranes. Quantitative estimates of the amounts of lipid loss showed that phagocytosis of red cells will occur when 10% to 20% of membrane lipid has been lost. These conclusions suggest that, for the red cell, susceptibility to phagocytosis is determined primarily by the state of the red cell membrane.

The medium.

Most experimental studies of phagocytosis are performed in the presence of serum. It is generally known that serum affects the phagocytic process. It was found that macrophages in culture could be maintained in a synthetic medium containing no serum or serum proteins, thus providing an opportunity to investigate the basic efforts of serum proteins upon phagocytosis. Using macrophages grown in serum free medium, it was shown that the phagocytic reaction to human red cells was more pronounced in the absence of serum than in its/

its presence. The depressive effect of serum, from both the mouse and man, occurred in fractions containing immunoglobulinG. The depressive effect of immunoglobulin fractions was both time and concentration dependent.

The Macrophage.

Experiments performed with fresh and injured red cells, and with serum containing and serum free medium, were extended to investigate the phagocytic reactivity of the macrophage towards the red cells of a variety of species. An analysis of the results obtained show that the macrophage is capable of three separate classes of reactivity. These have been defined as:

1. Recognition, or the ability of the macrophage to ingest red cells coated with haemolytic antibody. This class of reactivity is independent of the culture medium, and does not diminish in long term culture.

2. Heterogene discrimination, or the ability of the macrophage to ingest the red cells from genetically different sources. This class of activity is affected by the presence of serum, and is lost in long term culture.

3. Homogene discrimination, the ability of the macrophage to discriminate between healthy and injured red cells from the same species of donor. This class of activity is not affected by serum, but is lost in long term culture.

A theoretical survey of the likely nature of the reactions between macrophage and the red cell indicates that the process of phagocytosis, and the allied processes of recognition, homogene discrimination/

discrimination, and heterogene discrimination, are determined primarily by the reactivity of the macrophage membrane. The experimental results indicate that the macrophage membrane has the capacity to detect alterations in both its fluid and cellular environments, and to react to certain types of alteration by the process of phagocytosis.

CHAPTER 1

INTRODUCTION

CONTENTS.

1 - THE OBJECT OF THE PRESENT STUDIES.

Phagocytosis has been studied quantitatively for many years.
Most of this work has been concerned with the role of serum proteins
and antibody in promoting the phagocytosis of particles. Rather
less effort has been expended in the study of the phagocytic cell
itself. External factors affecting the phagocytic process are well
known, yet consideration for how these might act upon the phagocytic
cell is lacking. This is even more surprising in the light of
recent interest in the discriminatory processes of cells as they
affect cellular differentiation, organogenesis, and antibody
synthesis. The object of the present studies is to examine the
theoretical aspects of the interaction between the phagocytic cell
and particle, and to determine experimentally the phagocytic
potential of the macrophage towards red cells under varying con-
ditions of environment.

Phagocytosis is a widely distributed and well known biological phenomenon. It was first described by Lazzaro Spallanzani as occurring in "animalcules" generated in infusions of hay. In his book "OPUSCOLI FISICA ANIMALE ET VEGETABLE" he concludes that the phagocytic mechanism was concerned with feeding in these primitive animals. Interest in phagocytosis was reawakened by Haeckel (1857) with his description of the uptake of solid particles by the coelomic phagocytes of the crayfish. In 1862 Haeckel extended these studies by injecting carmine into the coelom of the sea hare (Tethys) and observing the maoebocytes with ingested the dyestuff. He related the phagocytic abilities of these cells to their capacity for amoeboid movement. In 1862, Von Recklinghausen observed the uptake of cinnabar particles by phagocytes in the lymph sac of the frog. Max Schultz in the same year observed and described phagocytosis of carmine by the slime fungus Polythalamica. Preyer (1864) used the dye indigo to study the phagocytes of the frogg and the salamander (Salamandra Maculata). He commented upon the time needed to complete the phagocytic act and observed pigment, blood cells and crystalline inclusions in the amoeboid cells. Ponfick (1869) demonstrated the localisation of the pigments carmine and vermillion in the liver, spleen, and lymph nodes of mammals after intravenous infection. Langhans (1870) showed that the removal of extravasated blood in the guinea pig and rabbit was associated with erythrophagocytosis, and the subsequent production of brown pigment by the phagocytic cells. Bizzozzero (1871; 1872) showed the/

the capacity of the macrophage to ingest polymorphonuclear leuko-
cytes in exudates. In 1874 Panum suggested that phagocytic cells
were responsible for the ingestion of bacteria which entered the
animal body. This was demonstrated in the classic paper by
Robert Koch (1876) in which he described the localisation,
phagocytosis and subsequent dissemination of anthrax bacilli by
the phagocytic cells of the frog. Gaule (1881) demonstrated the
phagocytosis of the gut parasite Drepanidium by the amoeboid cells
of the frog. Grawitz (1877), while studying a fungus growing upon
lily of the valley, injected some fungal spores into suspensions of
phagocytic cells, and observed their uptake. Roser in 1881 wrote
"The immunity of animals in absolute health depends in my opinion to
the relative quantities of salt contained in their fluids, and upon
the property of their contractile cells of ingesting the enemy which
enters the animal body". Metschnikoff in 1883 began his famous
researches upon the digestive capacity of phagocytic cells, and
postulated the mechanism of ingestion and digestion of bacteria by
phagocytes as the principal method of immunity in mammals. It is
also evident from later studies that he appreciated the role of the
macrophage in removing effete and dead cells from the whole organism
(1899). He described the removal of spermatozoa from the peritoneal
cavity of the guinea pig and described the increase in rate of
removal following previous immunisation. He also studied the
removal of red blood cells, and commented upon the effects of anti-
leukocytic serum upon both polymorphonuclear leukocytes and macro-
phages. He wrote "..... Nous avons mis en lumière le rôle
préponderant/

préponderant que les macrophages jouent dans la résorption des
cellules. Nous avons vû que ce sont surtout les phagocytes mono-
nucleaires qui s'émparent des élements morphologique introdiuts dans
l'organsim et l'example des spermatozoides nous a prouve que les
macrophages sont bien capables de saisir même des cellules vivantes".

The first attempt to explain the phagocytic act were due to
Haeckel (1862) and Preyer (1864) who related the capacity of certain
cells to phagocytose to the observation that such cells were always
capable of amoeboid movement. Partly because of the lack of good
culture techniques, and partly due to the discovery of the role of
opsonic substances, first described by Durham (1897), further
investigations into the nature of the phagocytic act were not under-
taken for many years. The first serious attempt to consider the
phagocytic process, from the point of view of the cellular changes
involved in the act, was due to Rhumbler (1914). In previous papers
Rhumbler (1889; 1910), had considered the role of surface tension
changes in the locomotion of amoebae, concluding that the production
of pseudopodia was the result of local lowering of the surface
tension in the membrane. In his paper "Das Protoplasma Als
Physikalisches System" written in 1914, he described four methods of
phagocytosis observed in the process. (Tait 1918; 1920) applied
Rhumbler's conclusions to the mammalian phagocyte, and critisised
Rhumbler's physical interpretation of the role of surface forces in
phagocytosis.

The classical studies of W.O. Fenn, in the years 1921 (a, b, & c)
1922,/

1922, and 1923 (a, b) remain to this day the most important single
contribution to the physiological investigation of the phagocytic
act. Ponder (1926; 1927), modified Fenn's treatment of the surface
forces in phagocytosis, and concluded that changes in cytoplasmic
viscosity were more important in phagocytosis than changes in surface
tension. He also considered the effects of surface charge, and
concluded that charge effects did not greatly contribute to the
process of phagocytosis.

Since this time two significant advances in the study of
phagocytosis have been made. The first was the proof of the
association of the phagocytic process with increased cellular
respiration by Baldridge and Gerrard in 1933). The second has been
the study of the dynamics of phagocytosis in vivo, by the technique
of measurement known as colloidal particle clearance, first described
by Halpern, Biozzi, Mene and Benecerraf in 1951. There now exist
a large body of knowledge of the biochemical events associated with
the phagocytic act. These events have been reviewed by Karnovsky
(1962; 1965) himself a major contributor in this field.

The clearance technique has already shown the importance of
opsonic substances in in vivo phagocytosis, and has allowed
measurements of the effect of such variables as particle size, the
effects of antibody, and of pharmocological agents upon the phagocytic
process in vivo. This work is an important addition allowing the
experimental testing in the whole animal of results obtained in vitro.
(See Halpern B.N., Editor, Physio-Pathology of the Reticulo-
Endothelial System, Blackwell, Oxford, 1957).

3 - DEFINITION OF PHAGOCYTOSIS.

Phagocytosis is the engulfment of a particle by a cell accompanied by the expenditure of energy. It is therefore an active process and a property only of living cells. The particle comes to lie within the phagocytic cell but enclosed upon all sides with the cell membrane.

The phagocytic reaction is usually said to occur in three stages: the approach and contact of the particle with the phagocyte, often resulting in adherence of the particle to the surface, engulfment of the particle either by the production of pseudopodia or by the invagination of the cell cytoplasm adjacent to the point of contact, and finally the formation of an enclosed vacuole in which digestion usually occurs. The mechanisms of the phagocytic process are unknown. Initiation of the event seems to involve changes in the plasma membrane of the macrophage (Essner 1960; Karrer 1960). There is an increase in respiration by phagocytosing cells (Baldridge & Gerrard 1933). The event itself uses A.T.P. (Greendyke et al 1964; North 1966) which is derived from either aerobic or anaerobic glycolysis. (Karnovsky & Sbarra 1960; Karnovsky 1962). A proportion of this energy is involved in synthetic mechanisms, especially the synthesis of glycogen and lipid during the phagocytic process. (Sbarra & Karnovsky 1960; Karnovsky 1964; Sastry & Hokin 1966). Some of the metabolic changes can be directly related to phospholipid turn over in the membrane itself during the process. (Sastry/

(Sastry & Hokin 1966).

4 - CLASSIFICATION AND DISTRIBUTION OF PHAGOCYTIC CELLS.

Phagocytic cells are widely distributed in nature. Many protozoa are capable of phagocytosis, and all metazoa possess phagocytic cells. In the Protozoa, Porifera, and Coelenterata, none of which possess a gastrointestinal tract, phagocytosis is a feeding mechanism. In the higher invertebrates, the Annelida, Insecta, Crustacea, and Cephalopoda, the phagocytic cells are well differentiated elements to be found in the blood or coelomic fluid. These phagocytes with well developed capacities to phagocytose cell debris and bacteria, constitute the primitive "immune" system. This is in some aspect the equivalent of the mammalian reticulo-endothelial system. (Huff 1940; Salt 1966; Stuart 1968).

The phagocytic cells of the mammal were originally classified into two main types, the microphages or polymorphonuclear leukocytes and the macrophages (Metschnikoff 1905). The macrophage in the mammal forms a system of phagocytic cells defined by Aschoff (1924) as the reticuloendothelial system. This system includes all the mononuclear phagocytic cells of the mammal. These can be broadly distinguished as three main classes of macrophage, endothelial macrophages, reticular macrophages, and free macrophages. The endothelial macrophages are represented by the Kupffer cells of the liver, and the phagocytic endothelium of the spleen and lymph nodes. Reticular macrophages constitute the macrophages of the germinal centres in spleen and lymph nodes, and the meduallry macrophages of the thymus. Free macrophages occur in peritoneal, pleural and pericardial/

pericardial sacs, and throughout connective tissues generally.
The macrophage of mammalian blood is called the monocyte. (For
reviews see Cappell 1929; Aschoff 1924; Marshall 1956). Cells
other than those of the R.E.S. (Gordon et al 1960; Rabinovitch
1969; Gropp 1963) and cells from some tumours can be induced to
phagocytose both in vitro and in vivo. That other cell types are
capable of phagocytosis in normal and abnormal environments, suggests
that the underlying mechanisms of phagocytosis may be common to all
mammalian cells (Gropp 1963). The phagocytic cell in the mammal
has evidently retained this primitive cellular function in a modified
and highly specialised form, whilst other tissue cells have, through
the course of evolution, lost this ability. The occurrence of pin-
ocytosis in nearly all mammalian tissue cells may reflect the residu-
um of an archaic phagocytic process.

A macrophage might therefore be expected to show morphological
features that distinguish it from neighbouring cells. By light and
electron microscopy the macrophage has no uniquely distinguishing
features other than frequent evidence of previous phagocytosis.
Macrophages can therefore be accurately defined only by their
functional characteristics, that is their ability to phagocytose
a variety of particles, and their capacity for pinocytosis in the
absence of specific inducer substances. (Carrel & Ebling 1926;
Nelson 1968; Rustad 1964). Morphological observations suggest
however that the macrophage has a somewhat larger surface area in
relation to its volume than the majority of other tissue cells.
The folded plasma membrane is frequently disposed as veils, pseudo-
podia/

podia, and fimbriae over the whole of the cell surface. There may
be a relationship between the convoluted membrane of the macrophage
and its exceptional phagocytic abilities. During the process of
phagocytosis, the surface area of the macrophage must increase, since
plasma membrane, originally part of the surface, encloses the particle
after engulfment. The cell has increased its volume whilst
decreasing its surface area, and the surface tension in the membrane
would rise. Since the work of phagocytosis must be done against the
tension in the membrane, the phagocytic process would soon stop
unless some method of increasing the surface area was available to
the macrophage. This is reflected in membrane flow. The metabolic
processes accompanying phagocytosis are in part concerned with the
synthesis of new membrane (Karnovsky 1964; Sastry & Hokin 1966;
Oren et al 1963). These synthetic processes probably enable the
macrophage to continue to phagocytose particles even of large
dimensions until not enough free cytoplasm remains to enclose a
single additional particle. The initial phagocytic response can
proceed to completion before significant amounts of membrane have
been synthesised because of the large reserve of plasma membrane
contained in the cytoplasmic veils, pseudopodia, and fimbriae. It
has been shown that supplying exogenous phospholipid, lysolecithin,
to phagocytes that have already ingested materail to the point of
inactivity, will re-establish phagocytosis by these cells. (Munder
& Fischer, 1965). These authors regard the action of lysolecithin
as affecting the interfacial tension between the membrane and the
medium. Macrophages inactive due to the repeated ingestion of
particles/

particles might well reach a state of inactivity because of an increase in membrane tension. The incorporation of phospholipid from the medium into the phagocyte membrane theoretically should re-establish phagocytosis by these cells.

Similar increases in phagocytic ability have been described when metabolites such as pyruvate, succinate, A.D.P., A.T.P., and ascorbic acid are added to the medium. These substances act by increasing the metabolic potential of the phagocytosing cell (Greendyke et al 1964), and hence the synthesis of new membrane material.

5 - THE NATURE OF THE REACTIONS BETWEEN CELLS.

If the assumption is made that phagocytosis is a phenomenon dependent ultimately upon the behaviour of the cell membrane, then those factors determining the process must act directly or indirectly upon the cell membrane. Since it is also likely that the phagocytic reaction is merely a variation of the reaction of any cell towards its environment, an examination of those factors involved in such reactions should illuminate the phagocytic process.

Cells react one with another to form the organised tissues so essential to the development of the multicellular organism. When tissues are observed with the electron microscope, the membranes of opposing cells are seen to be separated by a gap. This gap appears to be of almost constant width, the average separation of cell membranes being between 100 A and 200 A (Robertson 1961; Elbers 1965). A variety of methods have failed to reveal any clear cut evidence of a "cement" substance between cells, although some workers claim that a layer of intercellular material is present (Easty & Mercer 1962) (Elbers 1965). Specialised membrane structures such as the desmosomes and zonulae occludentes occur in regions of close contact between membranes. The constancy of the gap between cells, the absence of any demonstrable cementing substance, and the presence of these highly organised membrane structures suggest that the membranes of cells in opposition in some way react to one another at a distance. This mutual interaction results in cell adherence. Loss of this mutually adhesive capacity can be shown to occur in cells from/

from tumours. (Abercrombie 1962; Purdom & Ambrose 1958).

The fact of adherence requires that some attractive force exists between cells. The presence of a gap between cells requires that this attractive force be balanced by a repulsive force acting between cell membranes. The exact nature of these forces has been the object of intense enquiry for many years. A survey of the conclusions reached in these investigations (Curtis 1967) reveals that

1)   these forces arise in the plane of the cell surface.

2)   they are dependent upon the underlying structure of the cell membrane and

3)   they may be altered by changes in the aqueous environment of the cell. Forces such as these may be involved in phagocytosis. The adherence of a particle to the macrophage surface could be determined by those forces which between other cells cause adhesion. The act of phagocytosis, and the metabolic changes which accompany the act, may have a parallel in such phenomena as contact inhibition, and cellular differentiation. In such cellular reactions the membranes of the opposing cells are the primary reactants, and such reactions lead to changes in cell biochemistry, and morphology through the mediation of the reacting membranes. It is proposed that the phagocytic reaction is similar in its fundamental nature to these other types of cellular interaction. A study of the variable factors involved in the interactions between cells, that is a study of the surface, the cell membrane, and the environment is a necessary preliminary in the discussion of these variables as they affect the phagocytic process.

6 - THE VARIABLE FACTORS INVOLVED IN CELLULAR INTERACTIONS.

a.   The immediate environment of the cell: the cell surface.

The immediate environment of any cell is its surface.   The cell
surface can be defined as that region where the environment, and the
membrane of the cell meet.   The characteristics of this region are
therefore the products of the interaction of these two elements.
In other words, the properties of this surface are determined by the
structure of the cell membrane, and the composition of the external
environment of the cell.

The cell surface has a characteristic chemical structure.   The
surfaces of mammalian cells are coated with a layer of mucoprotein
together with adsorbed protein, which can be removed from the cell by
incubation with trypsin and neuraminidase.   (Heard & Seaman 1960;
Cook et al 1960).   The layers of protein and mucoprotein at the cell
surface were initially envisaged as being present as two layers, a
layer immediately adjacent to the lipid bilayer in the form of an
extended sheet and a second layer of globular protein lying outwith
this sheet.   This structure was proposed by Harvey & Danielli (1938)
to account for the very low interfacial tension existing in cell
membranes.   Recent work by Maddy & Malcolm (1965) indicates that the
postulated layer of protein in the form of an extended film, or sheet,
at the cell surface is not revealed when red cell membranes are
studied by infra red spectroscopy.   These workers found the proteins
at the cell surface to be mainly in their conventional globular con-
figurations.   The mucinous component of the mammalian cell surface
is/

is usually N acetylneuraminic acid, often found in combination with proteins, lipids and sugars such as fucose. This mucoid layer contributes the surface ionogenic groups which give rise to the surface charge. The carboxyl groups of N-acetyl neuraminic acid, are individually the most important contributors to the cell surface charge (Cook et al 1961). Removal of the mucoprotein or sialic acid from the cell surface/charge *reduces surface* by at least one third as measured by the subsequent fall in the electrophoretic mobility of the treated cells. (Cook et al 1961). The relationship between the electrophoretic mobility of a cell, and its surface charge is a complex one. At the cell surface an electrical double layer is formed, composed of a layer of ions firmly held to the surface, and a more diffuse mobile layer extending some distance into the aqueous environment of the cell. The fixed ionic layer is derived from the ionogenic groups of the surface layers of the cell. The mobile layer is composed of ions arranged in electrostatic combination or association with the ionogenic groups of the surface. These ions are called "counterions" and are complimentary in charge to those ionogenic groups with which they associate. These ions are hydrated, and have a finite size. (Tanford 1965). Their centres of charge will therefore not be coincident with the groups with which they associate, but will always lie some distance away. One part of the ionic double layer will therefore be occupied by space in which no ionic centres can be present. Because of the distribution of electrical charges at the surface, there is a difference of potential between the layer separating the fixed and the diffuse ionic layers, and the bulk environment./

environment. This potential is called the electrokinetic potential or zeta potential. In electrophoretic mobility studies, the application of an E.M.F." to the particle will cause a displacement of the oppositely charged layers relative to one another, and this displacement will occur through the layer separating the fixed and mobile charge layers, that is the layer in which no ionic centres can be present. This layer is called the "surface of shear". The zeta potential can be measured directly from measurements of the electrophoretic mobility, and the fixed cell surface charge can be computed from measurements of the zeta potential using the Goy-Chapman equation (See Cook et al 1961).

The fixed charges of the cell surface do not all lie within the same molecular plane, there may be convolutions of the cell surface leading to occlusion of at least a portion of the cell surface charge. In addition the surface may be penetrable to counterions from the surrounding medium. These two effects may lead to an underestimate of the thickness of the ionic double layer, and also of the cell surface charge. (Haydon 1961). The ionic structure of the cell surface may most properly be regarded as a three dimensional matrix of fixed charges, penetrable to some extent by counterions in the surrounding medium. The total thickness of this "electrokinetic cell surface" is in the order of some 20 to 30 A. The region in which the surface charge is measured that is the plane of shear, lies about 10A outwith the idealised "true" surface. The remaining 10 to 20 A, represents the ionic double layer, which is variable in thick-ness/

E.M.F." - Electromotive Force.

thickness depending on the ionic composition of the surrounding medium.

The chemical structure of the cell surface is important in that it determines directly the cell surface charge, and the thickness and composition of the ionic double layer. The electrokinetic structure of the cell surface is of importance in the consideration of cellular interactions. The electrokinetic structure cannot be analysed in the same way as the chemical structure of the cell surface. Its existance can be shown by electrophoretic mobility studies of cells, under varying environmental conditions (Heard & Seaman 1960; Seaman & Heard 1960; Cook et al 1961). It is now appreciated that the electrokinetic cell surface is functionally of great importance, in particular in determining the adhesiveness of cells (Purdie & Ambrose 1958), and accounting for the observed separations between cells. (Curtiss 1967).

Mammalian cells appear to bear an anionic surface charge. This has been shown to be due to the ionisation of the carboxyl groups of N acetyl neuraminic acid at physiological pH. The constancy of this anionic surface charge could account for the observed separations between cells. Derjaguin & Landau (1941) and Vervey & Overbeek (1948), suggested that the stability of colloidal solutions was a function of the surface potential of the particles in solution and derived equations relating the observed surface potential to the repulsive forces acting between particles. The data of Derjaguin, Landau, Vervey & Overbeek applied to mammalian cells, indicates that the repulsive forces due to the surface charge might acoount for the interactions of cells at distanced equivalent to twice the thickness of/

of the ionic double layer. The magnitude of this repulsive force will depend upon the environmental parameters of temperature, and the bulk dielectric constant of the medium. Interactions of cells over distances of the order of 50 to 60 A could theoretically occur. (Curtiss 1962). The observed fact that red cells can be agglutinated by suspending them in solutions containing divalent cations, of heavy metal ions which lower cell surface charge, and increase the thickness of the ionic double layer, emphasises the importance of the cell surface charge in providing the repulsive forces which normally keep cells apart. (Bangham et al 1958; Weck et al 1962).

It is more difficult to account for the attractive forces between cell and cell upon an examination of the surface potential alone. An attractive force between particles with surface potentials unlike in sign, or between particles with unequal surface potentials of the same sign, might exist. (Bierman 1955). Such attractive forces are likely to be weak, and ineffective over distances greater than about 15 A. (Pethica 1961). For this reason some workers have considered the adhesion of cells to result from the formation of "salt linkages" between a divalent ion, and the iogenic groups of two opposing membranes. (Pethica 1961; Easty & Mercer 1962). The specificity of cell adhesion, as described the embryologist, cannot be explained on the basis of salt linkages between opposing membranes. (Curtiss 1967). The adhesive forces acting between cells must therefore be explained with reference to some other quality of the interacting cell surfaces.

An interaction of cells at a distance can only occur if those forces/

forces acting at a distance are greater than the random thermal energy
of the molecules at the point of action. The random thermal energy
of the environment is usually expressed as $E = kT$, where E is the
random thermal energy, k is Boltzmanns constant and T is the absolute
temperature. This expression, and those governing the rates of
reaction in biological systems, are derived from the classical
statistical evaluation of the entropy of the system under considerat-
ion, (Dugdale 1966). These statistical methods are valid for large
volumes of molecules, or even for microstates containing small
numbers of identical molecules. The calculation of the surface
potential of cells from their electrophoretic mobility, involves
several concepts which may not be valid in microscopic terms.
Measurement of the rate of shear at the surface depends upon the
assumption that the bulk viscosity of the medium through which the
cells moves is equivalent to the viscosity in the plane of shear.
Again, the bulk ionic strength of the environment is assumed to be
constant up to the plane of shear, that is to within the thickness of
the ionic double layer. The measurement of the electrophoretic
mobility of cells cannot be related to the surface potential of those
cells without error; that is the measurement of cell surface charge
is an approximation. The theoretical evaluation of the cell surface
charge, from the consideration of the double layer theory, and the
idealised concept of the surface charge as a sheet of point charges,
or a uniform sheet of charge, is also an approximation. Again this
treatment of surface potential assumes that the distribution of
charges upon the cell surface, and in the immediate environment, can
be/

be described by statistical approximations. As an example, if the field due to the surface charge on the cell membrane is calculated on a statistical basis, using the Gouy-Chapman equation, it becomes uniform. The isopotential points in that field will all occur in planes parallel with the surface. The composition of the ionic double layer must also be uniform. It is evident that the cell surface, by reason of its chemical composition, is not a uniform sheet of charges, but represents rather a charge mosaic. The surface potential of a mosaic of fixed charges can be calculated by, methods known for some times, on a non-statistical basis. (Bitter 1956; Friedenberg 1967). These methods involved the theoretical quantitation of the field of each individual charge, and the summation of the fields of many such charges. The formulae describing the nature and characteristics of electric fields are not approximate, and they remain exact for interactions over any distance. (Bitter 1956; Duffin 1968). The need for more exact descriptions of the electrokinetic cell surface stems from the inadequacy of double layer theory in explaining the adhesion of cells, and in particular the specificity of cell adhesion, and other cellular interactions.

The electrostatic field structure of the cell surface.
An electric field consists, generally speaking, of lines of force originating on positive charges, and terminating on negative charges. An electrical field can therefore be said to exist whenever there is a charge inequality. For an ionised molecule, a field exists between that molecule (or between the centre of charge of that molecule) and its counterion.

For/

For a single point charge, for example the electron charge upon an ion, the field is radial in distribution, and decays as the inverse of the square of the distance from the charge. Even unionised molecules and individual atoms are not electrically neutral. Asymetrical molecules, such as water and sulphur dioxide, possess a dipole structure because of the asymetry of the interatomic bonds. This leads to the behaviour of the molecule as an electrical dipole, since the charge distribution of the molecule is not asymetrical. An electric field can be shown to exist as a consequence of the dipole moment of any asymetrical molecule of this type. This field is expressed at right angles to the vector describing the dipole orientation. This field is sometimes referred to as "Long Range Van der Waals Forces". This terminology has not been used to avoid confusion with the Van der Waals forces originating from the internal oscillating dipoles of individual atoms. (See review by Pethica 1961). Since it is a closed field, it decays inversley with the cube of the distance from the dipole. The lipids present in biological membranes, for example cholesterol and lecithin, have quite large dipole moments due to molecular assymetry. In addition the phospholipid molecules have polar head groups which under the influence of environmental pH, can ionise, and thus contribute one full electron charge to their field. The individual atom can also behave as a dipole, since the electrons in that atom cannot, by the uncertainty principle, be regarded as uniformly distributed around the nucleus. The positively charged nucleus, and the negatively charged electron can be regarded as an oscillating system, that is

as/

as an oscillating dipole. This dipole also produces a field, which can be shown to decay as the inverse of the seventh power of the distance from the dipole. These fields have been referred to as "Van der Waals" forces, in this thesis.

The field produced within biological membranes can be regarded as having three major components, the field produced by ionised molecules, the field produced by polar molecules with permanent internal dipole moments, and the field produced by the internal oscillating dipoles of the non-polar atoms.

The field due to the ionised molecules upon the surface of the cell membrane can be considered as of importance in providing the distractive forces which tend to keep cells apart. This occurs because of the similar anionic charge upon mammalian cell surfaces. The field arising from the internal oscillating dipoles of non-polar atoms, is responsible for the intermolecular interactions usually referred to as Van der Waals forces. These forces arise by the polarisation of the dipoles in neighbouring atoms, providing that these atoms are of similar molecular weight, and thus have similar frequencies of oscillation. These forces tend to bind like molecules together, and to act only at short range. The action of Van der Waals forces in contributing to cell adhesion has been considered by Curtiss (1960; 1962) and Jehle, Parke & Salyers (1964). The latter authors consider that the Van der Waals forces are of great importance in determining the specific association of one molecule with another. These forces for example act between the hydrocarbon side chains of phospholipid molecules. This will determine the packing of/

of lipid molecules in the cell membrane. Studies of artificially
prepared monolayers of lipid, show the lipid molecules to exist in
two "phases" depending upon the pressure applied to the monolayer.
Under conditions of high surface pressure, the molecules become
stacked almost perpendicular to the interface. In this phase, the
monolayer exhibits some of the characteristics of a crystal, and is
stated to be in the anisotopic or "crystalline" phase. As the
surface pressure is relaxed, the molecules become less tightly
packed, and less perpendicular to the interface. In this state they
are considered to be in the isotropic or "liquid" phase. (Dervichian
1949; Reiss Husson 1967; Chapman 1966). The lipid molecules
within cell membranes have recently been shown to exist on the
borderline of the transition between the liquid and crystalline
phases. (Luzzati et al 1962; Byrne & Chapman 1964). The asymet-
rical polar head groups of the phospholipid molecules composing the
cell membrane have a permanent dipole structure. The binding of
the hydrocarbon tails of these molecules within the membrane ensures
close packing of these phospholipids, and determines the orientation
of the polar head groups of the molecules at the membrane/medium
interface. For a layer of phospholipid molecules orientated
perpendicular to the interface, the field produced by these molecules
will lie in the plane of the membrane, and will be zero at any point
normal to it. (Bitter 1956). The field produced by the dipole
moment of the polar head group of a phospholipid molecule will decay
as the inverse of the cube of the distance from that molecule. The
effects of this field have therefore been neglected and considered to
be/

be of less importance than the field arising from the fixed surface charge on the cell membrane. Some features of the behaviour of artificial membranes, and of living cells cannot be explained upon the basis of surface charge alone.

It has been shown that changes in surface pressure applied to a lipid film will cause alterations in the surface potential of that film, and that the penetration and solvation of substances such as proteins in the film can be affected by alterations in the surface pressure. (Doty & Schulmann 1949). Similar effects can be shown by monolayers of non-polar lipids, such as cholesterol, and by polar lipids at their isoelectric point. These effects are therefore not simply due to the surface charge carried by the monolayer. Studies of the behaviour of a wide variety of living cells, including amoebae and red cells, in artificially generated electric fields, suggest that the observed effects cannot be explained on the basis of the cell surface charge. (Furedi & Valentine 1962; Furedi & Odah 1964) (Teixeira Pinto et al 1960). Charged inert particles show a different pattern of behaviour from that observed with living cells.

The lipid molecules of the cell membrane behave as molecular dipoles, generating a field at the membrane surface. If the lipid molecules are orientated at right angles to the interface, there will be no field at points normal to the membrane. The presence of macromolecules in the vicinity of the membrane will distort the orientation of the dipoles, which will align themselves along the local field lines arising from these accumulations of charged macromolecules. Even if the membrane is composed of a tightly packed bilayer of lipid, the/

the presence of macromolecules at the surface will ensure that a
field will exist at points normal to the membrane. If the lipid
molecules are treated as idealised point dipoles (an assumption
which is valid for a dipole length which is small compared with
intercellular distances), and the fields for the individual dipoles
are summed to points above and below the membrane, a field strength
several orders greater than "kT" can be shown to exist at distances
greater than 1,000 A from the membrane surface. If the field due
to the surface charges is computed in a similar manner, it can be
shown to have similar values at this distance from the cell surface.
Calculations of the field strength in the immediate vicinity of the
membrane, (up to 7 A from the surface) show probable values as high
as 10^9 newtons/ coulomb of charge. These values are high enough to
maintain water filled "pores" through the membrane, and are more than
sufficient to account for the selective adsorption and passage of ions
through the membrane. Moreover at distances consistent with the
thickness of the ionic double layer, the field strength due to the
dipoles exceeds the field due to the surface charges. (See Frieden-
berg et al 1966, a, b, c,; Friedenberg 1967).

From these considerations it can be shown that the rigorous
thermodynamic conditions obtaining in the vicinity of the cell surface
will result in an ordering of the molecular species, both solvents,
and solutes, at the cell border. This will apply not only to ions,
but to any molecule having a dipole structure, for example protein
molecules, and water. The action of the field at the membrane
surface upon water molecules in the immediate vicinity of the membrane
will/

kT" - The random thermal energy of the environment.

will be to polarise the dipoles of the water molecules which will
then move towards the membrane surface and stack against it. The
stacked water molecules are present in an ordered array, sometimes
termed "clathrate" or ice-like. The occurrence of such forms of
water in biological systems has already been postulated for the
behaviour of exchangeable and bound water in protein molecules.
(Jacobson 1955). One important feature of ordered structures of
water is the high viscosity the presence of even small amounts of
ordered water confers upon water in the bulk phase (Duryagin &
Churayev 1968). The recent interest in water "polymers" has shown
that anomalous water structures can be formed in glass or quartz
capillary tubes. This water has a viscosity fifteen times greater
than that of normal bulk water, a higher density than water, and a
lower freezing point (Willis et al 1969; Deryagin & Churayev 1968).
It is already known that the water inside the mammalian cell nucleus
exists in a form distinct from bulk water.

If water is present in an "anomalous" form in regions close to
the cell membrane, it could play a significant role in determining
cellular interactions. As an example, anomalous water existing in
the cell cytoplasm would tend to confer a high viscosity to the cyto-
plasm resulting in a "gel" structure. Since the presence of anomal-
ous water is likely to depend upon the proximity of a cell membrane,
and the field arising from it, a change in the membrane or its field
could result in a loss of the anomalous water structure, and its
reversion to water in the bulk phase. This would tend to decrease
cytoplasmic viscosity, and result in a gel to sol transformation.

In/

In summary the presence of an electric field at the cell surface can account for:

a. The interactions of cells at distances greater than 1,000 A.

b. The ordering of polar molecules at the cell surface, and the selective adsorption of proteins and ions by the cell membrane.

c. The redistribution of ions between the extracellular and intracellular phases.

d. The organisation of the internal molecular anatomy of the cell, its cytoplasm, and its organelles.

e. The specificity of the interactions between cells.

The redistribution of the polar molecular species in the vicinity of the cell surface is probably one of the adaptive mechanisms of cells, enabling them to survive and function under conditions differing from those obtaining in the healthy intact animal. The ability of cells to adjust to the in vitro environment, for example, reflects the capacity of the healthy membrane to adjust the local environment towards the optimum in those environmental conditions. Failure to so adjust whether from biochemical distur- bances, structural deficiencies in the cell membrane, or extreme conditions in the environment, will result in cell malfunction and eventual death.

b. The bulk environment.

It has been shown that the local environment of a cell is produced by the interaction of two parameters, the composition of the/

the bulk environment, and the structure of the cell membrane. It has also been suggested that small changes in the bulk environment, can be adjusted to by the cell membrane, and are unlikely to produce gross changes in the behaviour of cells.

The loss of this ability to adapt to changes in the bulk environment could occur through loss of membrane integrity, or through changes in the bulk environment too great for the local compensatory ability of the cell membrane. The loss of membrane integrity can be produced by the action of lipid solvents, heavy metal ions, organic molecules such as formaldehyde and so on. All these substances prove toxic to cells whatever their environment. An example of a gross change in the bulk environment, too great for the compensatory ability of the cell membrane, is shown by the effects of saline solutions of decreasing toxicity upon red cells. Such a medium is deficient in both ions and macromolecules, and yet some degree of adaption to these solutions is shown by the membrane, resulting in the well known osmotic fragility curve.

The bulk environment of the cell in vivo is regulated within very narrow limits by the concerted actions of all cells. The specialised organs of regulation are the lung and kidney, but all cells contribute directly to this regulation by the cellular processes of pinocytosis and ion transport. Thus the composition of the extracellular fluid is maintained in the face of a changing external environment.

The macrophage is one of the major contributors to the processes of homeostasis. It removes selectively the dead and damaged cells, which/

which by their presence prejudice the survival of others, and will also remove substances such as fibrin from areas of inflammation. Its role in the removal of bacteria and the detoxification of toxic materials is well known. It has recently been shown that the macrophages of the liver will selectively ingest damaged organelles in intact but injured parenchymal cells. (Stuart et al 1969).

The composition of the external environment is important for two principal reasons, firstly it contributes these ions and macro- molecules from which the cell orders and maintains the local environment, and, secondly, since the volume of the local environment of the cell is small compared with either the intracellular or extra- cellular volume, it provides an important "buffering" mechanism, reducing the degree of which cells have to adapt, thus extending their range of function.

c. <u>The structure of the membrane in relation to the environment.</u>

There is a tendency for lipid substances, in particular the phospholipids which are univeral constituents of cell membranes, to associate in micellar structures in the presence of water. These micelles are composed initially of small globules of lipid surrounded by water. The polar hydrophilic head groups of the phospholipid molecules dissolve in the aqueous environment, while the hydrophobic "tails" associate in the central region of the globule. Addition of further phospholipid to the system causes an enlargement of the area of contact between the aqueous and hydrophobic phase. The globule is therefore no longer the most stable form of lipid, and the/

the molecules reassociate to form a variety of tube-like structures, and eventually sheets. (Lucy & Glauert 1964). If these structures are fixed and sectioned in the usual way for electron microscopy a unit membrane corresponding in its dimensions to the plasma membrane of cells is seen. (Henn et al 1967; Bangham 1967; Fernadez-Moran 1964). Studies of these"myelin forms" of phospholipid suggest that they are energetically the most stable forms of phospholipid in the aqueous environment. (Haydon & Taylor 1963; Weiss 1963). To be stable this arrangement of phospholipid should exhibit a minimal interfacial tension along the plane of the leaflet. When the interfacial tension of the phospholipid bilayer reaches zero, the addition of further molecules of phospholipid will simply cause an enlargement of the areas of contact between the aqueous and hydro-phobic phase, that is an enlargement of the phospholipid sheet will occur. The interfacial tension is a complex function of such membranes. Unlike the surface tension in a simple liquid/air or lipid/water system, two separate sheets of molecules here interact through a hydrophobic phase to produce a single unit. The tension of this unit is therefore the algebraic sum of the two opposing surface tensions, and merely reflects slight differences in the aqueous environment between the two sides of the bilayer. The surface tensions of cells, have been shown to be of a very low order, in the region of 0.02 - 2 dynes/ cm. (See Harvey 1954). These actual tensions are much lower than those predicted for monolayers of lipid from the behaviour of lipid films at an oil water interface. This lead to the proposal by Harvey & Danielli (1938) that a layer of/

of adsorbed protein was needed to account for the low interfacial tensions observed for cells.   The data of Haydon and Taylor (1963) have shown that in a bimolecular membrane the tension developed along the plane of the leaflet facing the exterior of the cell will be almost exactly balanced by the tension developed by the second leaflet towards the cell interior.   The net tension, which is the algebraic sum of the two individual leaflet tensions, therefore, reflects merely the slight differences in composition between the external and internal environments.   Maddy & Malcolm (1965) have moreover confirmed the absence of the extended protein sheet, as proposed by Harvey & Danielli, at the surface of red cells.

All cell membranes so far examined contain proteins.   The role of proteins in the structure of cell membranes is uncertain.   It has been suggested that one possible type of membrane protein is composed of two hydrophilic peptide regions widely separated by a central hydrophobic region in the centre of the molecule.   (Wallach et al 1966).   The protein molecule is thus envisaged as penetrating the membrane, its polar groups remaining on or near the surfaces. The proteins of the red cell membrane have been extensively studied by Maddy (1966 a, b, c,; 1964).   The weight of protein present in the red cell membrane is one to two times the combined weight of the lipids in the membrane.   It is therefore quantitatively the most important portion of the cell membrane.   A proportion of this protein is enzyme protein.   The proteins of the red cell membrane can be extracted by butanol, suggesting that they may be hydrophobic in part, and that they probably contain lipid.   Proteins from the environment/

environment are also adsorbed to the surface of cells, and some are
so firmly bound they must be considered as a part of the membrane
proper. (Gramblich & Muller 1963). The study of frozen, fractured
cell membranes has revealed a structure consistent with the bilayer
structure originally proposed by Harvey & Danielli (1938). There
are some globular subunits revealed by this technique which could
be protein in nature. The numbers of these sub-units varies with
the cell and organelle from which they are obtained. (Branton 1966).

The present concept of the cell membrane is that of an extended
bilayer of phospholipid, protein, and cholesterol. Since the
phospholipid layers of the cell membrane contain the smallest
numbers of molecules theoretically necessary to form a coherent
layer, the term "paucimolecular" bilayer is often used. The polar
groups of the phospholipid molecules are directed towards the
internal and external aqueous environments. The hydrophobic fatty
acid side chains of the phospholipids are in the central region of
the membrane, which also holds the cholesterol in solution. Protein
molecules are present as adsorbed surface layers on both sides of
the membrane. These proteins are globular, very little protein is
present as an extended sheet. Some structural protein occurs
within the bilayer, either as penetrating strands, or as enclosed
globular sub-units within the hydrophobic layers of the membrane.
The lipid components of the membrane are packed in close array,
exhibiting the characteristics of monomolecular films of lipid under
high surface pressure. The interfacial tension of such a membrane
is low. (For reviews see Maddy 1966; Parsons 1967; Robertson 1960;
Stoeckgnius/

Stoeckenius & Engelman 1969).

In considering how two such membranes might interact at a
distance, it is necessary to examine some of the properties of the
membrane consistent with this structure. It has already been shown
that this structure should exhibit a low interfacial tension. This
interfacial tension is to some extent dependent upon the constitution
of the surrounding medium, and the cell membrane in consequence is
likely to be sensitive to the presence of surface active agents.
Thus the presence of aliphatic acids, alcohols, anionic or cationic
detergents in the vicinity of the membrane should produce changes in
membrane structure, and the nature of these changes can be predicted
from the structure. The action of saponin, a substance which will
react with cholesterol to form an insoluble complex, has been descri-
bed by Dourmashkin, Dougherty & Harris (1962). Other surface active
agents cause complete disruption of cell membranes. These substances
act by penetrating the lipid bilayer, and solvating in the hydrophobic
region of the membrane. Since they are also polar substances, water
molecules will be introduced into this region, causing the membrane
to disrupt into micelles with an hydrated interior. The predicted
behaviour of the membrane in the presence of materials capable of
penetrating the bilayer has been shown for α tocopherol acetate by
Lucy & Dingle (1964). It may therefore be concluded that the
mammalian membrane approximates well to the paucimolecular phospho-
lipid bilayer as far as surface energy considerations apply.

The surface structure of the membrane will posses the capacity
to bind ionic groups present in the environment. This capacity
determines/

determines the thickness and composition of the ionic double layer
at the membrane surface. The presence of bound "clathrate" water
structures at the surface will obviously alter the ionic concentration
at the surface, and the degrees of hydration of ions in the vicinity
of the membrane. (Freidman 1966; Jehle 1964).

d. Mechanisms of cellular interactions.

When two cell membranes approach one another, changes will occur
in the medium separating the cells, and in the membranes of the cells
themselves. The cell membrane has been described as a molecular
bilayer composed of closely packed polar phospholipid molecules,
and bearing upon its surface a layer of mucoprotein contributing an
anionic surface charge. Studies with physical systems have shown
that when two charged plates are brought together in a medium con-
taining electrolytes, the displacement of water and electrolytes
between the plates give rise to "streaming" potentials which tend to
resist the close approach of the two plates. These streaming
potentials occur because of the displacement of the ionic double
layers in relation to the fixed charge upon the surface. The
resisting force opposing this displacement is called an electroviscous
force. When the distances between the approaching plates are very
small, an additional viscous force due to the displacement of water
molecules will also occur. Both these forces will tend to slow the
approach of the two surfaces. Experiments using quartz and mica
sheets have shown that a considerable time is required for the estab-
lishment of equilibrium separation, when the distances separating the
plates/

plates is of the order of 100 A. (Elton 1948). These results indicate that for a cell system, the approach of two membranes within 200 A of one another would require:

a. A strong attractive force between the membranes.

b. A long time for the equilibrium separation to be reached. Obviously the system involving two cell membranes is more complex than that involving sheets of inorganic materials. In particular, the character of the viscous and electroviscous forces between two approaching cell membranes will be of importance in determing both the rate of approach and the final distance of separation. Unfortunately, the characterisation of the viscous and electroviscous forces acting between cell membranes is at present not technically feasible, and there has been very little consideration of their effects in intercellular interactions. (Curtiss 1967).

The attractive forces acting between cell membranes can be considered as originating from two sources, firstly the field produced by the Van der Waals-London oscillating atomic dipoles and secondly the much stronger field produced by the internal dipoles of the polar molecules, such as phospholipid, composing the membranes. The action of these fields is not necessarily always attractive. That due to independently rotating (free dipoles) is attractive, but the attractive nature of fixed molecular dipoles depends upon the mutual orientation of these dipoles in the opposed membranes. For the purpose of this discussion these forces are presumed to be attractive. The existance of long range Van der Waals forces of attraction has Titijevskaia, Abricossova, and Malkina. been shown and their effects measured by Derjaguin/(1954) for a physical/

physical system employing two glass plates. The forces between the plates were measured at small distances of separation, in a vacuum, using an elaborate microbalance. Their effect is most marked for slight separations, in the order of a few angstrom units (up to 100 A) and falls off rapidly at distances greater than this. The contribution of Van der Waals forces to the attraction between membranes is probably insignificant, although their contribution to the internal stability and arrangement of the molecules within the membrane is of paramount importance.

The major attractive force acting between cell membranes is almost certainly that due to the field originating in those molecules having a permanent dipole structure due to bond assymetry. These forces have been extensively investigated from the theoretical aspect by Friedenberg, Blatt and Galluchi (1966 a, b, c) and by Friedenberg (1967). From their investigations it appears that the fields produced at the membrane surface will begin to interact when the membranes are separated by distances of up to 2,000 A. The action of these fields will be to attract one membrane to the other. (See above.) At large separations the forces of attraction will be relatively weak, but increase in strength as the membranes approach one another. At close separations of the order of 100 to 200 A, the ionic double layers at the cell surfaces will begin to interact, and the viscous and electroviscous forces will slow the further approach of the membrane until an equilibrium position is reached where the attractive forces are balanced by the repulsive electrostatic forces due to the cell surface charge.

The/

The forces already considered, that is the viscous and electro-viscous forces, the electrostatic forces arising from the surface charge, and the field effects, determine the approach of particles, and the point at which equilibrium separation is reached. They do not initially account for the reaction on the part of one cell which follows the close approach of another. Examples of such reactions are the phenomena of contact inhibition, the membrane changes following fertilisation of the ovum, and the propagation of a nerve impulse across the synaptic gap. There are two important and related ways in which these effects can be considered without altering the basic assumptions relating the field effects to the approach and adherence of whole cells.

The lipid within the cell membrane is in a "liquid crystalline" state, on the point of transition between the liquid state and the crystalline state. (Byrne & Chapman 1964). The parameters of interfacial tension, surface potential and surface field strength, are all dependent upon the phase state of the lipid molecules within the membrane. Changes in the phase state of the membrane could therefore alter any of these parameters. As already discussed, the field strength, surface potential, and interfacial tension are all important in determining how one cell membrane reacts to another. Small changes in phase state, causing for example changes in the orientation of the polar groups of the phospholipid molecules, would modify the field produced by these molecules at the cell surface, and could greatly alter the rate of approach, or the position of equilibrium between two cell surfaces. The end result of an alteration/

alteration in the field at the surface of the cell could be
proportionally greater than the initial disturbance initiating the
change.   A system of this type, where small changes in one part
can cause large changes in the whole has been called a "co-operative
System" (Hill 1957).   Changeux, Thiery, Tung & Kittel (1967)
proposed that phase changes in a membrane might be brought about by
a change in the concentration of a substance at the cell surface.
These concentration changes might be reflected in, for example,
the adsorption or desorption of substances at the cell surface, or by
the combination of a substance with the membrane itself.   For this
hypothetical substance they introduced the term "ligand" to denote
any proton, ion, or specific chemical substance which by adsorption
desorption, or chemical combination with the membrane could cause a
phase transition in that membrane.   The types of commonly occurring
biological substances which could be called ligands, are the synaptic
transmitters acetyl-choline and nor-adrenaline, proteins, such as
insulin, and specific ions such as sodium and potassium.   These
molecules by combining with a membrane in one phase state could cause
either temporary or permanent phase transition in that membrane, and
by so doing could alter its properties.   Possible alterations could
be alterations in surface potential, induction of adsorption or
desorption of other substances in the vicinity of the membrane,
alterations in membrane permiability, and alterations in the field
structure of the membrane.   Contact between membrane and ligand
could result in either temporary or permanent alterations in membrane
structure and reactivity.   The membrane could also be regarded as a
transducer,/

transducer, or amplifier of a certain sensitivity to one particular substance or ligand. (Gingell 1968).

The second way in which the approach of a cell might produce changes in the membrane of an opposing cell, is by the induction of field changes between the two cells. If the field at the cell surface has a particular conformation and strength due to the orientation of the membrane dipoles, and the pattern of fixed surface charge, the approach of another particle or surface with a high field strength would result in changes both in the pattern of surface charge, and in dipole orientation. The mobile ions, and the molecular dipoles would polarise under the influence of the combined fields, and the direction of their dipole moment would orientate at right angles to the field lines. The field patterns between two such particles would vary in a complex manner as the membranes approached one another, and the direction of orientation of the dipoles in each membrane would follow the changes in field pattern. If the combined field strengths were great enough, the direction of the molecular dipoles could be sufficiently displaced to cause a phase change in the membrane. Alterations in field strength can also be caused by ligand binding, so in this sense the effects produced by ligand binding, or by alterations in field strength would be equivalent. (Hill 1957; 1967; Changeux et al 1967).

It can therefore be seen that the approach of a particle or a cell to another cell changes the reactivity of the membranes involved at a distance. The cell membrane is a "co-operative system", and therefore such changes are amplified by the membrane, and translated into/

into changes in metabolic activity (as in nerve synapses), changes in mobility (as in contact inhibition) or changes in surface tension (as in the fertilised egg). It can now be shown that the changes occurring in the macrophage in the process of phagocytosis can be explained with reference to membrane changes induced by particles or cells at a distance.

7 - THE CHARACTERISTICS OF THE PHAGOCYTIC PROCESS.

a. Selectivity.

The literature on the subject of phagocytosis reflects the
interest that this subject commands in biology. Major reviews of
the subject have been made by Mudd, McCutcheon, and Lucke (1934)
Suter (1956), and Berry and Spies (1949). The metabolic changes
occurring during phagocytosis have been reviewed by Karnovsky (1962).
Two main aspects of the phenomenon have received close attention.
The first has been concerned with the effects of alteration in the
bulk environment upon the phagocytic process. The studies in this
field have led to the investigations of such variables as pH, the
actions of ionic substances, and temperature upon the phagocytic
process. The second part has been concerned mainly with the
"selectivity" of the process, and studies in this field have con-
tributed much to the knowledge of opsonins, and the role of specific
antibodies in phagocytosis. Phagocytosis has been defined as the
engulfment of a particle by a cell accompanied by the expenditure
of energy. The mechanism of engulfment is the unique feature of the
phagocytic process. It has been shown that only a few specialised
types of cell can react to a particle or another cell by this
mechanism of engulfment. The other features of phagocytosis, its
"selectivity", is not unique as a cellular phenomenon. All the
common processes of cells are selective in nature. The term
"selectivity" is syntactically inaccurate, but hallowed by long and
repeated employment. The selectivity of phagocytosis is not an
active/

active process in the sense that term implies. Enzymes select their substrates by methods involving physiochemical interactions. The selectivity of the macrophage membrane for a particle is determined by exactly similar physicochemical processes, and is therefore not "purposeful" as an active selective process might be. The first really accurate descriptions of the phagocytic process were made by Rhumbler (1914). He described four mechanisms by which amoebae were seen to ingest food particles. These mechanisms can be listed as:

1. Sinking of the particle into the cell after contact with the membrane.

2. A flowing of the cell cytoplasm around the particle.

3. Phagocytosis by the production of pseudopodia, and the enclosing of a food cup containing the particle.

4. Invagination of the cell cytoplasm at the point of contact.

Mammalian macrophages ingest particles primarily by the first and second mechanisms. The ingestion of antibody coated particles occurs principally by the first mechanism. Marmont, Piuma, Negrini, and Maietta (1958) have described a further mechanism of phagocytosis of antibody coated particles in which the phagocytic act is preceded by the attachment of many particles to the surface of the macrophage, forming a rosette (en couronne). The subsequent uptake of these particles is always mutliple.

Bacteria have also been shown to be phagocytosed by the fourth method of cytoplasmic invagination at the point of contact (Hirst 1965). Fenn (1922) described a further type of phagocytosis in which the/

the particle is incompletely phagocytosed. This he called Partial phagocytosis. It appears to be a comparatively rare event.

The phagocytic mechanisms can be altered by the presence of substances called "Opsonins" in the medium. An opsonin can be defined as a substance of no particular chemical configuration or class which has the property of facilitating phagocytosis. This definition is too broad to be useful, and for the purposes of this thesis an opsonin will be defined as any substance occurring naturally, or as a result of specific immunisation, in body fluids, which facilitates phagocytosis by combining with the surface of the particle.

The phagocytic process is truly "selective". (Fenn 1922) showed that phagocytic cells would preferentially ingest manganese dioxide particles when presented with a mixture of manganese dioxide and quartz. They would also ingest carbon particles in preference to quartz (Fenn 1920).

Phagocytes in vitro will ingest aged and injured red cells in preference to fresh and healthy red cells. (Stuart & Cummings 1968). Foreign red cells are phagocytosed in preference to homologous red cells (Halpern et al 1957). Many workers have stressed the importance of opsonins in determining the selectivity of the phagocytic process. It has recently been shown that erythrocytes can selectively adhere to macrophages in the absence of serum proteins and hence of opsonins (Rabinovitch 1967 a; Habeshaw personal observations). The same author showed that aldehyde treated red cells coated with antibody, which has opsonic activity, were phagocytosed in the absence of serum (Rabinovitch 1967 b.). This implies that opsonins/

opsonins facilitate the act of phagocytosis itself, and play no part
in the "selectivity" of the process. The discriminatory ability of
the macrophage must still apply even if opsonins are required for
phagocytosis, the discrimination then being between a particle coated
with an opsonin, and one that is not. A study of erythrophagocytosis
in the absence of specific antisera showed the discriminatory, or
"selective" ability of mouse macrophages towards the red cells of
other species. The more distant the genetic relationship between
the erythrocyte donor, and the macrophage donor, the greater was the
percentage of erythrocytes ingested. (Perkins & Leonard 1963).

It can thus be established that phagocytosis is a truly selective
process, which in its sensitivity is equivalent to the other selective
processes of cells. The sensitivity of this process can be measured
in the sense that small genetic differences expressed in terms of
cellsurface structure can lead to phagocytosis (Perkins & Leonard
1963), minute amounts of antibody can be detected upon the surfaces
of cells (Mollison & Hughes Jones 1967), and degrees of membrane
injury undetectable by other simple means, will readily lead to
phagocytosis. (Stuart & Cummings 1967).

b.   The effects of the environment on phagocytosis.

The environment affects phagocytosis, and has been extensively
investigated. (See Berry et al, Mudd et al). Among the environ-
mental agents studied have been the hydrogen ion concentration, the
effects of cationic substances, and simple organic compounds.

The pH optimum for phagocytosis appears to lie between 6.5 and

8.5/

8.5. Some phagocytosis occurs outwith this range (Tucker et al 1963; Ouchi et al 1965). Particularly interesting were the observations of Evans (1922) who showed a decrease in phagocytosis with decreasing pH. Mineral acids were much less effective in decreasing phagocytosis, than organic acids at the same pH. This was thought to be due to the greater partition coefficients of the organic acids in the lipid membrane. Fenn (1923) showed maximal phagocytosis of quartz particles at a pH of 7.0, and carbon particles at a pH of 7.0 to 7.3.

The pH optimum for phagocytosis by leukocytes in suspension, as compared with a monolayer on glass, was on the acid side of neutrality at pH of 6.7. Fenn explained the effects of pH on phagocytosis by observing the depressant effect of pH changes, particularly on the acid side, upon the mobility of the leukocytes employed. (See also De Haan, 1921-22).

Temperature changes have also been shown to affect the rate of ingestion and the total quantity of particles ingested by macrophages. The rate of phagocytosis was optimal between temperatures of 20° and $27^{\circ}C$. for the ingestion of quartz particles by rat macrophages. (Fenn 1922). Harmon et al (1946), showed that phagocytosis of staphlococcus aureus by guinea pig polymorphs was optimal at a temperature of $43^{\circ}C$. decreasing thereafter.

Extensive studies of the role of ions and simple organic compounds in phagocytosis were made by Radsma (1918; 1919-1920). He showed that sodium iodide inhibited phagocytosis, but this effect was reversible by sodium bromide. He also studied the effects of the cationic metals upon phagocytosis, showing that their efficacy

in/

in promoting phagocytosis could be represented by a series Na - K - Rb - Cs - Li. Magnesium chloride was found to stimulate phagocytosis in some concentrations, inhibiting it in the higher concentration range. (Eggers 1909). The effects of most of the anionic compounds studied by Radsma is now thought to be due to their ability to inhibit the enzymic processes accompanying phagocytosis. Iodide certainly inhibits phagocytosis by its interference with cell metabolism. (Karnovsky 1962).

Similarly the effects of m any of the organic substances studied can now be explained with reference to their effects upon the cell membrane. Hamburger (1912) studied a series of compounds having the common property of high fat solubility. He found chloroform and idoform both increased phagocytosis, and had the property of restoring phagocytic ability to cells long exposed to sodium chloride solutions. The concentration in which these two substances affected phagocytosis was between 1 in 20,000, to 1 in 5 million.

Chloral hydrate, ethyl alcohol, N-butyric acid, and N-propionic acid all increased phagocytosis in low concentrations, and inhibited it in high concentrations. The results of Hamburger have been questioned in regard to the properties of chloral hydrate, and ethyl alcohol. (Arkin 1913; Di'arco 1922). Many other substances have been studied, including the sulphonamides, tannins, heavy metal salts, picric acid, formaldehyde and fatty acids. Most of these have been shown to be without significant effect. (See Berry & Spies 1949). Long chain fatty acids (C6 to C10) were found to increase phago-cytosis. (Fethke 1939). From these studies it appears that phagocytosis can be stimulated by trace amounts of lipid solvents (Chloroform/

(chloroform and idoform), monobasic aliphatic acids and certain ions.

Phagocytosis can be inhibited by organic acids, mineral acids, C2 to C6 aliphatic acids, and dibasic fatty acids. Lipid substances can be shown to stimulate phagocytosis. Munder & Fischer (1965) have shown that the addition of phospholipid to macrophages in an inactive state will stimulate phagocytosis. Cooper & West (1962) and Cooper (1964) have described the stimulatory effects of trigly-cerides upon the phagocytic process. Some lipid esters, notably cholesterol oleate, and ethyl palmitate will depress phagocytosis in vivo (Stuart 1962; Stuart, Biozzi, Stiffel, Halpern & Mouton 1960). The phagocytic reaction is relatively insensitive to changes in pH, and ionic concentration. Sodium ion, and some divalent cations seem to be essential to the process. (See De Haan 1921-22). (Metzger & Casarett 1967). The characteristics of the simple com-pounds inhibiting or stimulating phagocytosis suggest they act through a common mechanism. This mechanism may be the alteration of the structure or dynamics of the cell membrane. The insensitivity of the process to changes in pH and ionic concentration, may reflect the stability of the local environment at the macrophage surface.

c. The role of opsonic substances and macromolecules.

The term "bactero-opsonin" was coined by Almoth Wright to describe those factors in serum that facilitated the uptake of micro-organisms. The term was also used by Hektoen (1906) to describe the immune antibodies which facilitated the uptake of red cells. An opsonin is defined, for the purpose of this thesis, as any substance, occurring/

occurring naturally, or as a result of specific immunisation, in the body fluids, which will combine with a particle, and by so combining will promote phagocytosis of that particle. This definition excludes reactants such as the cytophilic antibodies, recently described by Jonas, Guerner, Nelson and Coombs (1965), and Berken & Benacerraf (1966), which will promote phagocytosis of a particle, but achieve this by combining with the macrophage surface prior to reaction with the particle. There are three principal ways in which opsonins are held to promote phagocytosis: firstly by chemotaxis or direction of the macrophage towards the particle, secondly by promoting the adherence of the particle to the macrophage, and thirdly by acting upon the membrane to favour a phagocytic reaction by altering interfacial tension or lowering the surface charge of the particle.

Chemotaxis by macrophages has been described by Jacoby (1937), Bessis et al (1965) Pulvertaft (1961) Harris (1953) and Keller & Sorkin (1968). Jacoby showed fowl monocytes to be attracted towards dead monocytes in culture. Pulvertaft showed chemotaxis of macrophages towards thyroid cells damaged by cytotoxic antibody and complement. Keller & Sorkin (1968) have reviewed the evidence for chemotaxis of leukocytes. Although it has been established that chemotaxis, or something like it, occurs withreference to the macrophage, it remains unproven that this reaction is mediated by any substance classifiable as an opsonin. The reaction between a particle and an opsonin may release from serum a chemotatic principle, or "cytotaxin" specific forthe macrophage. (Wilkinson et al 1969(.

Adherence between an opsonised particle and a macrophage can be studied/

studied in preparations in which the phagocytic part of the event
is inhibited by the presence of metabolic inhibitors. Thus if the
opsonin coats the particle thereby rendering it "sticky", particles
can be demonstrated to adhere to the macrophage surface in the
absence of any subsequent phagocytic reaction. Adherence of
particles to the surface of macrophages can be shown to occur with
the so-called "natural antibodies" (Brumfitt 1965), specific non-
cytophilic antibody (Uhr 1965), specific antibody and bound complement
(Nelson 1965; Hess et al 1968) and cytophilic antibodies (Berken &
Benecerraf 1966). This latter group has been excluded from the
definition of opsonins, since these antibodies bind first to the
surface of the macrophage and subsequently promote adherenèe of the
antigen to the macrophage surface. Adherence can occur between
certain particles and macrophages in the absence of serum protein.
Sheep red cells adhere to mouse macrophages in the absence of serum
(Nelson 1969), and aldehyde treated red cells will adhere to mouse
macrophages without the intervention of opsonins, but will not be
phagocytosed. (Rabinovitch 1967 a).

The uptake of colloidal particles in-vitro and in-vivo has been
shown to depénd upon serum factors which are not antibodies.
Depletion of these serum factors by repeated injection of one type
of particle, results in eventual failure of phagocytosis. This state
is called reticuloendothelial "blockade". Usually blockade is
specific for one type of particle, but occasionally competetive
blockade between two types of particle competing for one opsonin can
occur (Halpern 1967). Inorganic particles entering the blood stream
of/

of an animal become immediately coated with serum protein.

Knisely (1948) showed that carbon suspensions injected into the blood stream of the frog became immediately coated with fibrin, and were thereby opsonised. A similar coating of fibrin upon phago-cytosed bacteria has been described. The fibrin coating these particles is of itself rapidly phagocytosed in mammals, while fibrin-ogen is not. (Lee et al 1962). No opsonin was needed for the ingestion of polystyrene spheres (Sbarra et al 1959) or for denatured human serum albumin (Sbarra 1966). Bentonite particles appeared to be opsonised with gammaglobulin (Slopeck et al 1960) and this opsonisation is more effective in the presence of complement. (Potter and Stommerman 1961). Carbon and starch particles require the presence of serum for efficient phagocytosis, and uptake here is presumably opsonin dependent. (Nelson 1956). The Kupffer cells of the liver are more dependent upon opsonins for the efficient clearance of particles than the macrophages of the spleen (Biozzi et al 1955). Di Luzio (personal communication) has identified the opsonin for particles of barium sulphate as an alpha-globulin, in a system employing liver slices as a source of phagocytes.

The in vitro ability of the macrophage to recognise red cells coated with S. Typhi Vi, and O antigens is dependent upon opsonins characterised as gammaglobulins. Early post-immunisation serum, containing macroglobulin, promoted the adherence of bacteria to macrophages, while sera obtained in the later stages promoted phago-cytosis, in serum free media. (Levenson & Braude 1967). The opsonin for a variety of bacteria has been shown to be either macro-globulin/

globulin (Rowley et al 1964) or immune gamma globulin (IgG) (Lee et al 1968). It has been shown that free antibody will compete with bound antibody for receptor sites at the macrophage surface. (Huber et al 1969).

The role of opsonins in the uptake of effete homologous and heterologous red cells has been investigated by Perkins & Leonard (1963), Vaughan (1963; 1964; 1965 a, b). Lee and Cooper (1966) (Lee 1968) and Bennet 1963). Perkins showed that opsonins were unlikely to account for the behaviour of mouse macrophages towards a variety of foreign red cells by absorbing the serum carefully with the test particle before performing the experiments. Vaughan (1964; 1965 b) demonstrated that phagocytosis of foreign red cells by rabbit polymorphonuclear leukocytes and macrophages was dependent upon both heat stable and heat labile serum factors. The heat labile factor was C_I component of complement while the heat stable factor was a gamma globulin. Absorption of the sera with the test particle did not interfere with the subsequent uptake of other types of red cell. Vaughan and Boyden also showed that the uptake of homologous effete red cells by macrophages was dependent upon a cytophilic factor in serum, which was also present upon the macrophage surface. (Boyden & Vaughan 1964). Experiments by Stuart & Cummings (1969), confirmed the ability of the mouse macrophage to distinguish between the injured and healthy human red cell in a heterologous system. The opsonic factors were not characterised for this system.

The role of the opsonins in phagocytosis may be summarised as follows:

1. Opsonins are required for the phagocytosis of bacteria by poly-
morphonuclear/

morphonuclear leukocytes.

2. Opsonins for bacteria may be naturally occurring serum proteins, for example fibrin, naturally occurring antibody, or immune antibody of both classes. Macroglobulin antibody is a more efficient opsonin than IgG.

3. Inert particles may require no opsonin for uptake or require a coating of serum protein which may be fibrin, alpha globulin, or gamma globulin.

4. Opsonins are probably not required for the uptake of foreign or effete red cells by macrophages. The requirements of opsonins for other cell types has not been determined. (See Vaughan 1965 a, b).

Much of the evidence concerning the role of opsonins in phago-cytosis is confused and contradictory. Several sources of confusion can be identified in the literature. These arise from:

1. The type of phagocytic cell studied. It is clear that the polymorphonuclear leukocyte has an absolute requirement for opsonins, for the phagocytosis of any particle.

2. Results vary with the methods used to assess phagocytosis.

3. The requirement for opsonins varies with the in vitro methods for reacting the particles with the phagocytic cells. For example macrophages in suspension require opsonins for efficient phagocytosis. If allowed to settle and adhere to glass, for 18 to 24 hours, they are capable of phagocytosis of S.Typhi in the absence of serum (Suter et al 1964). This phagocytic capacity may persist for long periods, (Chang 1964) in the absence of serum (Fauve 1964).

4. The interpretation of the term "opsonin" is wide, and few workers have actually defined it. Cytophilic antibody is not here included

in/

in the definition of an opsonin, since it reacts primarily with the macrophage surface.

Despite the effort devoted to the study of opsonins there is remarkably little speculation as to how they might act. (Nungester 1952) suggested that opsonins might reduce the surface charge of the particle with which they combined, thus facilitating approach of that particle to the macrophage surface. He was unable however to obtain a consistent relationship between reduction of surface charge, and susceptibility to phagocytosis. Ponder (1927-1928) discussed the relationship between surface potential and phagocytosis, concluding that the surface charge was unimportant in promoting or facilitating phagocytosis of any but the smallest particles. He suggested that modification of the surface tension and cytoplasmic viscosity were effects more likely to facilitate phagocytosis. There is no direct evidence that opsonins lower the surface or interfacial tension between the particle and the macrophage, but surface active agents have been shown to stimulate phagocytosis in the appropriate con-centrations. (Berry & Spies 1949). It has recently been shown that anti-red cell antibodies will lower the surface charge of red blood cells, as studied by cell electrophoresis. (Sachtleben 1965). Both agglutinins and haemolysins (immune antibodies) were found to produce similar decreases in electrophoretic ability, while it has been shown that only immune antibodies will stimulate erythrophago-cytosis in vitro. (Stuart, Davidson & Cummings 1967).

d. The modes of action of these variables upon the phagocytic process.

It/

It has been shown how cell membranes could react with each other at a distance, and that the membrane orders the local extracellular environment. Both these properties are consequences of the structural and metabolic integrity of the membrane. In any phagocytic reaction the membrane of the macrophage may be considered the primary or principal reactant. It is essential to the study of phagocytosis to consider how membrane structure can mediate between changes in the external environment occasioned by the approach of a particle, and the biochemical changes which accompany phagocytosis.

Changeux, Thiery, Tung & Kittel have proposed that phase changes in a co-operative system result in modified membrane activity. They regard these changes as being produced by a specific substance or ligand which can interact with the membrane. (Hill 1967) proposes that the phase change in the membrane is secondary to a change in the field structure, although the resulting phase change from this will have equivalent effects to those produced by ligand binding. Ligand substances are conceived as molecules which by interacting with the membrane produce changes in the orientation of the lipid molecules composing that membrane. Their interaction will principally affect those characteristics of the membrane most dependent upon the structural arrangement of molecules within the membrane. The field strength, surface potential, and interfacial tension are the most likely parameters to be affected. Many possible ligands have been biologically identified. Willmer (1966) has shown how steroids of various configurations could interact with the lipid bilayers of cell membranes to produce different patterns of packing of the lipid molecules. Synthetic polypeptides such as alamethicin, and naturally occurring/

occurring polypeptides of uncertain structure, will interact with artificial and natural membranes to produce changes in electrical resistance, permiability and conductivity. (Mueller & Rudin 1968; Seufert 1965). Insulin will react with the cell membrane to increase its permeability to glucose.

A number of substances, termed inducers will stimulate pino-cytosis in a variety of tissue cells. Some have a specific inducing reaction for some cell types. (Rustad 1964). Phagocytosis is likewise stimulated by the presence of certain proteins. Tullis & Surgenor (1956) have isolated a preparation from serum which will stimulate the phagocytic activity of polymorphonuclear leukocytes in the absence of other serum proteins. This phagocytosis promoting substance is a mixture of alpha and beta globulins. Macroglobulin antibody will stimulate phagocytosis of the antigen to which it is formed, in this respect it is more reactive than immune IgG. Both the phagocytosis promoting substances and antibody could be called "ligands" in the same sense as that implied by Changeux, Thiery, Tung & Kittel. Cytophilic antibodies will also promote phagocytosis of antigens to which they are formed. This indeed is the only physiological role they seem to have. Cytophilic antibody is a ligand substance. It will combine with specific lipoprotein recep-tor sites within the cell membrane, and by so combining will alter membrane properties, causing firstly adherence of the particle to the membrane, and secondly initiating the phagocytic raction. (Davey & Asherson 1967).

Phase changes in lipid systems can be detected optically when the lipid system shows birefringency in the crystal state, or birefringency/

birefringency when flowing under increased pressure. (Chapman 1966). These changes have been described in naturally occuring phospholipids as a result of heating (Chapman 1966). Since any phase changes occurring in the cell membrane are likely to decrease the orderliness of lipid stacking in the bilayer, they should be visible as a change in the birefringency of the membrane studied under polarised light. Since changes in refractive index, and optical density are also likely to occur, membrane phase changes should be visible as optical phase changes by interference or phase contrast microscopy. (See Tanford 1965). Phase contrast observations of living cells show the occurrence of phase changes in the membranes of cells in motion, and during the processes of phagocytosis, and cellular interaction. These changes are most pronounced at the tip of a moving pseudopod, and at the points of contact between cells. They can also occur in two opposing membranes separated by a gap clearly visible by light microscopy, and thus greater than 0.2 microns, and probably nearer 1 micron, in width. (Dewar personal communication).

For a ligand to combine with its receptor site upon or within a cell membrane, it is necessary for the membrane to "select" and order the approach of that molecule from a distance, in the face of competition from other molecules of similar size and molecular weight. This "selection" could occur by chance if an abundance of ligand molecules were in the vicinity of the membrane. Studies on cytophilic antibody have shown that the adherence of these molecules to the macrophage surface can occur from very low serum concentrations. Occasionally, cytophilic antibody cannot be detected in the serum by such reactions as the Passive Cutaneous Anaphylaxis, and yet can be shown/

shown to be present by an in vitro reaction with macrophages.
Macrophages can also detect minute amounts of immune antibody
(Mollison & Hughes Jones 1967) and react to cells coated with them
(Lo-Buglio et al 1967). It is probable that the approach and binding
of a ligand to the macrophage surface is determined by the strength
and conformation of the field at the surface. The nature of the
field could account not only for the binding of the ligand, but also
for the appropriate phase change for that ligand, and the induction
of the appropriate cellular response.

The presence of a field at the surfaces of living cells can be
inferred from the anomalous behaviour of living cells in electro-
magnetic fields, when compared with inert particles. When inert
particles, such as grains of potato starch, are placed in an electric
field, they will show two types of behaviour; alignment in the
direction of the field, and condensation to form first chains of
particles, and then floccular precipitates. This behaviour can be
predicted from the theoretical examination of the flocculation of
colloids. (See Koelmans & Overbeek 1954). Living organsims or
living cells on the other hand show entirely different types of
behaviour, which colloidal stability theory does not predict.
Living cells will align parallel or at right angles to an applied
field dependent upon the frequency at which the field is generated.
Inert particles always align parallel with the direction of the field,
and never at right angles to it. Motile organisms are constrained
to travel always at right angles to the field, or parallel to its
direction, whatever their method of locomotion. With viable cells
chains of particles are formed less frequently than with inert
particles,/

particles, and often only under conditions of very low ionic strength.
Living cells show also two types of behaviour not observed with inert
particles, firstly rapid spinning or rotation of the cell which can
be prevented by the addition of electrolyte to the medium, and
secondly red cells will deform to accomodate their shape to the field
direction, regaining their normal appearance after deformation.
Rotation is not involved. (See Furedi & Valentine 1962; Furedi &
Odah 1964; Teixeria Pinto et al 1960).

These effects described above were all produced by a strong
alternating electromagnetic field acting over a distance of 2-5 mm.
An analogous situation could exist in regions close to the cell
membrane, although the field it generates is likely to be much less
strong. Protein molecules, ion and water molecules can be imagined
as behaving like inert particles in an applied field. In such a
case, the appreoch and selection of particles by the macrophage
membrane would be a function of the field produced by the membrane.
The conformation and strength of the field at the surface is
dependent upon both the surface charge, and the orientation of dipoles
within the membrane. Both these parameters are likewise dependent
upon the arrangement of packing of lipid and protein molecules within
the membrane. (Parsegian 1967). The selection of particles by the
macrophage might then be said to depend upon the molecular organisat-
ion of the membrane. Certain specific substances, "ligands", or
the close approach of another field producing surface may alter the
molecular anatomy of the membrane. This change may be reflected in
a change in the birefringency or of the optical phase state of the
membrane. The measurable results of these changes are the metabolic
accompaniments/

accompaniments of the phagocytic act. The binding of a ligand to
the macrophage membrane, or the approach of another membrane with
differing field characteristics can activate enzyme systems which
will:

a. Hydrolyse A.T.P. (North 1968).

b. Stimulate the synthesis of the acidic phospholipids phosphatidyl
inositol, phosphatidic acid, and phosphatidyl ethanolamine, and their
incorporation into the membrane. (Karnovsky 1964; Sastry & Hokin
1966).

c. Stimulate aerobic and anaerobic glycolysis, depending upon the
supply of oxygen.

d. Increase the rates of cellular synthesis of both glycogen and
protein. (Karnovsky 1964).

The observed reactions of a phagocytic cell towards a particle
suggest that changes in interfacial energy between particle and
macrophage occur. By analogy with physical systems, it appears as
though there were a decrease in the surface tension of the macrophage
membrane during phagocytosis. (Rhumbler 1914). Ponder (1927-1928)
interpreted this effect as resulting from a change not primarily in
surface tension, but in cytoplasmic viscosity. Pontin (1923-25),
in his classical studies upon locomotion in marine amoebae, also
concluded in contrast to Rhumbler (1898; 1910) that changes in cyto-
plasmic viscosity were more important than changes in surface tension
in pseudopod production. The surface tension in the macrophage
membrane is almost certainly of a very low order. This is apparent
on studying the numerous fine cytoplasmic processes produced by these
cells. It is theoretically impossible to maintain such processes
unless/

unless the surface tension at the cytoplasmic/fluid interface tends towards zero. In addition, from theoretical considerations it has been predicted that mammalian membranes by reason of their structure should exhibit very low interfacial tension. Therefore the apparent change in surface tension suggested by observation of the phagocytic process cannot be truly held to occur.

The process of phagocytosis can be explained without reference to any change in the tension at the interface between the particle and the macrophage membranes. A change in the viscosity of the cell cytoplasm, from the gel state to the sol state would result in the production of a pseudopod by the phagocytic cell. The field at the membrane surface would determine the shape and direction of movement of such a pseudopodium. A particle attached to the membrane in the vicinity of the local disturbance of cytoplasmic viscosity may be drawn into the cell, or engulfed by a pseudopod moving outwards. The surface tension in the membrane need not alter, providing either membrane flow, or synthesis of new membrane can occur. The change in cytoplasmic viscosity could occur in one of two ways:

a. firstly as a result of a local increase in membrane permeability following changes in the membrane.

b. as a result of a change in the nature of water in the vicinity of the cell membrane.

A decrease in cytoplasmic viscosity could be produced locally by increased the permeability of the cell membrane to water, and ions. These will then be osmotically imbibed by the cytoplasm, and a change from the gel state to a sol state will occur. Increases in membrane permeability to water and ions occur in several well known

physiological/

physiological situations. The best known in the temporary increase in the permeability of the axon membrane to sodium during the passage of a nerve impulse. The metabolic changes occurring in the phago- cytic cell during phagocytosis, especially those concerned with the phosphatidic acid cycle, are similar to those changes described for the secretion of sodium by the goose salt gland, (Hokin 1964) and for the transport of sodium by the membrane of the erythrocyte. (Hokin & Hokin 1961; Post et al 1960; Hokin & Hokin 1963; Hokin et al 1963). It could be concluded that the occurrence of the phosphatidic acid cycle in the phagocytic cell during the process of phagocytosis, combined with the activation of membrane bound A.T.P.'ase, reflects an increase in the permeability of the macro- phage membrane to sodium ion and to water. The other metabolic changes described are the natural consequence of using up membrane material to form a vacuole (Phospholipid synthesis) and the increased rate of sodium ion excretion which follows an influx of this ion into the cell (catabolism of A.T.P., increase in respiration). The resynthesis of protein and glycogen will follow the increase in catabolism occasioned by the reactions described above.

A change in the nature of water at the cell membrane surface could be caused directly by an alteration in the nature of the field at the surface. If stacked "clathrate" water layers were present above and beneath a reactive membrane, an alteration in the field produced by the membrane could cause either an alteration in the distribution of the water molecules, or the loss of the cohesion between individual molecules which would then behave as bulk water. The net results of these two effects would be a decrease in the high viscosity/

viscosity of the layers immediately above and below the membrane, and an increase in the ratio of free water molecules to ordered water molecules in the cytoplasm. This would have the effect of firstly lowering the apparent "surface tension" in the macrophage membrane, and secondly of causing a gel to sol transformation in the cytoplasm.

The presence of ordered molecular forms of water in the vicinity of the cell membrane has other important effects. A major one is the retardation of the effects of field change occurring during the approach and reaction of two membranes. Any change in the field structure at the surface is likely to occur with great rapidity, yet the reactions of the phagocytic cell with a particle occur sufficiently slowly to be observed directly (Gropp 1963). If a layer of water molecules at a surface is maintained by the presence of a field generated by that surface, a loss of the field will produce a change in the state of water in this layer. Loss of the field can occur, for all practical purposes, instantaneously. The rate of reversion of the organised structures of water into their bulk form will occur at a rate determined primarily by the environmental free energy. It will therefore take a significant time for the effects of a field change to alter the immediate environment of the cell membrane, and for a change in the cytoplasmic viscosity to occur.

In summary, it can be shown theoretically from a consideration of the physical state and the structure of the cell membrane, that the phagocytic act and its biochemical consequences can be explained with reference to a single variable, the molecular architecture of the cell membrane. All the observed features of phagocytosis are the natural/

natural and logical consequences of a primary change in the state or structure of the cell membrane. It is suggested that all the changes in cellular reactivity in this process can ultimately be related to an increase in membrane permeability, and to a change in the state of water in the immediate environment of the cell membrane.

8 - EXPERIMENTAL DESIGN.

From the theoretical considerations of the phagocytic process, it is clear that the study of this event in practice involves the study of three main variables.   These are:

1.   The nature of the particle and its surface.

2.   The composition of the bulk environment.

3.   The nature of the phagocytic cell and its state.

In the case of an approaching red cell, the pattern of charge upon its surface and the field arising therefrom will determine the rate, and closeness of its approach to the macrophage, the binding of opsonins to its surface and the degree of which it will change the macrophage membrane.   The total effects of these variables will determine whether that red cell will or will not be phagocytosed.

The bulk environment of the cell is important metabolically, chemically, and electrically.   The appropriate metabolites for the macrophage to survive must be present or phagocytosis will not occur.   Opsonic substances may be required by the macrophage, and the ionic composition of the medium must be osmotically adequate. The composition of the bulk environment will also determine the local field strength therefore the range at which cellular interactions can occur.

The macrophage varies both with its source and its state (in vivo or in vitro).   Since it is a dynamic and variable cell both in size and shape it cannot be as easily or as accurately characterised as the red cell.   Thus the practical study of the macrophage must be limited to the determination of its phagocytic abilities in the presence/

presence and absence of opsonic substances, and the study of its response to a variety of red cells from unrelated species.

The red blood cell is probably the ideal test particle. Red cells are easily obtained, and are constant in size, shape and composition from any single healthy donor. An additional advantage of this particle lies in the degree of understanding of its biochemistry, reactivity, structure and behaviour, under a wide variety of experimental conditions. Nearly all studies of phagocytosis to date have used serum-containing media. The presence of serum in a mixture interferes with the study of those factors which serum itself provides for the phagocytic reaction, (for example opsonins and complement). It should also be understood that mammalian sera are not interchangeable in experiments involving phagocytosis. The composition of serum varies widely from one species to another. One requirement for the study of phagocytosis is a chemically defined medium which will support the metabolic activity of the macrophages in the absence of any macromolecular species which could influence the reaction. The state and type of phagocytic cell used is of great importance. In the study of erythrophagocytosis, macrophages should be employed as the phagocytic cells. Polymorphonuclear leukocytes will rarely ingest red cells even under the most favourable conditions. Macrophages are by far the most active mammalian phagocytic cells (Nelson 1969). They can be easily grown from peritoneum or from lung and from a wide variety of animal species including man (Stuart 1967). The reactivity of macrophages from even a single source is dependent upon the site from which they are obtained. The pulmonary phagocytes are biochemically more active than those of the peritoneum, containing greater amounts of catabolic enzymes/

enzymes such as acid phosphatase (Dannenberg et al 1963). The reactivity of the macrophage is dependent upon the conditions under which it is maintained in vitro. Macrophages grown an monolayer upon glass are phagocytically much more reactive than those in suspension. It has been argued that macrophages in suspension are more akin to the physiological ideal than macrophages grown on glass.

This can be shown not to be the case, since the bulk of the phagocytically active macrophages in vivo are the fixed macrophages of the liver and spleen. Free macrophages in vivo, e.g. those in the circulation, or those in large effusions, show poor phagocytic ability unless they are present in tissue or tissue spaces where some kind of ground substance, such as fibrin deposited during an inflammatory process, will provide a foundation for active mobility and for spreading. It is therefore considered more physiological to study the activities of macrophages grown as monolayer upon glass. This has the additional advantage that macrophages so cultured can be maintained for days or even weeks, while those kept in suspension rapidly die.

CHAPTER 2

THE PARTICLE: THE RED CELL

PART 1 - <u>INTRODUCTION</u>.

CONTENTS.

THE PARTICLE: THE RED CELL.

      a.   Form and Structure of the red cell.

      b.   The surface and surface morphology of the red cell.

      c.   Serum proteins and red cell antigens.

      d.   The concepts of ageing and injury as applied to red cells.

THE PARTICLE: THE RED CELL.

a. Form and Structure of the red cell.

The human red cell is a biconcave disc with a mean diameter in plasma of 8.4 microns, and a mean thickness of 2.4 microns. It is enclosed by a membrane 163 square microns in area. The volume of the interior of the cell is 87 cubic microns. (Albritton 1952).

Most mammalian red cells have a similar biconcave shape. The red cells of other mammals may not be circular in cross section. Those of the camel are ellipsoid. The peculiar shape of the red cell is considered to be a property of its membrane. Rice (1914) showed that the biconcave shape, which he also observed in micellar suspensions of lecithin, has less surface energy than any surface obtained from it by small deformations consistent with constant volume. This conclusion has received support from the demonstration of the very low surface energies to be found in biological membranes. Arey (1917) in a disucssion of red cell shape, concluded that changes from the normal biconcave disc form to cup shapes or spheres, were always associated with an increase in cellular volume. The maintainance of the biconcave shape is dependent upon the metabolism of the erythrocyte (See Cartier & Leroux 1952). Sphering of red cells readily occurs in the presence of metabolic poisons. Red cells from patients with well characterised enzymes deficiencies, such as glucose 6 phosphate dehydrogenase deficiency, will readily sphere in the presence of otherwise innocuous substances. These observations serve to confirm the relationship between an intact metabolism and cell shape.

In/

In view of Rice's conclusions it seems probable that the
relationship between red cell shape and metabolism, is determined by
the cell volume. The bulk of the metabolic activity of the red
cell occurs within the membrane, and is concerned chiefly with the
maintainance of the osmotic balance between the red cell interior
and the surrounding medium. This is achieved through the secretion
of sodium ion into the extracellular fluid, the so-called "Na/K pump".
This activity is dependent upon the membrane enzymes adenosine tri-
phosphatase, and acetyl cholinesterase. (Judah et al 1962; Schata-
mann 1962; Ahmed & Judah 1964; Heinz & Hoffman 1965). The enzyme
adenosine triphosphatase can be inactivated in the erythrocytes by
the cardiac glycosides digoxin and strophanthine. (Dunhan & Glynn
1961). After such inactivation rather slow swelling of the erythro-
cyte can be observed in isotonic media.

Another viewpoint is that the red cell shape depends upon some
structural arrangement of internal fibres, or contractile protein
(Onishi 1962). Two aspects of the behaviour of erythrocytes make
these conclusions unlikely. Firstly lysed red cell will regain
their biconcave shape when returned to isotonic media after lysis,
and secondly, red cells are highly deformable yet not elastic.
They will pass through pores 2.4 microns in diameter, (Gregersen
1967) without loss of their biconcave shape. The shape is also
retained after deformation in an electric field (Furedi & Gdah 1964)
and after assorted forms of mechanical deformation. (Mudd & Mudd
1931; Ponder 1936). If structural organisation within the membrane
was primarily responsible for the maintainance of shape, the red cell
would be more likely to exhibit "Elastic" rather than "Plastic"
behaviour/

behaviour in response to an applied force. (Gregersen 1967; Nicolau et al 1964). Abnormal red cells, or red cells that have been damaged chemically (as by fixation), loose their plasticity and behave as rigid particles. (Jandl 1958; Gregersen 1967; Nicolau et al 1964). The plasticity of the red cell membrane also accounts for the low internal viscosity of the red cell, which is of importance in lowering the bulk viscosity of the red cell, and in lowering the bulk viscosity of the blood. (Ditenfass 1964; 1968). The important characteristics of the morphology of the red cell are its shape, the constancy of its internal volume, a low transmembrane pressure diff- erential and hence a low internal viscosity, and a low interfacial tension in the membrane. These characteristics describe a cell which is highly deformable, passing through pores 2.4 microns in width, yet of little elasticity, being readily ruptured by stretching between needles or by osmotic lysis. These characteristics account for the low viscosity of the blood, and the resistance of the red cell to the traumatic conditions incurred in rapid circulation.

The membrane of the red cell is important in maintaining the deformability and low viscosity of the healthy cell. The membrane of the red cell is a bimolecular unit membrane composed of lipid and protein, exactly similar in electron microscopic appearance to the plasma membranes of other cells. Chemical analysis shows it to contain rather more lipid, especially neutral lipid and cholesterol, than other cell membranes. The chemical structure of the red cell membrane in respect of its lipid content has been extensively studied (Erickson et al 1937; Dawson et al 1960; De Gier et al 1961; Dodge et al 1963; Ways & Hanahan 1964; De Gier et al 1964). The red cell ghost/

ghost contains 45% by weight of lipid, 50% by weight of protein,
and small amounts of carbohydrate, and sialic acid. (Doge et al
1963).

The principal phospholipid constituents of the red cell membrane,
which amount to one half of the total lipid content, are phosphatidic
acid (1%), Phosphatidyl ethanolamine (27%), phosphatidyl serine (15%),
phosphatidyl inositol monophosphate (3%), sphingomyelin (27%) and
lecithin (27%). Free cholesterol amounts to approximately 29% of
the total red cell lipid, free fatty acids and their glycerol esters
(Neutral lipids) to 19%. Small amounts of cholesterol esters do
occur (1.5%). (Ways and Hanahan 1964; Dodge et al 1963; De Gier
et al 1964).

The phospholipids in the red cell membrane probably occur in
complex forms with sugars, proteins and carbohydrates. There are
probably no "free" phospholipids in the red cell membrane. Chemical
analysis does not reveal in what states phospholipids exist in the
intact membrane, although the isolation of cerebrosides (a complex
of sphingosine with fatty acids and a sugar residue), ganglisoides
(a complex of sphingosine, fatty acid, sugar, and N acetyl neuraminic
acid) and globosides (ganglioside lacking sialic acid) suggest that
most, if not all the lipid in the red cell membrane, exists in a
complex form. (See Dawson 1966).

The proportions of the individual phsopholipids in the membranes
of red cells from sources other than human, differs considerably.
The red cells of ruminants contain very little lecithin, but much
more sphingomyelin than the red cells of other species. (Dawson
et al 1960). The fatty acid content of human and animal erythrocytes
is/

is variable, and can be altered by changing the dietary intake of fatty acid. (De Gier et al 1961). This evidence is suggestive of an exchange between the plasma fatty acids and those of the erythrocyte membrane. (Oliviera & Vaughan 1964). (Lovelock et al 1960). The red cell does not synthesise phospholipid de novo, although it does possess the capacity to de-acylate, and re-acylate phospholipid moeities, that is to remove the fatty acid side chains from phospholipids, and re-esterify these fatty acids to other phospholipids. Phospholipid from the lipoproteins of plasma is rapidly exchanged with phospholipid in or on the red cell membrane. (Sakagmi et al 1965 a, b, Lovelock et al 1960; Polonovski and Paysant 1963). It has been assumed that this exchange is between plasma lipoproteins and a loosely attached layer of phospholipid or lipoprotein material on the cell surface, rather than material intimately concerned with the function of the cell. (Dawson 1966). This view may not be correct, particularly as it has been shown that cholesterol, which has a very short half life, and is deeply located within the membrane, is rapidly transferred between serum and the red cell membrane (Murphy 1962 a, b). On the other hand, the red cells of ruminants contain very little lecithin, while this appears as the major phospholipid in the plasma of these animals. Lecithin, like the other phospholipids of the cell membrane, forms stable laminates in solution in the presence of salt. Once phospholipid becomes organised in such a form, no exchange of phospholipid between the solution and the leaflet occurs. (Dawson 1964; 1966). The lipid composition of the red cell membrane can be shown to be of central importance of the integrity of the red cell. The lipid from the red cells of patients with/

with paroxysmal nocturnal haemoglobinuria has been shown to be more susceptible to peroxidation than the lipid from the red cells of healthy individuals. (Meriwether & Mengel 1967). The lipids from the membranes of cells deficient in the enzyme glucose 6 phosphate dehydrogenase, are similar to the lipids derived from normal red cells. (Szeinberg et al 1965). Changes in the lipid content of the red cell membrane occur after storage, and during the ageing process in red cells. (Rimon et al 1965).

The structural proteins of human and bovine red cells have been investigated by Moskowitz & Calvin (1952), Maddy (1964 a, b,; 1966). Maddy and Malcolm (1965), Poulik & lauf (1969, 1965) and Mitchel and Hanahan (1966). The antigenic differences in the structural proteins of adult and infant human red cells had been investigated by Vulpis (1962). The gentle extraction of ox red cells with butanol after removal of salt, (Maddy, 1965 a, b) or human R.B.C. with hypertonic saline solutions. (Mitchell & Hanahan (1966), produces, high yields of a mixture of proteins. Paper chromatography reveals three, or possibly four components. The major protein obtained by Maddy (1964)b, has a molecular weight of 160,000. Most of the protein isolated from the ox red cell ghost contains sialic acid. (Maddy 1964 b). All the sialic acid of the red cell membrane can be removed by treating intact red cells with the enzyme neuraminidase, which does not penetrate the cell membrane. (Eylar et al 1962). This suggests that there is a continuous layer of protein on, or within the lipid bilayer of the erythrocyte membrane. This conclusion supports, in part, the theoretical consideration by Wallach & Zahler (1966) that membrane proteins are hydrophobic in the central portion/

portion of the molecule, and penetrate the lipid bilayer, with their polar portions exposed upon the cell surface. (See also Leonard & Singer 1966). In this case the sialic acid at the membrane surface would be attached to protein rather than lipid, a suggestion that is supported by the ease with which trypsin will remove sialic acid from cells (Seaman et al 1963).

The earlier studies on the red cell proteins (Jorpes 1932; Moskowitz & Calvin 1952) were performed upon the residue left after extracting the lipid from red cell ghosts. After extracting the ghost with alcohol and ether, the resulting residue was called "stromatin" (Jorpes 1932). Red cell ghosts washed at a pH of 9, and then extracted with dry ether, leave a residue called "Elenin". This protein preparation contains rhesus antigen. Removal of the lipid from elenin with alcohol leaves a further residue "stromin" (Moscowitz & Calvin 1952). These proteins are insoluble in aqueous media and are almost certainly denatured. The native red cell protein appears to be a lipoprotein, only partially soluble in the presence of salt, and containing sialic acid. The molecular form, and exact location of this protein in the cell membrane is not known.

It has however been shown that the ghost protein obtained by Maddy (1964) is in the $\propto$ helical, or globular form, in dried red cells, and does not occur in the extended sheet-like β configuration (Maddy & Malcolm 1965). This also applies to the protein, adsorbed or native, upon the surfaces of red cells. Mitchell & Hanahan, by salt fractionation of the erythrocyte ghost, obtained two proteins. One was a low density lipoprotein containing nearly all the membrane phospholipid, and showing acetyl cholinesterase activity. This enzyme/

enzyme was shown not to be removed from the red cell by simple washing, and was therefore considered to represent a true membrane protein, and not one adsorbed or bound to the surface. (Mitchell & Hanahan 1966).

b. The surface and surface morphology of the red cell.

The quantitative analysis of phospholipid extracted from red cells shows that enough lipid is present to form a bilayer. The amounts of cholesterol present are just insufficient to form a mono-layer around the cell (Gorter & Grendel 1925; Ponder 1949). From such analysis it was concluded that the membrane of the red cell was composed of a lipid bilayer coated on both polar surfaces with protein. Electrophoretic studies reveal that the red blood cell behaves as a macropolyanion, that is as a large polymer bearing an anionic surface charge. (Seaman & Heard 1960 b., Cook et al 1961). This anionic surface charge is due to the carboxyl groups of sialic acid present-ing at the cell surface. Removal of sialic acid by trypsinisation, or with neuraminidase will reduce the electrophoretic mobility of the cell considerably. (Cook et al 1961). The electrophoretic mo-bility of red cells can also be reduced by lowering the ionic strength of the suspending medium (Seaman & Heard 1960 a.) and by the incor-poration of additional divalent cations (such as copper) in the medium. (Bangham et al 1958). The effect of both these procedures is to increase the thickness of the ionic double layer, and to reduce the surface charge. Divalent cations will produce these effects by "diluting" the monovalent ions in the double layer, and by combining with the charge determining groups at the membrane surface. Uranyl ions/

ions produce similar charge depression, but may achieve this effect by combining with and removing from the surface lipoid material complexing with uranyl and other heavy metal ions; for example mercury (Weed et al 1962). Since these ions show an affinity for phospholipid, their site of action may well be within the membrane rather than upon its surface. The role of metallic cations in the agglutination of red cell has been studied by Jandl & Simmons (1957). Many multivalent cations, including those of chromium, iron, beryllium, thorium, and aluminium, will agglutinate red cells. Agglutinates of red cells formed in the presence of these metals are morphologically indistinguishable from those formed by the interaction of antibodies with red cells. The presence of metal binding agents, such as the polycarboxylic acids, and proteins, inhibited the effects of agglutination by heavy metal ions. This suggests that the red cell agglutinating ability of metallic cations depends upon the reactivity of the free carboxyl groups at the red cell surface, and is therefore dependent upon both pH, and ionic strength. Since the surface carboxyl groups are the main contribution to the cell surface charge (Seaman et al 1963) the agglutination of red cells may depend upon their capacity to lower the surface charge. Agglutination of this type occurs because of a lowering of the surface potential below a certain value. This allows particles to approach closely, and to flocculate, or precipitate, since the surface potential supplies the electrostatic repulsive forces which normally distract particles. The point at which particles will begin to flocculate is called the "critical point", and the potential at the particle surface is the "critical potential".

The/

The negative surface charge is of importance in preventing the agglutination of red cells. Cells will however agglutinate under conditions in which the surface charges of the cells are not reduced or altered. Red cells suspended in hydrophilic polymers, such as dextran, ficoll, or polyvinylpyrollidine will agglutinate without loss of surface potential. Kwiatkowska & Morawiecki (1965) showed that polyvinyl alcohol, polyvinylpyrollidine, and dextran interacted with the red cell surface through hydrophobid bonding. They suggested that this interaction took place between the neutral polymer and a "lipid like" substance derived from the cell surface. Neutral polymers and antibodies do not greatly decrease the surface potential. They seem to exert their agglutinating ability by "diluting" the ionic double layer, that is by the exclusion of ions from the vicinity of the cell surface by occupying the space normally occupied by the ions of the double layer. Agglutination of particles by double layer dilution has been theoretically treated by Derjaguin (1954). Antibodies will reduce the electrophoretic mobilities of red cells, and this may in part account for their agglutinating ability. (Sachleben 1965). Antibodies may also agglutinate by providing chemical linkages from one red cell to another. The true outer surface of the red cell is the adsorbed serum protein in which it is suspended, and the sialic acid residues which contribute the bulk of the cell surface charge. Whether this layer can be morphologically demonstrated by present techniques is doubtful, but it is certainly of functional singificance. The exchange of cell membrane material, lipid and phospholipd, occurs through this layer, and it is this layer that contributes to the cell surface charge, and determines the composition of the ionic double layer. Below this outer/

outer layer lies the membrane proper. The surface of the erythrocyte would be morphologically difficult to demonstrate for two reasons; firstly it would be a labile structure due to its chemical nature and situation, and secondly it would not be identifiable as a definite layer in the accepted sense. It is not possible to define accurately a membrane or layer of unimolecular dimensions, unless it has a repeated periodic structure with the ability to scatter either x-rays or electrons. The "unimolecular layer" of the surface is too thin to do either, and would have no recognisable repeat structure because of its lability. The moprhology of the red cell surface has been studied by conventional electron microscopy (Glaeser et al 1966; Hillier and Hoffman 1953), and more recently by scanning electron microscopy. (Salsbury & Clark 1967; McDonald & Hayes 1969). (Hillier and Hoffman (1953) studied shadowed ghosts by conventional techniques. Glaeser studied the surfaces of red cell ghosts which had first been coated with a cast of carbon, which was then shadowed with palladium after digesting away the residue to the ghost. The surface of the red cell revealed by these techniques is composed of an outer layer of plaques, with approximate maximum diameters of 200 A., situated on a fibrous network. It has been suggested that the plaques are composed of elenin, cemented together by lipid which is removed during the preparation of the specimen. (Glaeser et al 1966; Hillier & Hoffman 1953). The scanning electron microscope reveals a smooth surface on normal red cells. A slight "graininess" is suggested at maximal resolution (200 A) (Salsbury & Clark 1967). Abnormal red cells show pits, and defects in the surface, and some of these are similar to pits produced in the red cell membrane by

haemolytic/

haemolytic antibody. Cells exposed to saponin for a short time reveal an irregular surface, which no longer appears smooth. (Salsbury & Clark 1967). Red cells from blood clots, show occasional surface defects, sometimes of large size, revealing an inner sheet of membrane material. (McDonald & Hayes 1969). It is not certain whether the surface revealed by these techniques corresponds to the chemical and electrophoretic outer layer, or to the outer leaflet of the lipid membrane.

c. Serum proteins and red cell antigens.

The serum proteins comprise the bulk of the physiological mileau of the red cell, apart from water. The total protein concentration in plasma is between 5.7 and 8 g. per 100 ml. of serum. Traces of the various plasma proteins can be demonstrated upon the surfaces of red cells after washing. Gramblich & Muller (1963) have shown that some serum proteins are adsorbed to the red cell surface in different proportions than their quantitative ratios in plasma. In particular, gammaglobulin, lipoprotein, and pre-albumin, were 2.42, 5.83, and 9.13 times as concentrated at the red cell surface than they were in plasma. This adsorbed protein layer constitutes in part the physiological surface of the red blood cell.

The blood group substances are also parts of the red cell surface, which by their reactivity, can be readily identified. The more common antigens are displaced from the red cell by mild physical and chemical manipulations. The ease with which this may be achieved is an indication of their superficial location. The Land0 steiner/

steiner antigens are composed of polysaccharide and aminoacids.
All these antigens are composed of the sugars D-galactose, L-fucose,
D-glucosamine, and D-galactoseamine, together with the amino acids
arginnine, lysine, serine, threonine, proline, glycine, alanine,
valine, leucine, aspartic and glutamic acid. Complexed with these
components is a small and variable proportion of sialic acid.
The specificity of the Landsteiner antigens resides in the poly-
saccharide component. Loss of fucose does not alter their reactivity.
(Trankerd 1961).

The Lewis and H antigens lose their antigenicity when fucose
is removed, and the reactivity of these antigens is not determined
by the polysaccharide portion of the molecule. The antigenicity of
the M & N blood group substances has been shown to depend upon the
presence of sialic acid (Hotta 1964; Romanowska 1961). The
physiological role of blood group substances is not known.

d. <u>The concept of ageing and injury as applied to red
cells.</u>

The red cell is a living cell, with a life span from 110-120 days
in the circulation. At the end of this time the red cell is
sequestered in the spleen and destroyed by phagocytosis.

Assessing the age of red cells, and understanding the processes
of ageing are of importance in improving blood storage facilities
for transfusion. There is indisputable evidence that red cells age,
and it is evident that the processes concerned with ageing ultimately
determine cell destruction. Simple physical studies reveal an
increase in red cell specific gravity with age (Piomelli 1967) and a
decrease in resistance to haemolysis in mechanical and osmotic
fragility/

fragility tests. (Stewart et al 1950). Biochemical changes especially in enzyme content and metabolism, electrolyte content, and proteins have been shown to occur with increasing age (See Prankher 1961). The composition of the membrane lipid has also been shown to alter with increasing red cell age. (Rimon et al (1965). One of the principal changes described in enzymes is the decrease in both A.T.Pase activity, and in A.T.P. levels with increasing age, and these parameters correlate well with survival studies in vivo using artificially-aged red cells. (Dern et al 1967). Red cell acetyl-choline esterase activity also decreases during storage (Anderson & Pethica 1955) and this decrease also correlates with in vivo survival. Changes in various aspects of red cell metabolism during storage have been observed by Gomberts (1967). This author finds phospholipid loss from the red cell membrane, and a decrease in red cell A.T.P., to be associated with an increase in inorganic phosphate in red cells and storage medium. Loss of erythrocyte cholesterol from stored cells has also been described. (Murphy 1962 a.) Investigations into red cell injury, or the ageing process have in general been hampered by the lack of a convenient test to assess the degree of cell damage. The most commonly applied technique has been the measurement of in vivo survival after radiochromium labelling of the test red cells. The chromium labelling technique is cumbersome, expensive, but accurate. Increases in osmotic fragility are much too approximate to characterise a membrane lesion, and furthermore it has yet to be shown that there is a positive correlation between the in vivo half life/

life, and the osmotic fragility of a red cell.

Stuart & Cummings (1967) using an in vitro method, employed mouse macrophages grown in human serum to detect damage to red cells as a consequence of ageing and antibody sensitisation. They reported that red cells suspended in saline for 18-24 hours were rapidly and completely phagocytosed. They used the term "saline injury" to describe this state. Unlike other forms of injury, such as incubation with heavy metals (Jandl 1957), heat, lipid solvents etc., saline injury is "physiological" in the sense that no active agent damages the erythrocyte. Saline itself will cause erythrocyte damage especially when present in high concentrations, as occurs at the surface of the red cell during the process of freezing. (Lovelock 1955). Cells injured by freezing show loss of phospholipid from the surface. An equivalent loss can be produced by shaking red cells with an adsorbent, such as alumina. Loss of about 20% of the membrane phospholipid is followed by haemolysis. (Lovelock 1954; 1955). Substances such as polyvinylpyrrollidine and glycerol exert a protective effect upon red cells during freezing. Phospholipid in the form of egg lecithin is also effective. These substances may act by reducing the concentration of water, and hence of sodium and chloride ion, at the cell surface. They may act by "diluting" the ionic double layer (Farrant 1969). The exact mechanism of membrane damage in the presence of excess salt is unknown.

In summary, the ageing of red cells is associated with lipid loss from membranes, a decrease in the levels of A.T.P., and in the activity of the enzyme A.T.P.ase. Choline-esterase activity also decreases, and an increase in the cell inorganic phosphate can be demonstrated.

demonstrated. These changes render the red cell susceptible to phagocytosis.

Injury to the red cell can be produced by a variety of agents, lipid solvents, heavy metals, enzymes, alcohols, and heat. Injury to the red cell can also occur following the incubation of red cells in isotonic saline, and during the freezing of red cells without haemolysis. Cells injured by freezing have been shown to lose phospholipid, and this is thought to be due to a local increase in salt concentration at the membrane surface during the freezing process.

These forms of injury can most conveniently be detected by the method of Stuart & Cummings (1967); that is by the employment of living macrophages in an in vitro system. The fact that red cells can be so injured is also of value in the investigation of the process of phagocytosis, since this form of red cell damage does not involve reaction with an antibody, which will act as an opsonin, nor is the injury severe enough to produce haemolysis, sphering, or other changes which of themselves might lead to phagocytosis of the red cell. Finally since the injury may be considered analagous to the normal ageing processes of red cells, its early detection and investigation by the use of macrophage might add to the knowledge of the normal physiology of the red cell.

PART II - THE EXPERIMENTAL INVESTIGATION
OF RED CELL INJURY

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7. DISCUSSION.

1 - THE RESPONSE OF MOUSE MACROPHAGES TO NORMAL HUMAN RED CELLS IN THE PRESENCE OF SERUM.

Mice of Tucks TO strain (suppliers Tuck & Son Rayleigh, Essex) weighing between 20 and 25 g. were used as macrophage donors. Macrophages were seeded into rimless pyrex glass test tubes at a concentration of 1 million to 1.5 million cells per ml. Human serum was added to give a final concentration of 10%. (For details of the methods of culture and standardisations of culture see Chapter 7, Appendix). Cultures prepared in this manner were ready for use after 18 to 24 hours of incubation at 37°C.

Red Cells.

Red cells were obtained from the South East Regional Blood Transfusion service (Courtesy of Dr. R.A. Cummings) and from the medical and technical staff of the Pathology Department, University of Edinburgh. Blood groups, if not known were determined for A.B.O. and Rh antigens. Compatible serum was always used for macrophage cultures. Red cells of not more than 6 hours old were used throughout. Red cells were collected in either A.C.D. or Heparin as anticoagulant. After collection red cells were separated from serum by centrifugation at 800xg. The top third of the deposit was discarded as it was contaminated by leukocytes and platelets. The remaining cells were washed four times in ten times their own volume of saline. After the final wash the cells were packed by centrifugation at 800 g. for 15 minutes. From the packed cells a suspension of 5% by volume was made in saline. 0.1 ml. of this suspension was added to each culture, shaken to ensure an even distribution of cells, and/

and incubated for two hours. After incubation the coverslips were removed, rinsed twice in normal saline to remove adherent red cells, and then fixed in a mixture of acetone and methanol 50/50 by volume. The coverslips were stained with haematoxylin and eosin, or Giemsa stains.

Assessment of phagocytic index.

The phagocytic index was defined as the ratio:

$$\frac{\text{Number of macrophages containing one or more red cells}}{\text{Total number of macrophages counted}} \times 100$$

Slides were counted using a high magnification (x600) which allowed between 50 and 70 cells per field. At this magnification it proved quite easy, with experience, to differentiate between those cells adherent to macrophages, and those cells which had been phagocytosed. In the former case, the red cell outline was clear, and the individual cells were easily defined. Phagocytosed red cells were often in well defined vacuoles, but even if the vacuoles were not easily visible, the outlines of phagocytosed cells were blurred, and groups of cells appeared to merge one with another. At least 1,000 cells were counted from three separated areas of the coverslip. Observer error was determined by recounting batches of slides at an interval of 3 to 6 months. For one observer this was never greater than 3%. of the average phagocytic index for the counted slides.

Results.

Normal fresh red cells of all blood groups showed a phagocytic index of less than 10%. Some adherence of red cells to macrophages and/

and occasionally seen.   This occurred with red cells of group AB
more frequently than with other groups.   It also occured with red
cells obtained without the use of anticoagulant.   The range of the
phagocytic index for over 100 separate determinations was between
1.0% and 8.5% with a mean of 5.5% (± SD 3.2).   A few experiments
were performed upon human macrophages obtained from ascitic fluid,
and from peritoneal dialysis fluid.   These showed a lower range of
uptake of compatible human red cells on the four occasions tested.
In each case the donor was of blood group O.   Red cells of O Rh +
and O Rh - donors in compatible serum gave a phagocytic index of
less than 1%.   The range was between 0.02% to 0.8%.

The fresh human red cell is therefore not phagocytosed by
either mouse or human macrophages in the presence of compatible
human serum.

2 - RESPONSE OF MACROPHAGES TO SALINE INJURED CELLS IN THE PRESENCE
OF HUMAN SERUM.

Red cells from the sources already mentioned were injured by
incubation in normal saline for 18-24 hours.   Prior to injury the
red cells were prepared as before, and made up to a 5% suspension in
normal saline.   This suspension was incubated under sterile condit-
ions at 37°C. on a Gallenkamp oscillating shaker.   From each blood
sample so treated samples were innoculated in broth and on blood agar.
After the incubation period, red cells were reprecipitated by
centrifugation, and washed once in ten times their own volume of
saline.   They were packed at 800 g. and resuspended in a final
concentration of 5% by volume.   0.1 ml. of this suspension was
added to monolayers of macrophages, and reincubated, after shaking,
for a further two hours.   Coverslips were harvested, fixed and
stained as before.

Results.

Red cells treated in this manner always gave a high phagocytic
index.   All blood groups proved equally susceptible to injury in
this manner.   The phagocytic index varied between 61% and 90%, with
a mean of 75.2%.   These figures were obtained from twenty eight
samples of blood studied over forty experiments.

It was therefore concluded that fresh human red cells after
28-24 hours incubation in normal saline become susceptible to
phagocytosis.   Incubation in saline therefore alters the constitution
of the red cell in a manner which renders it susceptible to phago-
cytosis./

3 - THE EFFECTS OF SALINE INJURY UPON THE HUMAN RED CELL.

Incubation in saline renders the red cell susceptible to phago-
cytosis. Investigation was therefore directed into the mechanisms
and the nature of saline injury.

a. The effects of temperature upon saline injury.

Washed samples of fresh human red cells were stored as a 5%
suspension in an incubation at 37°C., and incubator at 45°C., a
water bath at 56°C., and in a refrigerator at 4°C. Cells were
removed at 18, 24, 36 and 48 hours, washed once, and tested for
injury in monolayers of macrophages. The phagocytic index varies
as illustrated below. Cells stored at 56°C. and at 45°C. became
injured by 18 hours. Haemolysis was marked in these samples.

TABLE I.

THE EFFECTS OF TEMPERATURE UPON THE RATE OF RED CELL INJURY.

	4°C.	37°C.
18 hours	18%	56%
24 hours	22%	62%
36 hours	46%	74%
48 hours	68%	76%

It is therefore evident that saline injury is only slightly
temperature dependent. Incubation at 4°C. retards the onset of
injury, but significant injury was apparent within 24 hours.
Incubation/

Incubation at 45°C. and 56°C. produces maximal injury within a few hours. At 56°C. lysis is marked at 18 hours, and the remaining red cells clump and turn brown. These clumps are rapidly and completely phagocytosed.

b. The relationship between injury and the time of
incubation of red cells in saline.

A 5% suspension of washed fresh human red cells was incubated in saline for 2,4,6,8,12,18,24 and 36 hours. They were afterwards washed once, and the phagocytic index determined in monolayers of mouse macrophages grown in compatible serum. Phagocytosis increased with increasing time of incubation after 6 hours.

TABLE II.

THE RELATIONSHIP BETWEEN TIME OF INCUBATION OF RED CELLS IN SALINE AND THE DEGREE OF INJURY AS ASSESSED BY THE ERYTHROPHAGOCYTOSIS TEST.

Time (hours)	2	4	6	8	12	18	24	36
Phagocytic Index.	3%	4%	8%	18%	34%	63%	68%	74%

c. Effects of serum dilution upon saline injury.

Red cells stored in a 5% suspension in whole serum do not show any evidence of injury as assessed by the erythrophagocytosis test, after 24 hours of storage at 37°C. If red cells are stored under the same conditions in serum which has been diluted with saline, the red cells become injured. Injury is most pronounced in saline/serum mixtures in which the serum concentrations are below 12%.

TABLE III./

TABLE III.

THE EFFECTS OF SERUM CONCENTRATION UPON SALINE INJURY OF RED CELLS
STORED IN SERUM/SALINE MIXTURES FOR 24 HOURS.

AT 37°C.

Serum Concentration	neat	50% serum	25% serum	12½% serum	6% serum	3% serum
Phagocytosis Index	2%	4%	11%	20%	44%	66%

Red cells incubated in saline alone
gave an uptake of 72%

Serum protects the red cell against injury during storage under
these experimental conditions.   Dilution of serum reduces its
protective effect upon the red cell.   At this point it was thought
that loss of serum proteins adsorbed to the red cell surface might
precipitate the uptake of red cells incubated in saline;   that is
injury might be caused by loss of adsorbed serum proteins from the
red cell surface.   Serum proteins adsorbed to the surface of red
cells after washing, and after saline injury were studied qualitat-
ively by immunoelectrophoresis, agglutination with specific antisera,
and by gel diffusion.

d.   Serum proteins adsorbed to the surfaces of fresh
and injured red cells.

8   Red cells were washed four times in saline.   After the final
wash they were suspended in a 50% concentration by volume in saline,
and incubated for 30 minutes at 37°C.

The supernatant, obtained after centrifugation of these cells,
was/

was concentrated by dialysis against polythene glycol (Koch-Light
Ltd.) and its serum protein content studied by gel diffusion, and
immunoelectrophoresis. Suspensions of washed red cells were also
tested for agglutination with specific antisera to the following
serum proteins: Albumin, alpha 2 Macroglobulin, Transferrin, Alpha
1 lipoprotein, beta lipoprotein, IgM, IgG, IgA, and prealbumin
(Acidic alpha 1 globulin, "serumucoid") Antisera were supplied by
Behringwerke.

The experiment was repeated using saline injured red cells
instead of fresh washed human red cells. The results were as
follows:

a. Gel diffusion, and immunoelectrophoresis.

The supernatant from washed red cells showed the presence of nearly
all the serum proteins by immunoelectrophoresis and gel diffusion.
The supernatant from saline-injured cells was shown to contain
albumin, transferrin, alpha and beta lipoproteins, alpha 2 macro-
globulin, and a trace of IgG, by gel diffusion. Immunoelectrophoresis
revealed albumin, transferrin, alpha and beta lipoproteins, and alpha
2 macroglobulin.

b. Agglutination.

Fresh red cells were agglutinated by antisera against alpha and beta
lipoproteins to a titre of 1/250, and by antisera against alpha 2
macroglobulin to a titre of 1/7. Saline injured human red cells
were agglutinated by anti-alpha and anti-beta lipoprotein antisera
to a titre of 1/50. Only microscopic agglutination by anti-alpha
2 macroglobulin serum was shown, up to a dilution of 1/4. Antisera
against other serum proteins produced no visible agglutination in neat
concentrations./

concentrations. This experiment shows:

a. that alpha and beta lipoproteins are firmly bound to the red cell surface, and are slowly lost during saline injury.

b. that small quantities of many normal serum proteins are difficult to remove from the red cell by four washes in ten times their own volume of saline.

e. The effect of reincubation of injured red cells in compatible serum.

Saline injured red cells were washed once and reincubated in compatible serum for 24 hours in a 5% suspension. Washed fresh red cells incubated as a 5% suspension in serum were used as controls. Reincubation of injured red cells in serum produced no change in the phagocytic index. This proves that once injury is established it cannot be reversed by re-incubation, in serum.

TABLE IV.

THE EFFECT OF RE-INCUBATION OF INJURED RED CELLS IN SERUM AT 37°C. FOR 24 HOURS.

Fresh red cells	Injured red cells	Injured red cells in serum.
P = 45%	64%	62%

f. The effect of aerobic and anaerobic incubation of red cells in physiological salt solutions.

The red cell incubated in saline might become injured by lack of its main substrate, glucose. Deficiencies in the ionic constitution/

constitution of the incubating medium might affect enzymic processes in the membrane. These deficiencies could lead to the accumulation of harmful metabolites, such as lactic acid which could directly damage the membrane. Storage of red cells under anaerobic conditions might either accentuate, or suppress the development of metabolic lesions in the cell membrane.

The following salt solutions were used to assess the dependance of saline injury upon the ionic, and metabolic composition of the incubating media.

a. Saline soluttion with glucose; 9.45 g. sodium chloride, 53.4 g. glucose.

b. Ringers solutions, Sodium chloride 9.00 g., Potassium chloride 0.4 g. Calcium chloride $(6H_2O)$ 0.25 g., Sodium bicarbonate 0.20 g.

c. Ringers Lactate solution, Sodium chloride 6 g., potassium chloride 0.4 g., calcium chloride $(6H_2O)$, 0.20 g., sodium lactate 3.05 g.

The quantities given above are for making up 1 litre of solution. All the reagents used were Analar grade reagents supplied by B.D.H. Glass distilled water was used to make up the solution.

d. Hanks Balanced Salt Solution (Glaxo).

e. Eagles Medium (Glaxo).

f. Medium 199 (Glaxo).

The pH of all these solutions was adjusted to 7.4 by the addition of a solution of sodium bicarbonate (14 g./litre) if required. Cells were stored in these conditions as 5% suspensions for 24 hours at $37^{o}C$. under both aerobic and anaerobic conditions. Anaerobic conditions/

conditions were provided by gassing the media for 1 hour with nitrogen before adding the cells, and then sealing the ampoules in an atmosphere of nitrogen.

Results.

There was no significant protective effect by the first five solutions tested under either aerobic or anaerobic conditions. Medium 199 offered better protection than any of the other media under aerobic conditions.

TABLE V.

THE EFFECTS UPON SALINE INJURY OF AEROBIC AND ANAEROBIC INCUBATION OF RED CELLS IN PHYSIOLOGICAL SALT SOLUTIONS.

Medium	Phagocytic Index (Aerobic)	Phagocytic Index (Anaerobic)
Saline + Glucose	78%	82%
Hanks BSS.	66%	85%
Ringers Soln.	78%	78%
Ringers + Lactate	62%	70%
Eagles Medium	35%	48%
Medium 199	26%	43%

f.   Effects of additional chemical agents upon the injury of red cells in saline

A variety of chemical agents, including co-enzymes, vitamins, metabolic inhibitors, and a stimulant of red cell metabolism were added to saline in an attempt to prevent saline injury.

Co-enzymes.

Adenosine/

Adenosine triphosphate (0.1 mg./ml.) Nicotinamide adenine dinucleotide (N.A.D.H.). Nicotinamide adenine dinucleotide phosphate (N.A.D.P.H.), (Nutritional Biochemical Corp.) (0.1 mg./ml.) were added to saline. Fresh washed red cells as a 5. suspension were added to saline, and incubated in these mixtures for 24 hours at 37°C. The phagocytic index was determined as before.

TABLE VI.

THE EFFECT OF CO-ENZYMES UPON INJURY TO RED CELLS INCUBATED IN SALINE AT 37° CENTIGRADE FOR 24 HOURS.

Medium	Phagocytic Index
Saline + A.T.P.	65.
Saline + NADH.	30.
Saline + NADPH.	35.

The two co-enzymes nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate had some effect upon reducing the degree of red cell injury in saline. This fact may explain the protective effect observed for medium 199, which contains nucleotides. ATP had no protective effect, possibly because extracellular ATP might injure red cells by causing peroxidative reactions in the membrane lipids.

Vitamins.

Nicotinamide (60 mg./ml.), Riboflavine (1.8 mg./ml.) and Pantothenic acid (as calcium pantothonate 1 mg./ml.) were made up in these concentrations in saline. The reagents were analar grade.
Riboflavine/

Riboflavine was supplied by Roche pharmaceuticals, and Nicotinamide and Calcium pantothenate from British Drug Houses. Fresh washed red cells were added to these solutions, as a 5% suspension, and incubated at 37°C. for 24 hours. After incubation the red cells were washed once in saline and added to macrophage monolayers in compatible serum. Phagocytic index was determined as before.

TABLE VII.

THE EFFECT OF VITAMINS UPON THE INJURY TO RED CELLS INCUBATED IN SALINE AT 37° CENTIGRADE FOR 24 HOURS.

Medium	Phagocytic Index
Saline + Nicotinamide	60%
Saline + Riboflavine	48%
Saline + Pantothenic acid	30%

Results.

Of the vitamins, pantothenic acid was the only one to affect the degree of cell membrane injury, affording a "partial protection". Pantothenic acid is necessary for the synthesis of co-enzyme A, an enzyme which is involved in intracellular lipid synthesis, and in the aceytlation reactions involving lipids (Biesele 1955; Mahler & Cordes 1967) - reactions which are known to occur in the red cell membrane. Even in the large concentrations used here the vitamin did not entirely prevent saline injury.

The evidence suggests that disturbance in metabolism were in part associated with red cell membrane injury, following incubation in saline, since these effects could be partially prevented by nucleotides and/

and pantothenic acid. If metabolic lesions in the red cell membrane were responsible for the development of saline injury, and if these lesions were the result of uncompensated metabolic processes within the membrane, due for example to the accumulation of lactic acid from the metabolism of glucose, injury might be prevented by the potent metabolic inhibitors of cell respiration. Methylene blue, which increases the oxygen consumption of red cells and stimulates the metabolism of glucose to CO_2 rather than to lactate, (Brin & Yonemoto) (1958) might prevent injury by preventing lactate accumulation. The major part of the energy produced by the red cell is employed in the cation transport system of the membrane. Inhibition of the sodium potassium pump mechanism with Digoxin, which has been shown maximally to inhibit potassium influx into the red cell (Glynn 1961), might prevent saline injury.

Metabolic inhibitors.

Cysteinamine (beta mercaptoethylamine (Koch-Light)) was made up in a concentration of 0.01 mg./ml. in saline. A 5% suspension of fresh washed red cells was incubated in this mixture for 24 hours at $37^{\circ}C.$, washed once, and the phagocytic index determined as before. The phagocytic index for red cells treated in this manner was 62%.

D.P.P.D. (Diphenylparaphenylamine diamine) was made up in saline to a concentration of 0.01 mg./ml. A 5% suspension of washed red cells was incubated for 24 hours at $37^{\circ}C.$ in this solution. The phagocytic index was determined as before (P=58%).

Methylene blue.

A/

A 5% suspension of washed fresh red cells were suspended in glucose saline containing 0.1 mg./ml. of methylene blue (Gurr.) and buffered to a pH of 7.4 with sodium bicarbonate solution. Incubation was continued for 24 hours at 37°C. and the phagocytic index determined as before. The phagocytic index for cells treated so was 68.

Digoxin.

Red cells were incubated as a 5% suspension in saline containing 10^{-2} mg./ml. of purified digoxin (Koch-Light) (concentration of 7.7 x 10^{-5} molar). This concentration was found by Glyhn (1957) to maximally inhibit the influx of potassium in normal red cells. Incubation was continued for 24 hours, and the phagocytic index determined as before. Digoxin did not prevent the expected increase in phagocytic index resulting from the incubation of red cells in saline. (Phagocytic index - 66%).

TABLE VIII.

THE EFFECT OF METABOLIC INHIBITORS UPON THE INJURY TO RED CELLS
INCUBATED IN SALINE FOR 24 HOURS AT 37° CENTIGRADE.

Medium	Phagocytic Index
Saline + Cysteinamine	62%
Saline + D.P.P.D.	58%
Saline + Methylene blue	68%
Saline + Digoxin	66%

In/

In summary it has been shown that saline injury occurs in physiological salt solutions, containing adequate glucose, and all the major ions present in extracellular fluid. Salt solutions containing nucleotides, or pantothenic acid, partially prevent saline injury as judged by the estimation of the phagocytic index. Metabolic inhibitors had no effect upon saline injury. Thus the change that occurs in the red cell membrane after 24 hours incubation in saline is not the result solely of metabolic disturbances taking place within that membrane. The injury appears to be independent of the ionic composition of the salt solution in which the red cell is incubated. Since normal serum protects the red cell from saline injury, and the crystalloid constituents of serum have little effect upon the process, the protective effects of serum must lie in the protein component. The previous studies have indicated that the serum lipoproteins are strongly bound to the red cell surface, and can be detected in this situation even after injury. It has also been shown that the lipoproteins of serum can exchange their phospholipid with phospholipid in the red cell membrane. (Sakagmi et al 1965 a, b). This evidence suggests that the lipoproteins of serum may be involved in saline injury. The effect upon red cells of incubation in defatted serum and plasma was therefore studied.

h. The effects of incubating red cells in normal and defatted serum and plasma.

Serum or plasma was defatted by repeated extraction with an equal volume of ice cold ether for 2 hours at 4°C. The extracted serum/

serum was separated from the ether in a separating funnel, and the remaining ether removed by vacuum pump. Serum treated in this manner showed considerable denaturation (a white floccular precipitate) which was removed by centrifugation before incubating the red cells in the serum. Immunoelectrophoresis studies of serum defatted by this method showed absence of prealbumin lines, and diminished alpha and beta globulins. Red cells incubated in serum or plasma defatted by this method became injured (P = 68.).

There are two fundamental objections attending this method of preparation of serum, firstly traces of ether present in the serum could cause direct injury to the red cell membrane, and secondly marked denaturation of serum, even under the most favourable conditions still occurred. A second method of defatting serum was devised. The starting material (serum or plasma) was freeze dried under high vacuum. It was then further dried under vacuum in a dessicator over phosphorous pentoxide, sealed, and stored in a depp freeze at -14° C. This dried material was then extracted with either Methanol/ chloroform (6/4 by volume) or methanol/acetone (1/1) by volume. These mixtures were dried by the addition of 6G of anhydrous sodium sulphate per 500 ml. or anhydrous calcium chloride 6g per 500 ml., and pre-chilled to -14°C. before being added to the dried serum. The whole extraction procedure was performed without agitation of the serum mixture, and at low temperatures (-14°C.). The extraction procedure was performed by decanting the lipid solvents from the serum residue once or twice daily, and replacing by the same pre-chilled solvents. After 7 to 14 changes of solvent, the freeze dried serum or plasma was separated from the solvents by filtration, and/

and all solvent vapour removed under high vacuum (40 microns
mercury). When reconstituted to its original volume with distilled
water denaturation was slight, and the serum gave a normal immuno-
electrophoretic pattern. Specific antisera still reacted with
material antigenically alpha and beta lipoprotein, but no trace of
lipid could be detected in these lines by staining with sudan black.
The reconstituted defatted serum was glass clear and almost colour-
less. If plasma had been used as the starting material, small
"spiders web" clots developed on storage after reconstitution.

Fresh red cells were washed four times and added to reconstituted
serum or plasma which had been defatted by this procedure. They were
then incubated as a 5. suspension, at 37°C. for 24 hours. After
washing once in saline, the red cells were incubated in macrophage
monolayers for 2 hours, and the phagocytic index determined as before.
Fresh red cells incubated in normal serum were used as controls.

TABLE IX.

THE OCCURRENCE OF INJURY TO RED CELLS INCUBATED IN DEFATTED SERUM OR
PLASMA FOR 24 HOURS AT 37° CENTIGRADE.

Medium	Phagocytic Index
Defatted serum (ether)	68%
Defatted serum (dry extraction)	58%
Defatted plasma	60%
Normal serum	4%

All samples of red cells became injured in defatted serum or
plasma./

plasma. As a further check that this preparation of serum was not rendered directly toxic by virtue of the extraction process, it was used as a medium supplement in unrelated tissue culture experiments. Cells grown in defatted serum proved normal in every respect.

It was therefore concluded that the lipid in serum exerted a protective effect upon the red cell during incubation. Removal of lipid from serum or plasma diminished this protective effect. These experiments also suggested that a common mechanism of red cell injury might result from incubation in saline, and in lipid free plasma, and that both these forms of injury are a consequence of a lack of exchangeable lipid in the incubation medium. Red cells incubated in saline alone might show loss of membrane lipid into the supernatant, and investigation was therefore directed into the examination of the contents of the saline supernatant from saline injured cells.

4 - THE BIOCHEMICAL INVESTIGATION OF SALINE INJURY.

a. Lipids.

The supernatant from saline injured cells was studied with regard to its content of protein, haemoglobin, and lipid. The red cell suspensions for this investigation were prepared as follows.

Fresh red cells were packed at 800x g., the serum was removed, and the top one third of the cell suspension, containing platelets and leukocytes was aspirated and discarded. 25 ml. of red cells were washed three times in normal saline, in the ratio of 100 ml. of saline for each 5 ml. of red cells. Cells prepared in this manner were then incubated in 475 ml. of sterile saline under sterile conditions for periods of 6, 18 and 24 hours at 37°C. Red cells were removed at these times and tested for injury as judged by the erythrophagocytosis test.

	6 hours	18 hours	24 hours
Phagocytic Index	8%	34%	68%

To study the supernatant, the red cells were removed by centrifugation at 1500 g. for 20 minutes. The decanted supernatant was then centrifuged for a further period of 2 hours at 20,000 g., to remove any ghost membranes due to haemolysis. Haemoglobin estimations were carried out on this supernatant, by comparative photodesitometry with haemoglobin solutions prepared by lysis of known numbers of red cells in distilled water. An E.E.L. colorimeter was used with a green (0.G.R.I.) filter. By concentration of the supernatant, the/

the total number of lysed red cells per ml. of unconcentrated
supernatant was estimated at between 100,000 and 250,000 cells/ml.,
with a mean of 110,000 cells/per ml. (six estimations).

The remaining supernatant was freeze dried under high vacuum at
low temperature, and was then extracted for lipid with a mixture of
cyclohexane 50 ml. chloroform 50 ml., and methanol 50 ml., at low
temperature using chilled solvents. The extract was then filtered
through filter papers pre-washed with methanol and chloroform to re-
move any contaminating phospholipid. These filter papers were then
re-extracted three times with fresh solvents. The total lipid
extracts were pooled, and dried in vacuo. They were then re-extrac-
ted with cyclohexane, 20 ml., and methanol 20 ml. This extraction
was repeated twice more, and the final volume reduced to 10 ml. in
vacuo. Methanol will boil off the mixture at room temperature
during the reduction procedure, and the latent heat lost cools the
cyclohexane to its freezing point. The lipid extract is stored in
the frozen state, in the non-polar cyclohexane, and under nitrogen
until used. This method of storage proved necessary, since the
phsopholipids in the extract proved extremely labile; for example
large amounts of lysolecithin will occur in lecithin containing
mixtures stored in polar solvents in air, even at low temperature.

The lipids in the extract were identified by thin layer chromato-
graphy on silica gel (Merck, grades G and H) in a solvent system of
chloroform methanol, acetic acid, and water (50/25/7/4) or (85/15/10/4).
A second solvent system was used for the identification of the non-
polar lipids, comprising petroleum ether, ethyl ether, and acetic
acid/

acid (50/35/5). The individual spots on the plates were identified with reference to their Rf values, comparison with standard preparations, and by staining reactions. It was found that not all the lipid extractable from the supernatant was soluble in cyclohexane alone, or in cyclohexane/benzene mixtures. A small quantity of methanol was required for the complete solubilisation of the extracted lipids. The colour reactions used to detect the phospholipids occurring in these extracts were as follows.

1. Rhodamine 6 G.

An aqueous solution of Rhodamine 6 G (Gurr) approximately of 0.5% concentration, was diluted with 20 parts by volume of anhydrous methanol. The resulting solution was faintly pink in colour. Plates were sprayed with this solution, and allowed to dry, before being examined in ultra-violet light for fluorescence. The majority of the phospholipids will give a yellow fluorescence with this dye, but phosphatidic acid, phosphatidyl isositol, and phsophatidyl serine fluoresce blue. The reaction is not very sensitive, a minimum of 5 micrograms of phospholipid per spot is required before this method will detect it.

2. Iodine.

The chromatography plates were allowed to stand for up to 20 minutes in an atmosphere of iodine vapour generated by placing crystals of iodine in an enclosed jar. All lipids stained with a brownish colouration which enabled their position on the plate to be marked and photographed. Plates so treated could subsequently be bleached by/

by exposure to air, and thereafter could be sprayed with the ninhydrin
reagents. Iodine proved a very sensitive method of detecting the
lipids in this extract, between 0.5 and 1 micrograms of lipid per spot
could be easily shown by this reaction.

3. Ninhydrin.

Ninhydrin (B.D.H.) was made up as a 0.25% solution, in com-
mercial grade lutidine (Eastman Chemicals Ltd.) and water 1/9 by
volume. After spraying with this solution the plates were incubated
for 20 minutes in a hot air oven at 90°C., removed, and the full colour
reaction allowed to develop at room temperature over the next two
hours. Phospholipid containing an amino group gave a pink or purple
colouration. Spingomyelin gave a peculiar reaction in that only the
"head" of the spot stained with ninhydrin. This portion of the
sphingomyelin spot was called "sphingomyelin S". The ninhydrin stain
could reveal the appropriate phospholipids in concentrations as low as
1 microgram per spot.

4. The Schiff reaction.

Both direct and indirect Schiff reactions were applied to the
plates after chromatographic development. In the direct reaction,
the plates were first reduced by exposure for 20 minutes to an atmos-
phere of sulphur dioxide generated by the action of water upon crystals
of potassium metabisulphite. They were then sprayed with Schiff
reagent, and the direct colour reaction allowed to develop in an
atmosphere of sulphur dioxide. The plasmalogen forms of phospholipid
gave a blue or purple colouration with the direct Schiff reaction.
The/

The indirect Schiff reaction was performed as above, but after first spraying the plates with periodic acid solution (0.5 ml. of commercial "strong solution", T. Gurr, in 100 ml. of 50% methanol). With this reaction phosphatidic acid and phosphatidyl inositol in addition to the plasmalogens gave a purple or blue colour. This reaction was the most sensitive, revealing 0.2 micrograms of the appropriate phospholipid per spot.

Standards.

Standard lecithin was prepared from crude ovolecithin (Lever Bros. Research Labs., Welwyn, Herts.), by preparative thin layer chromatography on silica gel. The crude lecithin was run in a solvent system of chloroform, methanol, and water 50/35/5. The major component was identified by strip staining of the edge of the plate with phosphotungstic acid, and eluted from the gel with a mixture of methanol and ethyl ether 50/50. The purified component, lecithin, after storage, showed only one major spot with iodine staining. A second, very much weaker and slower moving spot was identified as lysolecithin, and some minor contamination with fatty acids was observed at the solvent front. Other standard preparations used were examples of cholesterol (courtesy of Dr. G. Boyd) cholesterol esters (cholesterol oleate B.D.H.) and examples of neutral lipids and fatty acids. (Unilever Research Laboratories, Herts.).

Table of Results.

Standards used in the identification of these lipids included lecithin, distearin, triolein, ethyl stearate, ethyl palmitate, palmitic/

mitic acid, oleic acid, and stearic acid (all supplied by Lever Bros.

Experimental Laboratories, Welwyn, Herts.), Cholesterol (Courtesy of

Dr. G. Boyd, Department of Biochemistry, University of Edinburgh) and

Cholesterol oleate (B.D.H.).

TABLE Xa) and b).

THE SOLVENT SYSTEM USED WAS CHLOROFORM 50, METHANOL 25, ACETIC ACID 7,
AND WATER 4 PARTS, EACH BY VOLUME.

a).

Spot	Present	Rf value	Staining Reaction	Identification
1	6H	0.11	Iodine	Unknown
2	All	0.17	Schiff +	Phosphatidyl Inositol
3	All	0.24	Ninhydrin	Phosphatidyl Serine
4	All	0.31	Direct Schiff+	? Plasmalogen
5	All	0.31	Iodine	Sphingomyelin B
6	All	0.36	Ninhydrin	Sphingomyelin A
7	18 & 24 H	0.4	Direct Schiff+	? Plasmalogen
8	All	0.46	Ninhydrin	Phosphatidyl Ethanolamine
9	All	0.54	Standard	Lecithin
10	6H	0.6	Direct Schiff+	Lysoplasmalogen of ?Lecithin
11	18 & 24 H	0.86	Iodine	Unknown
12	18 & 24 H	0.9	Iodine	Unknown
13	18 & 24 H	0.96	Rhodamine 6G	Phosphatidic acid

b).

b).

NON POLAR LIPIDS IDENTIFIED USING PETROLEUM ETHER, ETHYL ETHER, ACETIC ACID 50/35/5 PARTS EACH BY VOLUME.

Spot	Present	Rf value	Staining Reaction	Identification
14	18 & 24H	0.64	Standard	Cholesterol
15	Trace 6H 18 & 24H	0.70) to 0.96)	Standards	Triolein, Distearin, various fatty acids, incl. Palmitic.
16	18 & 24H	Front	Standards	Cholesterol Esters

Thin layer chromatography on silica gel revealed sixteen components (Table X) in the lipid extract, including the fatty acids and fatty acid esters. These components were identified as far as possible by their staining reactions, Rf values, and with reference to standards. Of these sixteen components, eight were present in the lipid extracts made at 6, 18 and 24 hours, two were present only at 6 hours, and six present only at 18 and 24 hours. At 6 hours an unidentified polar lipid staining with iodine and with an Rf value of 0.11 was present. This lipid was not identified at 18 and 24 hours. Another unidentified lipid, staining with Schiff reagent and iodine was also present at 6 hours. This lipid had an Rf value of 0.6 and by its staining reactions was thought likely to be a lysoplasmalogen of lecithin. Of the lipids present only at 18 and 24 hours, the most noteworthy occurrence was the presence of phosphatidic acid, which was not found after six hours incubation. In addition, only traces of neutral lipid were present in the extract at 6 hours, while these components were easily identified at 18 and 24 hours. The neutral lipid/

lipid occurring in these extracts contained triolein and distearin and many unidentified components. Cholesterol was found to be present only at 18 and 24 hours, it was not found at 6 hours. Traces of cholesterol esters were also found at 18 and 24 hours, but not at 6 hours.

Control extractions of the total phospholipid from 25 ml. of packed freeze dried red cells were made by the same method as applied to the freeze dried supernatant from saline injured cells. Thin layer chromatography of the total red cell lipid showed the same lipids to be present as were found in the extracts of the supernatant from saline injured cells. The quantities of all classes of lipid were much greater than those obtained from the supernatant. In particular greater quantities of cholesterol, cholesterol esters, and neutral lipids were present.

The qualitative analysis of the lipids contained in the supernatant from saline injured cells showed the presence of the major red cell membrane phospholipids. Phosphatidic acid, cholesterol, and the cholesterol esters were not present at 6 hours, and only traces of neutral lipid were found at this time. Similar lipids were shown to be present in much greater quantity in the lipid extract from whole red cells. Phosphatidyl monophosphoinositol was present at 6, 18 and 24 hours, although this lipid is quantitatively only 5 of the total red cell phospholipid. A highly polar lipid containing substance was present in the extract of supernatant at 6 hours. By staining reactions this could not be shown to contain either amines, or aldehyde linkages. This material was thought to be a complex of phospholipid with/

with possibly protein, or mucoprotien which would account for its low
mobility. This complex is not present at 18 or 24 hours. In
summary, these findings can be held to illustrate a gradual break-up
of the membrane structure of the red cell incubated in saline. This
may begin with the loss of the outer components of the membrane,
including a major part of the membrane phosphatidyl inositol, and
complexed phospholipid. After 18 hours incubation, this complexed
material is further degraded, and is accompanied by the additional
loss of "deeper" membrane components such as fatty acids, neutral
lipid and cholesterol. This simplified picture of red cell injury
is complicated by the physico-chemical and enzymic degradation of the
released membrane material in the supernatant during incubation. The
presence of any particular lipid or phospholipid in the supernatant
from injured cells cannot be held to indicate the presence of that
lipid in the membrane in the detected form. Phospholipid material
in the red cell membrane must, on a quantitative basis, be closely
associated with protein, and it is probably released from the membrane
during injury in a complex of protein and lipid. These complexes are
likely to be degraded during incubation and during the lipid extraction
procedure. After extraction of the lipid from the dried supernatant,
a residue composed mainly of salt remained. If water was added to
the residue, an insoluble precipitate remained. This white material
was shown to consist largely of protein by Micro-Keldjahl analysis.
An investigation into the nature of this protein was carried out by
immunodiffusion techniques, and by analysis for lipid and protein
content.

b. Proteins.✓

b. Prot&ins.

Prot&in was isolated from the supernatant of 25 ml. of packed
red cells, injured for 24 hours in 475 ml. of saline at 37°C. by
freeze drying the supernatant, adding 50 ml. of distilled water, and
dialysing the suspended protein against running tap water for 48 hours,
and subsequently distilled water for a further 24 hours. By this
procedure a small quantity of the protein could be solubilised. The
preparation was then freeze dried, or concentrated by dialysis against
polyethylene glycol.

Gel diffusion.

Antiserum was prepared against human red cell ghosts, by injecting
rabbits with 40 mgm. (dry weight) of ghost material intramuscularly
in saline containing 0.5 by weight of polyvinylpyrollidine as adjuvant.
The antiserum was harvested after twelve injections over a period of
three weeks. Antisera so prepared were adsorbed with haemoglobin,
prepared from lysed red cells by colmn chromatography on Sephadex G200
(Pharmacia), in borax phosphate buffer at pH 8.6. Adsorbed antiserum
was reacted with the concentrated dialysed supernatant, in agar plates.
After 48 hours at room temperature, a single line appeared which was
not haemoglobin-antibody complex, and which did not react with
univalent antisera against human serum protein. This confirmed that
the supernatant from saline injured cells contained a protein also
present in the human red cell ghost.

The protein component from saline injured cells proved poorly
soluble in water, or in saline, but was rather more soluble in aqaeous
butanol (10% by volume). In the freeze dried state, this protein was
white/

white, or pale green in colour. The isionic point was at a pH of
7.8 to 8.2 (2 samples).

MicroKeldjahl analysis of this protein was performed. Weighed
samples of dry protein were digested in concentrated sulphuric acid
in the presence of sodium sulphate. The digest was then steam
distilled after the neutralisation of the acid with sodium hydroxide,
and the vapour collected in 2 ml. of saturated boric acid solution,
containing 0.01 ml. methyl red as indicator. The liberated ammonia
was back titrated with 0.07 Normal hydrochloric acid, prepared by
weighing concentrated hydrochloric acid, (Kabat & Meyer 1961) to
neutrality. Assuming that $N = 16\%$ protein, the protein content of
the substance isolated from the supernatant was found to be 79.85%
($\pm$ 2.2%) (6 samples). The lipid content of the substance from
the supernatant was determined by extracting a weighed sample of protein
with methanol/chloroform mixtures on a weighed filter pad in a Buchner
funnel. The extracted material and filter pad together were weighed
after removal of solvent in vacuo. By repeated extraction to con-
stant weight, and assuming that lipid only was extracted by the dry
solvents used, the lipid content of the protein was found to be 8.3%
($\pm$ 4%) (6 samples).

The protein deposit was found to be Schiff positive. This
suggested the presence of mucoprotein in the sample. Sialic acid
was measured qualitatively by the colour reaction given with Ehrlich's
reagent in glacial acetic acid, after boiling with sodium bicarbonate
solution. The purple colour occurring after allowing 2 hours to
develop showed absorption maximum at 550 mμ in a Bechman spectro-
photometer./

photometer. This was taken to indicate the presence of sialic acid.

By subtraction the percentage composition of the protein isolated from the supernatant from saline injured cells had the following composition:

Protein	79.85%
Lipid	8.3%
Sialic Acid	11.85%

5 - THE QUANTITATION OF SALINE INJURY.

It has been shown that red cells lose phospholipid and protein from their membranes during incubation at 37°C. in saline for 18 to 24 hours. Very few cells can be shown to have lysed during this incubation, as estimated by haemoglobinometry, being much less than 1% of the total number of red cells present. Quantitation of the lipid loss from the red cell membrane, if the loss due to lysed red cells is neglected, should reveal the injury in the light of the percentage of the total membrane lipid lost. Red cells are not injured by incubation in saline for six hours, but are injured after 18 or 24 hours incubation. By the accurate quantitation of lipid loss it was thought possible to relate injury to the percentage of red cell membrane material appearing in the supernatant. Red cell injury was quantitated by chemical estimation of phospholipid in the extracts of of supernatant from saline injured cells, and relating this quantity to the total amounts of phospholipid extractable from the red cell.

Red cell suspensions were prepared as before. After washing, the red cells were packed at 800 g. by centrifugation, and two equal aliquots of 25 ml. of red cells were prepared. One aliquot was freeze dried, and the lipids extracted with methanol/chloroform, and Methanol/cyclohexane as before. The remaining red cells were injured in saline for 18 and 24 hours, and the supernatant freeze dried and extracted as before.

Lipid was quantitated in these extracts by the following procedure. The staining reactions used to detect lipids in thin layer chromatography/

graphy are of known sensitivity (Marinetti 1964). Samples of lipid extract of known volume were diluted with a measured volume of methanol. After mixing, small aliquots of the diluted samples were applied to the chromatography plate with a microsyringe. After development of the plate, the lipids were identified by their staining reactions. By repeated dilution of the sample, a point was reached where the quantities of lipid present were below the sensitivity of the staining reaction used. Taking this as the end point, and knowing the dilution of the original extract, the volume applied to the plate, and the sensitivity of the staining reactions used, a simple proportion sum gave the quantity of phospholipid in the original extract. The sensitivity of the staining reactions are taken from Marinetti (1964). This method was applied to three separate samples of red cells. The quantities estimated in these samples are given below.

TABLE XI.

THE QUANTITIY OF LIPID IN THE SUPERNATANT FROM SALINE INJURED CELLS COMPARED WITH THE TOTAL EXTRACTABLE LIPID FROM THE SAME VOLUME OF WHOLE RED CELLS.

Total red cell lipid	Total lipid in supernatant	Percentage
102 mg.	18.8 mg. (at 18 hrs.)	13.7%
186 mg.	32.8 mg. (at 18 hrs.)	17.6%
124 mg.	24.2 mg. (at 24 hrs.)	19.4%

The percentages obtained by this method vary considerably, and the nature/

nature of the estimation renders it inaccurate. It is a useful guide, in that red cells can be shown to become injured when they have lost approximately 16 ± 3 of the membrane lipid. More accurate estimations were made using red cells labelled with P 32.

Quantitation of saline injury using P 32.

Red cells labelled with the P32 radioisotope of phosphorous, were obtained from two patients undergoing treatment for polycythaemia vera, 24 and 48 hours after receiving clinical treatment with the isotope. (Courtesy of Dr. McCurk, Radiotherapy Department, Western General Hospital, Edinburgh). These red cells were packed by centrifugation, and the serum aspirated. The top three quarters of the red cell deposit was aspiratedand discarded. The remaining red cells were washed, as before, four times in ten times their own volume of saline, and finally packed at 800 g. A 1 ml. aliquot of red cells was removed for counting to establish the baseline radio-activity of the red cell sample. This sample of red cells was stored and counted at the completion of the experiment. An additio-nal sample of 1 ml. of red cells was lysed by dropwise addition to 100 ml. of distilled water, and then centrifuged at 20,000 g. for 2 hours to precipitate the ghosts. The ghost sample was stored as before, to be counted at the completion of the experiment. Of the remaining red cells, an aliquot of 25 ml. was made up as a 5% suspension in 475 ml. of saline, and incubated under sterile con-ditions for 24 hours at 37°C. Samples of saline were removed at 2, 6, 18 and 24 hours, and the red cells removed by centrifugation at 1,500 g. The samples were then centrifuged at 20,000 g. for 2 hours and/

and the supernatant stored until counted.

Counting was performed using a twin channel scintillation counter (Packard). The scintillation fluid was N.E. 572 ("Scinstant") (Nuclear Enterprises Ltd.), in dioxan. All samples were counted at the same time, since P 32 has a short half life, (12.7 days). A blank control sample was counted simultaneously with each test. From the first two samples, consisting of whole red cells, and red cell ghosts, and assuming the red cell ghosts to be membrane only, the proportion of whole red cell radioactivity due to the membrane alone was calculated (as counts/min.). From these results, the total radioactivity, expressed as counts/minute, in the red cells added to the saline before injury could be calculated. Assuming that this radioactivity was uniformly distributed in every 1 ml. of the red cell/saline mixture, the maximum counts obtainable could be predicted. This predicted value was compared with the actual value obtained from counting, and by applying the correction for the percentage distribution of radioactivity between ghost and lysate, the percentage of membrane radioactivity appearing in the supernatant was calculated. The results are given below.

Total count (whole R.B.E. 1 ml.) - 25890 c/min.

Total count predicted per 1 ml. saline - 1079 c/min.

The ratio of radioactivity between the red cell membrane and the radioactivity of the whole red cell sample was 2/3, expressed as counts/min.

TABLE XII √

TABLE XII.

THE RATE OF LOSS OF P^{32} FROM LABELLED RED CELLS INCUBATED IN SALINE AS A 5% SUSPENSION AT 37°C.

Counts/Min.	Percentage whole R.B.C.	Percentage Membrane	
1079	100%	66%	
24	2.2%	1.4%	2 hours
260	49%	16%	6 hours
536	24%	32.6%	13 hours
605	55%	36.6%	24 hours

This experiment shows that the red cell becomes injured by the loss of approximately 30% of the membrane lipid. This conclusion is based upon the assumption that all the radioactivity isolated from the saline was due to labelled phospholipid. This conclusion is probably unjustified, since some membrane radioactivity might result from phosphorous incorporated into protein, or bound inorganic phosphorous. Chemical analysis and quantitation of lipid loss showed between 13% and 19% of membrane lipid lost during injury. The true evaluation of the phospholipid and lipid loss lies probably between the two estimates of 30-37% and 13-19%.

The discovery that injury to red cells caused by incubation in saline is associated with lipid loss and protein loss from the red cell membrane, raises several important questions.

1. Why is the membrane unstable in the absence of serum lipoprotein?

2. Is the transfer of lipid between lipoprotein and the red cell membrane of importance in the prevention of injury to the membrane?

3√

3.   In what way does damage to the red cell membrane predispose the affected red cell to be phagocytosed?

The transfer of lipid between the plasma lipoproteins and the red cells membranes of normal and injured red cells was investigated using the serum from the samples of blood labelled with P32, used in the previous experiments.   The plasma was removed from the sample of radiolabelled blood, and dialysed for 24 hours against normal saline containing 0.25 g. of Calcium chloride (hydrated) per litre.   This achieved two things, removal of free inorganic radiophorphorous, and the promotion of clotting in the plasma sample.   After dialysis, the clot was removed by centrifugation, and the serum stored.

Normal fresh red cells were washed four times in ten times their own volume of saline, and then injured for 24 hours by incubation in saline.   After injury they were washed once more, packed at 800 g., and 0.1 ml. aliquots of packed red cells incubated with a 0.5 ml. aliquot of radio-labelled serum for 10 minutes, 30 minutes, 2 hours, and 4 hours.   After incubation, the red cells were removed, washed four times in a large excess of saline, and transferred quantitatively to counting pots containing 10 ml. N.E. 572 in dioxan.   Controls consisted of blank pots containing scintillation fluid, 0.5 ml. of serum labelled with P32, and a series of normal red cells incubated in labelled serum for the same time, and treated in exactly the same way as the saline injured cells.

TABLE XIII./

TABLE XIII.

THE RATE OF INCORPORATION OF P^{32} INTO FRESH AND INJURED RED CELLS
INCUBATED IN P^{32} LABELLED SERUM AT 37°C.

Sample	Counts per minute.	Percentage P32 Transferred
0.5 ml. serum	3987	100%
Red cells 10 mins.	74	1.86%
Red cells 30 mins.	86	2.16%
Red cells 2 hours.	190	4.78%
Red cells 4 hours.	387	9.71%
Saline injured red cells in P32 serum.		
0.5 ml. serum	3987	100%
Red cells 10 mins.	17	0.43%
Red cells 30 mins.	49	1.23%
Red cells 2 hours.	70	1.84%
Red cells 4 hours.	123	3.1%

The very low counts obtained are corrected for background count
using the blank control, counting on the second channel of the Pack-
hard scintillation counter simultaneously with each sample.   The
figures given above were obtained by subtracting the bakcground count
from the total count.

These results show that the saline injured red cell has lost the
capacity of the normal red cell to bind phospholipid from serum lipo-
protein.   These results seem to indicate that the transfer of phosp-
holipid between the serum lipoprotein and the red cell membrane is of
importance/

importance in the maintainance of membrane integrity. The role of
the serum liporproteins has not been directly investigated, due to
technical difficulties involved in the preparation of these proteins
in a pure form. Good preparations of beta lipoprotein can be made
using the method of Machbeof (1949), but the preparation contains
traces of ammonia, which itself proved toxic to the red cell.

6 - A SUMMARY OF THE EXPERIMENTAL INVESTIGATIONS INTO SALINE INJURY.

Red cells incubated as a 5. suspension in saline for 18 to 24 hours become injured. Injury is apparent after 8 to 12 hours incubation. This injury does not occur in the presence of serum, and the protective effect of serum lies in the lipoprotein it contains. Injury is independent of metabolism, to a great extent, the only metabolisable substances affecting injury being the nucleotides N.A.D.H. and N.A.D.P.H., and the vitamin pantothenic acid. These substances do not prevent injury occurring, but lessen the degree of injury.

Injury is associated with the loss of lipid and protein from the red cell membrane, and this loss is irreversible.

7 - DISCUSSION.

The investigation of this membrane lesion was performed using the macrophage as an indicator of red cell damage. This method was chosen, since all other criteria for the assessment of injury, such as sphereing, haemolysis, morphology, and osmotic fragility tests proved of little value. The osmotic fragility was slightly increased, but still within the limits acceptable as "normal". One additional observation, which may prove significant, was that saline-injured cells could be readily induced to form rouleaux in the presence of calcium. Solutions of saline, containing 0.15 m calcium chloride, caused roleaux formation with saline-injured cells, but not with normal cells. This indicated that saline injury had caused an alteration in the surface properties of the erythrocyte, causing them to become sensitive to agglutination with divalent cations. As already discussed, agglutination by divalent cations occurs because of the ability of these substances to lower the surface charge, and hence the critical potential, at the cell surface. (Bangham et al 1958; Weed et al 1962). It may therefore be concluded that saline injury results in a loss of surface charge, insufficient in itself to cause agglutination, but sufficient to allow agglutination of injured cells by low concentrations of divalent cations. This observation may also correlate with the suceptibility of the injured cell to phagocytosis, the lowered surface charge allowing a closer approach of the injured cell to the macrophage membrane.

High concentrations of salt have been shown to be injurious to red/

red cells, and to red cell ghosts. (Lovelock 1955; Mitchell & Hanahan 1966). The lesions descirbed as being causedby saline have been associated with the loss of membrane lipid (Lovelock 1954; 1955) and of protein (Mitchell & H anahan 1966). In these respects the injury to red cells caused by incubation in saline is similar to that descirbed following freezing, adsorption of red cells with alumina, and extraction of ghost membranes with hypertonic salt solutions. From the results of the present series of experiments it appears that saline dissolves the red cell membrane in part, and that in vivo membrane loss is made good by the transfer of lipid to the membrane by the serum lipoproteins. However the constituents of the membrane are poorly soluble by their nature. Maddy described the protein isolated from ox red cell ghosts, as being nearly insoluble in the presence of salt. (1964 b.). Phospholipids of the type found in membranes form stable lamellae in salt containing media, with very little exchange of lipid occurring between the lamellar phase, and the aqueous phase (Dawson 1966). The same author cast doubt upon the ability of lipid to be transferred from lipoprotein to such a membrane through an aqueous phase, assuming that the transferred lipid identified by Sakagmi et al, was not an integral part of the membrane structure, but merely present as an adsorbed outer layer. This view is in conflict with the experimental observations already described, in that red cells in serum become injured in the absence of transferrable lipid. Moreover this injury can be shown to lead directly to the phagocytosis of the affected cells.

One way of reconciling these apparently conflicting observations is/

is to suggest that lipid exchange between the membrane proper, and the lipoprotein of serum does occur, but through a hydrophobic rather than an aqueous phase. This suggests that at the surface of the normal red cell in vivo, there exists a hydrophobic layer through which the exchange of plasma lipoprotein lipid with cell membrane lipid occurs. This layer is essential to the integrity of the red cell, and its absence diminishes the rate of lipid exchange between plasma and cell membrane. Saline injured red cells have lost membrane lipid. They have also lost at least partially, the ability to bind phospholipid present in the serum or plasma. Saline injury also proves irreversible. This evidence suggests that a common mechanism, that is the loss of a theoretical hydrophobic layer, from the surface of the red cell, could explain all the observed features of saline injury.

The nature of this hydrophobic layer is quite unknown. It might be composed of a layer of loosely bound lipoid material (See Dawson 1966), adsorbed serum proteins, or even organised "anomalous" structures of water at the cell surface. Attempts to reduce the local concentration of water at the cell surface, by employing such agents as Dextran 250, Dextran 100, and Ficoll, have not resulted in any improvement of the survival of red cells in saline, or even in distilled water. Very high concentrations of these substances (70% w/v) will partially prevent "saline injury", and injury in distilled water. (Habeshaw personal observations). There is not as yet enough experimental evidence to prove or disprove the theory that a local decrease in the concentration of water and ions at the red cell surface will prevent or inhibit saline injury.

CHAPTER 3

THE MEDIUM AND ITS EFFECTS

CONTENTS.

e. The kinetics of the reaction between the serum fractions and the mouse macrophage/human red cell system.

8. SUMMARY OF RESULTS.

9. DISCUSSION.

1 - INTRODUCTION.

In phagocytic reactions occurring in vitro, the medium in which the macrophages are maintained is one of the major variable factors affecting the process. In order for the phagocytic process to occur, the surrounding fluids must support the growth of the macrophage, and maintain its metabolic processes. In addition the medium may supply additional factors, such as opsonins, which influence the basal phagocytic activity of the macrophage towards certain types of particle.

Most in vitro experiments employing macrophages, are performed in media containing serum, or serum proteins. With one notable exception, (Fauve 1964), experiments performed in the absence of serum, or of serum proteins, have been short term experiments, lasting from 30 minutes to 6 hours. (Lee & Cooper 1966; Levenson & Braude 1967); (Rabinovitch 1967). The principle reason for the short term experiments performed in the absence of serum, has been the difficulty of promoting the survival of the macrophage in serum free medium beyond a few hours. (Fauve (1964) using a medium containing bovine serum albumin, was successful in maintaining cultures of macrophages for periods of up to 2 weeks, and demonstrated that such cultures were still phagocytically active. This was also demonstrated by Chang (1964) for macrophages cultures in horse serum and beef embryo extract for up to 220 days. It has been noted that the phagocytic potential of the macrophage towards red cells is optimal only after 24 hours of culture. The phagocytic index increases with increasing time of incubation up to this period. The phagocytic activity declines after 48 hours in culture in serum-containing media. (Stuart 1968; Stuart

&/

& Cummings 1963). Short term cultures are therefore unreliable, if a true or absolute value for the phagocytic activity of the macrophage is required. In the same way the use of cultures older than 48 hours is also likely to produce an unreliable estimate of macrophage activity.

The media employed for the maintainence of macrophages in culture are usually adequate in their content of ionic substances. Radsma (1919-1920) showed that the phagocytic ability of peripheral blood leukocytes was dependent upon the presence of certain ions, notably those of sodium and potassium. The divalent ions of calcium or magnesium were found to be of equivalent importance by Eggers (1909) and De Haan (1921-1922). The two most commonly employed tissue culture media are complete Eagle's medium, and Medium 199. Both these media contain adequate amounts of sodium, potassium, and calcium. (See Rabinovitch 1967).

The serum used as a medium supplement in most experiments involving the culture of macrophages, has been found necessary primarily to ensure the survival of macrophages. Usually homologous sera have been used, but in the system employed by Stuart & Cummings, mouse macrophages grown in human serum were used. Vaughan & Boyden (1964) employed rabbit macrophages grown in a variety of heterologous sera for the quantitation of erythrocphagocytosis. It has been observed that the serum employed will not stimulate phagocytosis of the test particle, providing that the naturally occurring antibodies to the test particle have been removed by adsorption. (Perkins & Leonard 1963). It has not been determined that serum has or has not an effect upon the basal phagocytic activity of the macrophage. An obvious/

obvious requirement therefore for the accurate quantitation of erythrophagocytosis, and the estimation of the basal activity of the macrophage in the absence of opsonins and antibodies, is an adequate tissue culture medium supplemented by either pure proteins, or by protein free supplements.

2 - THE PREPARATION OF A SERUM PROTEIN FREE MEDIUM.

Macrophages, harvested from mice by peritoneal lavage, will adhere to coverslips, and will spread in the absence of serum. After 6-8 hours of culture in medium 199 (Glaxo) without serum, the cells round up but remain adherent to glass. After 24 hours culture in medium 199 alone, only a very small percentage of the remaining macrophages are capable of phagocytosing red cells coated with haemolytic antibody. Macrophages grown under similar conditions, but in the presence of serum from the mouse, rabbit, horse, calf, or human, will normally react to antibody coated red cells maximally (the phagocytic index $>$ 90.).

It was found empirically that the addition of glycogen to tissue cultures of macrophages in medium 199 produced better functional survival of the cells than occurred with medium 199 alone, as assessed by the phagocytic index for antibody coated red cells. For macrophages grown in medium 199 containing 0.20 g. of glycogen per 100 ml. (Oyster glycogen B.D.H.), the number of macrophages ingesting antibody coated red cells after 24 hours of culture was 15%, while only 2-3% of macrophages cultured in medium 199 alone were capable of this reaction. Increasing the concentration of glycogen did not increase the numbers of phagocytically reactive cells. It is obvious that even under optimal conditions, medium 199 supplemented with glycogen alone, was inadequate as a medium for the study of basal macrophage reactivity.

The addition of lactalbumin hydrolysate (Difco T.C. lactalbumin) to/

to glycogen solution in distilled water improved the performance
of the cells as assessed by measurement of the phagocytic index.
Using a medium supplement containing Glycogen (Oyster B.D.H.) 1 g.,
and lactalbumin hydrolysate 4 g. in distilled water (100 ml.),. at
a concentration of 10% or 20% in medium 199, the maximal phagocytic
index at 24 hours reached 80%. Solutions containing these con-
centrations of lactalbumin and glycogen were hypotonic, as assessed
by sphering of red cells incubated for 5-10 minutes in them. By the
addition of sucrose (Analar grade B.D.H.), and retesting for osmotic
adequacy using the red cell as an osmometer, the medium was found
to be further improved. In general concentrations of sucrose of
between 0.5 and 1 g. per 100 ml. of supplement were adequate to
maintain the osmotic balance.

An adequate supplementary medium, which supported both the
morphological and functional existance of macrophages for at least
24 hours in culture, was therefore devised, consisting of

Lactalbumin Hydrolysate (Difco)	4.7 g.
Oyster Glycogen (B.D.H.)	1.0 g.
Sucrose (Analar grade B.D.H.)	0.5 g.
Glass distilled water	100 ml.

The medium was found to keep poorly, and required to be made up
in fresh batches every 2-3 days. No reason could be advanced for
this lability. It was found that the addition of small amounts of
immunoelectrophoretically pure human serum albumin improved the
keeping qualities and the general usefulness of the supplement.
The albumin used was salt poor human serum albumin prepared by the
South/

South East Regional Blood Transfusion Serkiee, and supplied through
the courtesy of Dr. R.A. Cummings. Commercially available albumins
are unsatisfactory, and have been found to be toxic to macrophage
cultures. Albumin solution, equivalent to a final concentration of
0.65 g. per 100 ml. was added to the other ingredients in distilled
water. The final composition of the medium for general use was

Lactalbumin Hydrolysate (Difco)   4.7 g.

Oyster Glycogen (B.D.H.)   1.0 g.

Sucrose (analar grade B.D.H.)   0.5-1.0 g.

Salt poor Human Serum Albumin   0.65 g.

For the study of some aspects of macrophage reactivity, medium
supplements containing albumin were unsuitable, especially in circum-
stances where the albumin itself might influence the phagocytic reaction.
A further modification of the basic medium was therefore employed in
these experiments. The modified medium was used mainly in experiments
concerned with the reactivity of the macrophage in long term culture.
The addition of both L glutamine and pantothenic acid to the medium,
improved the morphology and functional ability of macrophages in long
term culture. Polyvinylpyrollidine (Koch-Light Ltd.) was employed
instead of albumin as a stabilising agent for the medium supplement.
The final composition of the medium supplement for long term cultures,
and for cultures used to investigate the role of serum proteins in
phagocytosis was a s follows:

Lactalbumin Hydrolysate   4.7 g.

Oyster Glycogen   1.0 g.

Sucrose   0.5 g.

Polyvinylpyrollidine/

Polyvinylpyrollidine	0.75 g.
L-glutamine	20 mg.
Calcium Pantothenate	10 mg.
Water	to 100 ml.

The labtalbumin hydrolysate is prepared from milk protein by enzymic hydrolysis. The commercially available hydrolysate contains a little undialisable material, which probably represents partially degraded protein. In order to eliminate this source of macro-molecular material, the dialysable fraction of lactalbumin hydrolysate only was used to prepare the medium.

Fresh red cells incubated for four hours at 37°C. in lactalbumin medium and lactalbumin 10% in medium 199 showed no evidence of injury.

3 - THE REACTIVITY OF MOUSE PERITONEAL MACROPHAGES TOWARDS FRESH
HUMAN RED CELLS IN SERUM PROTEIN FREE MEDIA.

Mouse macrophages were obtained by peritoneal lavage, and
cultured in tissue culture medium 199 (Glaxo). After 24 hours in
culture, the monolayers were assessed for functional activity and
appearance. (See appendix). Fresh human cells were obtained from
a variety of donors. Sufficient red cells could be obtained from
a finger prick, but care was needed in taking the blood since the
presence of alcohol on the skin surface, or the application of
excessive pressure to the finger, caused the red cells so obtained to
adhere to monolayers, and rendered accurate assessment of phagocytosis
difficult. The cells were washed four times in physiological saline
and reconstituted as a 5% suspension in saline. They were then
added to the monolayers in quantities of 0.1 ml. of suspension for
each monolayer. Phagocytosis occurred during two hours of incubation
at 37°C. The coverslips were then removed, washed and fixed as
before.

RESULTS.

Fresh human red cells were phagocytosed by mouse macrophages
grown in serum protein free medium. The phagocytic index was rather
more variable than that determined by previous experience with saline
injured human red cells and mouse macrophages in human serum. In a
series of 28 separate experiments, involving over 100 separate cultures,
the phagocytic index varied between 18% minimum to a maximum of 70%.
The average values for all the results was 37.4% ± S.D. 9.3%. The
cells/

cells from one particular donor blood group AB+, showed marked adherence to the macrophage monolayer in four of the experiments, and slight adherence in one. The phagocytic index for the combination of this donors cells and mouse macrophages grown in serum protein free medium was above the average, varying from 52% to 66%. Donors of blood group O were found to give consistently lower than average phagocytic index, varying from 18% to 29%. This evidence is suggestive, but by no means conclusive, that the mouse macrophage in the absence of human serum can detect A.B.O. antigens on the surfaces of red cells.

In comparing these results with those obtained from macrophages grown in human serum, it is obvious that the presence of human serum decreases the phagocytic activity of the mouse macrophage towards the human red cell. Other combinations of serum and cells were therefore explored in order to elucidate this effect.

4 - THE REACTIVITY OF MOUSE PERITONEAL MACROPHAGES TOWARDS MOUSE
RED CELLS IN SERUM PROTEIN FREE MEDIA.

Mouse macrophage monolayers were prepared as before. Fresh

mouse red cells were obtained by bleeding from the retroorbital plexus,

and were suspended in saline containing 0.5 units of heparin per ml.

They were then washed four times in heparinised saline, and reconsti-

tuted as a 5. suspension in physiological saline. 0.1 ml. of this

suspension was added to each monolayer. Incubation was continued

for 2 hours at 37°C., the coverslips were rinsed in saline and fixed

in the usual manner.

RESULTS.

Fresh mouse cells were not phagocytosed by mouse macrophages

grown in serum protein free medium. For 15 separate experiments,

the average phagocytic index was 1.21 with a range of 0.1 to 2.5.

The range of variability was small, despite the large numbers of cells

counted. In six of the fifteen experiments the phagocytic index

was less than 1. The macrophages used were derived from the pooled

peritoneal fluids of outbred mice. The red cells were obtained from

a single individual for each experiment. This suggests that the

mouse macrophage is not capable of distinguishing individual

differences between the red cells of mice of the same strain.

5 - THE REACTION OF MOUSE MACROPHAGES TOWARDS FRESH MOUSE AND FRESH HUMAN RED CELLS IN MEDIA CONTAINING SERA FROM THESE SPECIES.

a. The reaction to mouse red cells in mouse serum.

Macrophages grown in serum protein free medium show no phagocytic reaction towards fresh mouse red cells. The reactivity of mouse macrophages grown in mouse serum towards mouse red cells was therefore investigated.

Mouse serum was obtained by bleeding from the retroorbital plexus. From 0.5 to 2 ml. of blood was obtained from each mouse. The pooled samples were allowed to clot in polypropylene tubes, and the serum separated after 30 minutes by centrifugation. Glass containers, and some plastic containers were found unsuitable for the collection of mouse blood, since haemolysis readily occurred. The presence of serum heavily contaminated with haemoglobin leads to unsatisfactory macrophage cultures, or impaired phagocytic ability. (See appendix).

Fresh red cells were obtained from mice by the method outlined previously. Macrophages, obtained by peritoneal lavage, were cultured in medium 199 (Glaxo) containing 10% by volume of serile mouse serum that had been stored in the refrigerator for at least 2 days. Fresh mouse sera are on occasions toxic to macrophage monolayers, and this toxicity decreases rapidly on storage. Monolayers were used after 24 hours culture. Fresh mouse sera are prepared as outlined previously, were added to the monolayers as 0.1 ml. of a 5% suspension by volume of packed cells. The macrophages and red cells were incubated together for two hours, and the coverslips washed,/

washed, fixed and stained in the usual manner.

RESULTS.

Mouse macrophages grown in mouse serum do not react to the presence of fresh mouse red cells. The phagocytic index for a series of 11 experiments was between 0.3 and 3 with an average of 1.1.

b. The reaction to human red cells in mouse serum.

Macrophages grown in serum free medium will ingest human red cells. The reaction between mouse macrophages grown in mouse serum towards human red cells was therefore investigated.

Mouse macrophages were grown in 10% pooled mouse serum obtained by bleeding mice from the retroorbital plexus. Cultures 24 hours old were used.

Fresh human red cells were obtained from blood collected from a finger prick. After washing four times in saline, the red cells were reconstituted as a 5% suspension in saline, and added to the monolayers in 0.1 ml. quantities. After two hours incubation the coverslips were washed, fixed and stained as before.

RESULTS.

The mouse serum used in the culture of the monolayers was tested against the human red cell samples added to these monolayers for the presence of naturally occurring heteroagglutinins or lysins. As a rule, these did not occur, although some combinations of mouse sera with individual human red cell samples caused agglutination. In such cases/

cases, either "compatible" human red cells were used, or the mouse
serum was adsorbed with the test particle before being used for
culture. Mouse macrophages grown in mouse serum showed a variable
response to the human red cell, but the phagocytic index was always
low. This varied between 5% and 14% with an average for 10 experiments of 9.5%.

c. The reaction to mouse red cells in human serum.

Mouse macrophages obtained by peritoneal lavage were cultured
for 24 hours in pooled group O human serum, in a 10% concentration in
medium 199.

Mouse red cells obtained by bleeding one mouse from the retroorbital plexus, and prepared as before. These red cells were then
tested for agglutination or lysis, by the human serum used in the
preparation of the monolayer cultures. This occurred on only two
occasions, and the sera causing agglutination were discarded, as were
the cultures grown in them. The remaining combinations of human
serum, mouse red cells, and mouse macrophages were reacted in culture
for 2 hours at 37°C. The coverslips were harvested, washed, and
fixed as before.

RESULTS.

Human serum did not appear to greatly increase the uptake of
fresh mouse red cells by mouse macrophages grown for 24 hours in
it. The phagocytic index varied between 3% and 9% with a mean value
of 6.6% for eight experiments.

d./

d. The reaction to fresh human red cells in human serum.

The results of many such experiments have already been reported. (See section 1., Chapter 2., THE RED CELL).

For 100 separate determinations, the phagocytic index varied between 1.0% and 8.5% with a mean of 5.5%.

Summary of results.

a. Mouse macrophages reacting with human red cells in serum protein free medium.

Phagocytic index.	Range	Number of experiments.
37.4% (± SD 9.2)	18% to 70%	28

b. Mouse macrophages reacting with mouse red cells in serum protein free medium.

1.21% (± SD 1)	0.1% to 2.5%	15

c. Mouse macrophages reacting with mouse red cells in medium supplemented with mouse serum.

1.1% (± SD 0.75)	0.5% to 3%	11

d. Mouse macrophages reacting with human red cells in medium supplemented with mouse serum.

9.9% (± SD 5.26)	5% to 14%	10

e. Mouse macrophages reacting with mouse red cells in medium supplemented with human serum.

6.6% (± SD 2.05)	3% to 9%	8

f. Mouse macrophages reacting with human red cells in medium supplemented with human serum.

5.5%/

5.5 (± SD 3.22)   1.0 to 8.5   100

The serum protein content of the medium used to maintain the macrophage cultures, obviously affects the phagocytic ability of the macrophages cultured in it. Both mouse serum and human serum depress the phagocytic reaction of the mouse macrophage towards the human red cell. In the absence of human serum or mouse serum, the macrophage exhibits considerable reactivity towards the human red cell. Mouse red cells are not ingested in either mouse or human serum, or in serum protein free medium. This suggests that the effect of serum upon the phagocytic process is limited to a depression of the innate phagocytic ability of the mouse macrophage towards the human red cell. Heterologous serum does not greatly increase the response of the macrophage towards homologous red cells. The phagocytic reaction is therefore serum dependent, but in the absence of specific antibody, the effect of serum is to depress the innate phagocytic potential of the macrophage. This series of experiments also illustrates that the macrophage itself is capable of specific phagocytic activity towards heterologous red cells in the absence of serum proteins, and hence of both opsonins and antibody.

Study of the depressive effects of serum upon the reaction between the mouse macrophage and the human red cell was extended to consider the kinetics of the effect, and to study the role of the individual serum proteins in producing this effect.

6.- THE KINETICS OF PHAGOCYTIC DEPRESSED BY SERUM.

Two aspects of the kinetics of the depressive effect of serum upon the phagocytic response of the mouse macrophage towards the human red cell were considered, the effect of dilution of serum, and the time required for serum to produce depression.

a. The dependence of phagocytic depression upon serum concentrations.

Cultures of mouse macrophages were prepared in mouse and human serum in dilutions ranging from 20% to 1% by volume, in medium 199. Fresh washed human red cells were prepared as before, and added to cultures 24 hours old. Incubation was continued for 2 hours, and the coverslips washed, fixed and stained as before. Both mouse and human serum were found to have similar effects.

TABLE IV.

(a) THE EFFECT OF SERUM DILUTION ON PHAGOCYTOSIS OF FRESH HUMAN RED CELLS BY MOUSE MACROPHAGES.

Results	20%	10%	5%	2.5%	1%	(Dilutions)
Mouse serum	12	11.8	10	12.8	13	
Human serum	5	3.5	3.4	4.0	6	Phagocytic Index
Lactalbumin	29	27	26	24	18	

Since the phagocytic index for the particular human cells used was slightly greater than expected, the experiment was repeated with a) a different sample of human red cells and b) with mouse serum adsorbed/

adsorbed with the original test red cell.

TABLE XIV. (contd.)

(a). THE EFFECTS OF SERUM DILUTION ON PHAGOCYTOSIS OF FRESH HUMAN
RED CELLS BY MOUSE MACROPHAGES.

Results	20	10	5	2.5	1	
Mouse serum	6	4	7	2	5	Phagocytic Index.
Adsorbed serum.	6	8	2	3	8	

The lactalbumin results were control values.

Serum from both the mouse and man retains its depressive effects
in concentrations of 1 on medium 199. Serum concentrations less
than this will not support the macrophage at a level of functional
activity sufficient to give meaningful results. This difficulty can
be overcome by the dilution of serum with lactalbumin medium and using
the combination of lactalbumin and serum to give a total supplement
concentration of 10. The final concentrations of serum used in the
medium ranged from 1 to 0.1.

TABLE XIV.

(b) THE EFFECTS OF SERUM DILTUION ON PHAGOCYTOSIS OF FRESH HUMAN
RED CELLS BY MOUSE MACROPHAGES.

Serum Concentration	1	0.9	0.5	0.1	0
Human serum	5	13	28	32	40

It can be seen that as the serum concentration decreases, the
macrophage regains some phagocytic potential towards the human red
cell✓

The effects of Serum Dilution upon the Phagocytic Response of Mouse Macrophages to Human Red Cells in Vitro.

+ Control in dilutions of Lactalbumin.

● Test in dilutions of Serum.

⊕ Control in 10 % Lactalbumin.
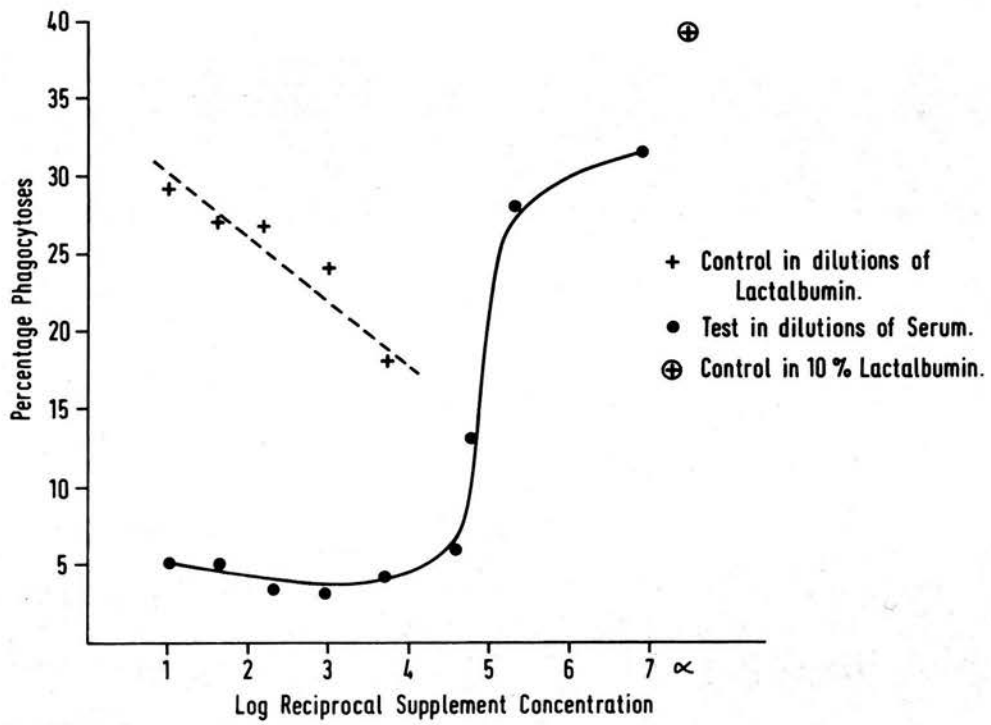
cell. The dependence of this effect upon concentration of serum indicates that the effect is more likely to be due to a direct action by the serum proteins upon the macrophage, than an indirect action such as altering the composition of the medium. The reason for assuming this, is the independence of the effect from serum concentrations above 1%. A well defined threshold effect occurs at concentrations below 1%, and effect unlikely to arise other than by a direct action upon the macrophage.

b. The dependence of the depressive effect of serum upon the time of incubation of the macrophage with serum.

Macrophages incubated for 24 hours in serum protein free medium will react to the presence of fresh human red cells. The addition of serum to monolayers of active cells should depress their reactivity. The time of contact between serum and macrophage necessary to produce the depression was investigated as follows.

As a preliminary experiment it was necessary to ascertain that the depressed macrophage, that is one cultured in human or mouse serum, retained its depressed state for periods at least greater than 2 hours. Macrophage monolayers grown for 24 hours in human serum were therefore transferred to the serum protein free medium, and incubated for varying periods of time. At these set times, each monolayer was tested for its phagocytic abtivity towards the fresh human red cell. The results were as follows.

TABLE XV./

TABLE XV.

THE DURATION OF THE DEPRESSIVE EFFECTS OF SERUM ON THE PHAGOCYTOSIS
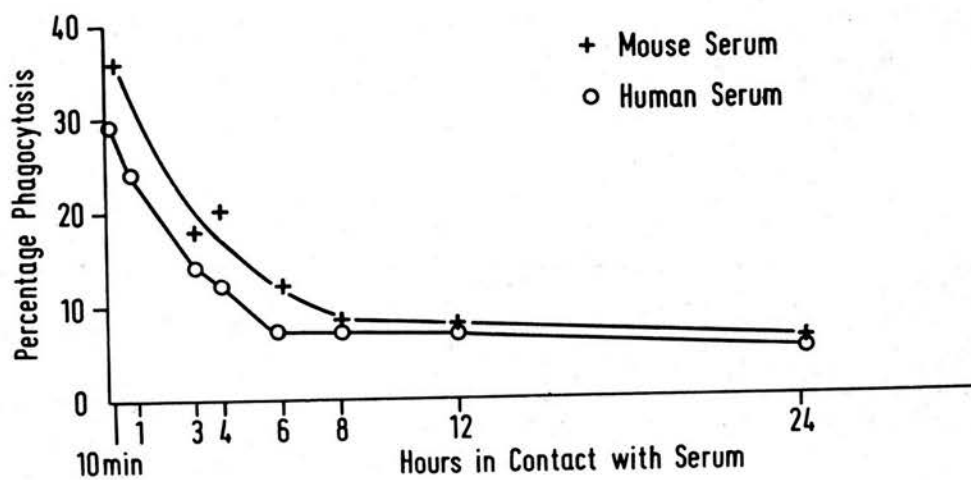OF HUMAN RED CELLS BY MOUSE MACROPHAGES TRANSFERRED TO SERUM FREE
MEDIUM.

Time of culture in lactalbumin	2 hours	4 hours	6 hours
Phagocytic index (fresh human cells)	5%	11%	24%

The depressive effect of serum diminishes with increasing time
of incubation in serum protein free medium. This effect is not
marked at 2 hours, and would not interfere with the investigation of
the time required to produce phagocytic depression in macrophages
exposed to human and mouse serum.

Mouse macrophages were grown in medium 199 supplemented with
lactalbumin. After 24 hours in culture, the medium was removed, the
cultures washed in medium 199 alone, and then transferred to a medium
containing 10% of human serum. The cultures were then incubated in
this medium for periods of 10 minutes, 1 hour, 3 hours, 12 hours and
24 hours. After the appropriate incubation period, the monolayers
were washed again in medium 199 supplemented with lactalbumin. The
cultures were then incubated for a further 30 minutes to allow recovery
from the manipulations involved in washing the cells and changing the
medium. After this time, 0.1 ml. of a 5% suspension of washed fresh
human red cells were added. Incubation was then continued for a
further two hours. Coverslips were then harvested, washed, fixed
and stained as before.

RESULTS./

The dependence of the depressive effect of serum on the phagocytosis of fresh human red cells, on the time of contact of mouse macrophages with serum.

RESULTS.

TABLE XVI (a).

THE VARIATION OF THE PHAGOCYTIC RESPONSE OF MOUSE MACROPHAGES TO FRESH HUMAN RED CELLS WITH TIME OF CONTACT OF THE MACROPHAGES WITH SERUM.

Time	10 mins.	1 hour	3 hours	12 hours	24 hours
Phagocytic Index	28%	24%	11%	8%	5%

These results show that the depressive effect of serum requires between three and twelve hours to become established. The serum used in this experiment was human serum. The experiment was repeated with mouse serum, and human serum with incubation times of 3 hours, 4 hours, 6 hours, 8 hours, and 12 hours.

TABLE XVI (b).

THE VARIATION OF THE PHAGOCYTIC RESPONSE OF MOUSE MACROPHAGES TO FRESH HUMAN RED CELLS WITH TIME OF CONTACT OF THE MACROPHAGES WITH SERUM.

Results	3 hours	4 hours	6 hours	8 hours	12 hours
Human serum	14%	12%	7%	8%	7%
Mouse serum	18%	12%	20%	8%	6%

These experiments show a) Serum from either mouse or human sources requires to act in culture for some hours in order to depress the phagocytic reaction of the macrophage towards the human red cell.

b) Human serum takes less time to depress the phagocytic response to the human cell than does mouse serum.

c) The experiment proves that the serum must react with the macrophage to produce this effect, and that it cannot/

cannot be entirely due to modification of the physical and chemical constitution of the medium.

7 - THE EFFECT OF SERUM FRACTIONS UPON THE PHAGOCYTIC RESPONSE OF THE MOUSE MACROPHAGE TOWARDS THE HUMAN RED CELL.

The previous data relating the concentration of human serum, and the time of contact between the macrophage and human serum to the depressive effect of that serum upon the phagocytosis of human red cells, indicates that these effects are probably related to a direct action upon the macrophage by one or more serum proteins. The effects of serum fractions, and of serum proteins upon the reaction between the mouse macrophage and the human red cell were therefore studied.

a. Fractionation of serum.

Human serum was obtained from several sources, pooled serum from blood donors of group O, and individual sera from a donor of blood group B. The groups were known in order to facilitate the selection of red cell donors compatible with the sera used as the starting material for fractionation.

The sera were centrifuged at high speed (20,000 g.) for 2 hours to remove any cell debris. They were then equilibrated by dialysis against the buffer used in the fractionation process, for 24 hours.

Serum was fractionated by gel filtration using "Sephadex" G 200 (Pharmacia). Two kinds of fractionation were employed. For the first pilot experiments small quantities (5 ml.) of serum were separated on an analytical column 100 cm. in length. The buffer employed was the Borax/phosphate buffer of Kolthoff, with the addition of 0.5 molar sodium chloride. The pH at which the buffer was employed/

employed was 8.6 (See Documentia Geigy pp. 314). The flow rate through the column was maintained at 0.2 ml. minute. The eluate was divided into ten fractions. Each fraction was collected, and dialysed for 48 hours against running tap water, and then for a further 24 hours against phosphate buffered saline at a pH of 7.4, containing 0.45 molar sodium chloride. Each fraction was freeze dried, and reconstituted to its original volume by the addition of 5 ml. of glass distilled water. The fractions were then sterilised by filtration, and stored at 4°C., until required.

For large scale preparations, human serum was fractionated in 60 ml. quantities on a K 100/100 column, containing "Sephadex" G 200 gel. The buffer employed was a 0.1 Molar phosphate buffer at pH of 8.3 containing 1% butanol as a bacteriostatic agent. The large scale fractions were prepared by Dr. Smith, South East Region Blood Transfusion Service. Large scale fractionation of pooled group O sera and of a single serum of blood group B was performed. Thirteen fractions were prepared from each batch of serum. The fractions were freeze dried, and reconstituted to their original volume with distilled water. The dialysis step was omitted from the preparation of these fractions. After reconstitution, the fractions were sterilised by filtration, and stored in 5 ml. quantities at 4°C.

Mouse serum.

Mouse serum was obtained from mice of Tucks TO strain, by bleeding from the retroorbital plexus. The pooled serum was fractionated in 2 ml. quantities on "Sephadex" G 200 gel. Ten fractions were prepared, sterilised and stored after reconstitution in the manner outlined above./

above.

b. The protein content of the serum fractions.

The protein content of each fraction of both mouse and human serum was assessed from the elution curve, by gel diffusion using specific antisera, by immunoelectrophoresis and cellulose acetate electrophoresis.
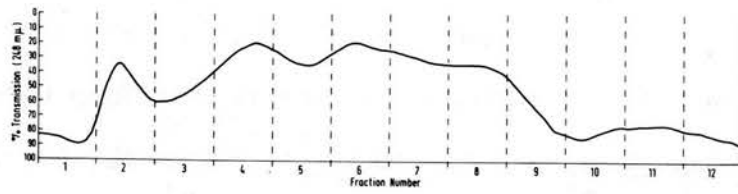
Antisera.

Specific antisera against human serum proteins were obtained commercially. (Behringwerke). These antisera were anti-prealbumin, antialbumin, antitransferrin, anti-alpha-lipoprotein, anti-beta-lipoprotein, anti IgA, anti IgG, and anti IgM, and anti alpha 2 macro-globulin. Horse antiGc serum was also employed. Whole anti-human serum from the horse (Supplied by Institute Pasteur) and from the rabbit were used in electrophoresis.

Whole rabbit anti-mouse serum was prepared by the injection of two rabbits with 0.2 ml. quantities of mouse serum on each alternate day for a period of six weeks. The rabbits were then bled out, and the separated sera pooled after sterilisation. These antisera were used in immunoelectrophoresis only.

Results.

From the elution diagram on sephadex G 200, three major and one minor peak were obtained, with human serum. Mouse serum showed three peaks only. The fractions collected from both mouse and human serum after fractionation on the analytical column were selected to cover similar/

ELUTION CURVE FOR 50 ml Gp B SERUM ON G 200 SEPHADEX IN 0.01 MOLAR PHOSPHATE BUFFER

PROTEIN CONTENT OF HUMAN SERUM FRACTIONS 1 to 12

CELLULOSE ACETATE ELECTROPHORESIS	IMMUNO ELECTROPHORESIS USING MONOVALENT AND POLYVALENT ANTISERA	GEL DIFFUSION USING MONOVALENT AND POLYVALENT ANTISERA	ULTRACENTRIFUGATION (S20w VALVES)	FRACTION NUMBER
β_2 GLOBULIN, TRACE α_2 GLOBULIN	γ M GLOBULIN, α_2 MACROGLOBULIN, β LIPOPROTEIN	γ M GLOBULIN α_2 MACROGLOBULIN β LIPOPROTEIN	S 19.30	1
β_2 GLOBULIN	γ M GLOBULIN α_2 MACROGLOBULIN	γ M GLOBULIN α_2 MACROGLOBULIN. TRACE OF LIPOPROTEIN	19.30	2
TRACE OF γ GLOBULIN, β_2 GLOBULIN	TRACES OF γ A GLOBULIN AND γ M GLOBULIN	γ A GLOBULIN. TRACE OF γ G GLOBULIN AND α_2 MACROGLOBULIN	—	3
γ GLOBULIN, β_2 GLOBULIN (TRACE)	γ A GLOBULIN γ G GLOBULIN TRACE OF TRANSFERRIN	γ A GLOBULIN, γ G GLOBULIN. TRACE α LIPOPROTEIN, TRANSFERRIN, AND GC GLOBULIN	7.046	4
α_1 GLOBULIN, γ GLOBULIN, α_2 GLOBULIN (TRACE)	γ G GLOBULIN α LIPOPROTEIN TRACE OF TRANSFERRIN	α LIPOPROTEIN, γ G GLOBULIN, γ A GLOBULIN. TRACE OF GC COMPONENT AND TRANSFERRIN	7.14 and 5.54	5
α_2 GLOBULIN (TRACE), α_1 GLOBULIN (TRACE)	TRANSFERRIN TRACE GC GLOBULIN α_1 LIPOPROTEIN	TRANSFERRIN. TRACE OF γ A GLOBULIN, GC GLOBULIN, AND HAPTOGLOBULIN. α_1 LIPOPROTEIN	5.5	6
α_1 GLOBULIN, α_2 GLOBULIN	GC GLOBULIN α_1 LIPOPROTEIN. TRANSFERRIN. HAPTOGLOBULIN.	GC GLOBULIN. TRACE α LIPOPROTEIN. HAPTOGLOBULIN. TRANSFERRIN.	—	7
α_1 GLOBULIN, α_2 GLOBULIN, β GLOBULIN	PREALBUMIN I. TRANSFERRIN HAPTOGLOBULIN WITH POLYVALENT ANTISERUM	ALBUMIN, GC GLOBULIN, TRACE OF PREALBUMIN I	4.5	8
ALBUMIN, α GLOBULIN, β GLOBULIN	PREALBUMIN I TRACES OF ALBUMIN	ALBUMIN PREALBUMIN I	4.5	9
PREALBUMIN, α_1 GLOBULIN, ALBUMIN, β GLOBULIN	ALBUMIN PREALBUMINS I AND II (ACIDIC α_1 GLYCOPROTEIN)	ALBUMIN PREALBUMIN II (ACIDIC α_1 GLYCO-PROTEIN)	4.2	10
α_1 GLOBULIN, α_2 GLOBULIN, PREALBUMIN (TRACE)	PREALBUMIN II α_1 GLOBULIN (?α_1 ANTITRYPSIN)	ALBUMIN (TRACE) PREALBUMIN II PREALBUMIN I	—	11
TRACES α_1 GLOBULIN	PREALBUMIN II α_1 GLOBULIN (TRACE)	PREALBUMIN II	3.3	12

similar regions of the elution curve. Each fraction should therefore contain serum proteins of approximately the same molecular weight from both mouse and human serum. The fractions collected from human serum during large scale fractionation, were fairly well matched with the analytical fractions. Thirteen fractions were taken using a guide region of the elution curve covered by the ten fractions of human serum prepared on the analytical column. The three major peaks obtained from both mouse and human serum corresponded to the macroglobulin, gammaglobulin, and albumin fractions of these sera. All the proteins occurring in each peak are of approximately the same molecular weight. The minor peak occurring towards the end of the elution curve in the large scale preparations, was found to contain dialysable polypeptides.

The protein content of each serum fraction of the human serum samples is shown in the accompanying table.

c. Effect of human serum fractions upon the phagocytosis
of human red cells by the mouse macrophage.

Mouse macrophages were cultured in serum protein free medium 10% in medium 199. After 2 hours in culture, 0.1 ml. of each serum fraction was added to each monolayer. The cultures were then incubated for a further 24 hours. Fresh human red cells were then added to the cultures, and incubation continued for a further two hours. The monolayers were then washed, fixed and stained in the usual manner. The phagocytic index was determined for each serum sample, and for each fraction.

Results./

Results.

TABLE XVII.

THE DEPRESSIVE EFFECT OF HUMAN SERUM FRACTIONS UPON THE PHAGOCYTOSIS
OF FRESH HUMAN RED CELLS BY MOUSE MACROPHAGES.

Fraction Number	Phagocytic Index	
	Pooled Serum	Group B Serum
1	42	40
2	32.5	31%
3	22.7	25%
4	12	5
5	14%	12%
6	31	31.5
7	36	34
8	50	43.75
9	54	54.75
10	48	45.75
11	41.5	41.25
12	42	42
13	48	54
Controls	5.5%	3.0%
Lactalbumin Control	64.5%	64.5%

As these results demonstrate, the main depressive effect occurred
with the early fractions four and five. These depressed the phago-
cytosis of human red cells to nearly the same extent as whole serum.
The depression observed with pooled serum was less impressive than
that obtained with group B serum. The results are the average results
of four determinations.

d./

d. The effect of mouse serum fractions upon the phagocytosis of fresh human red cells by the mouse macrophage.

Mouse serum fractions were prepared as outlined previously. Macrophages were grown in serum protein free medium containing 10% of the serum fraction to be studied. After 24 hours incubation, 0.1 ml. of a 5% suspension of fresh human red cells were added to each culture. Incubation was continued for 2 hours, and the monolayers washed, fixed and stained as before. Seven of the initial ten fractions prepared from mouse serum were employed in this test. The three fractions ommitted were those occurring towards the end of the elution curve. Experience with human serum had shown that the active fraction was unlikely to be located in these terminal fractions.

Results.

TABLE XVIII.

THE DEPRESSIVE EFFECT OF MOUSE SERUM FRACTIONS UPON THE PHAGOCYTOSIS OF FRESH HUMAN RED CELLS BY MOUSE MACROPHAGES.

Fraction Number	Phagocytic Index in mouse Serum.
1	24%
2	21%
3	15%
4	5%
5	32%
6	38%
7	35%
Control in mouse serum	2.1%
Control in lactalbumin	42%

It was therefore demonstrated that the active material in serum, causing a depression of the reactivity of the mouse macrophage towards human cells, was contained in similar fractions in both mouse and human sera. Other serum fractions also produced some depression of the phagocytic response, in particular fractions, 2,3,5 and 6 of human serum, and fractions 1,2,3 of mouse serum. Thus the total depressive effect observed with whole serum could be due in part to the contribution of serum fractions other than the most active ones. It is also possible that the fractionation procedure itself disturbed the activity of whole serum in other ways. In order to prove that the active fractions were responsible for the depression of phago-cytosis, the kinetics of the reaction between the serum fractions and the macrophage were re-explored.

e. The kinetics of the reaction between the serum fractions and the mouse macrophage human red cell system.

From the studies with whole serum, the depression observed takes four to six hours to develop with human serum, and 8 hours to develop with mouse serum. If the active fractions, that is fractions 4 and 5 of human serum and fraction 4 of mouse serum, require similar time intervals for the production of this effect, it is likely that the activity of whole serum is the result of the activity of the active fraction alone.

Mouse macrophage monolayers were prepared in serum protein free medium, and incubated overnight at $37^{\circ}C$. (i.e. for 18 hours). Human and mouse serum fractions were added in 0.1 ml. quantities to each monolayer.

monolayer. Incubation was continued for four hours in those mono-
layers exposed to human serum, and for four hours and six hours in
those monolayers receiving the mouse serum fractions. After these
times of incubation, 0.1 ml. of a suspension of fresh washed human
red cells was added to each culture. The phagocytic reaction was
assessed after a further two hours incubation in the usual way.

Results.

TABLE XIX.

THE VARIATION WITH TIME OF THE DEPRESSIVE EFFECTS OF MOUSE AND HUMAN
SERUM FRACTIONS UPON THE PHAGOCYTOSIS OF FRESH HUMAN RED CELLS BY
MOUSE MACROPHAGES.

Control monolayer mouse serum	4 hours	6 hours
Phagocytic Index	28%	8%
Control monolayer in human serum at 4 hours		5%

(The times given refer to the time of incubation of the macrophages
with the serum fraction PRIOR to the addition of the human red cell.
The total time of contact between the macrophage and serum fraction
is therefore the stated time + 2 hours).

TABLE XIX./

TABLE XIX.

THE VARIATION WITH TIME OF THE DEPRESSIVE EFFECTS OF MOUSE AND HUMAN
SERUM FRACTIONS UPON THE PHAGOCYTOSIS OF FRESH HUMAN RED CELLS BY
MOUSE MACROPHAGES.

For mouse serum.

Time in contact	4 hours	6 hours
Fraction 1	32%	23%
2	30%	21%
3	26%	18%
4	22%	8%
5	41%	34%
6	40%	38%
7	36%	36%
Control in lactalbumin	42%	40%

TABLE XIX./

TABLE XIX.

THE VARIATION WITH TIME OF THE DEPRESSIVE EFFECTS OF MOUSE AND HUMAN
SERUM FRACTIONS UPON THE PHAGOCYTOSIS OF FRESH HUMAN RED CELLS BY
MOUSE MACROPHAGES.

Fraction Number	Mouse Serum		Human Group O serum	Human Group B serum
	4 hrs.	6 hrs.	4 hrs.	4 hrs.
1	32%	23%	-	40%
2	30%	21%	33%	41%
3	26%	18%	18%	25%
4	22%	8%	8%	6%
5	41%	34%	18%	11%
6	40%	38%	42%	18%
7	36%	36%	50%	26%
8	-	-	45%	32%
9	-	-	54%	56%
10	-	-	50%	52%
11	-	-	40%	53%
12	-	-	-	52%
Control monolayer in mouse serum.	28%	8%	-	-
Control monolayer in human serum.	-	-	5.5%	3.5%
Control monolayer lactalbumin.	42%	46%	62%	62%

The control values for 4 hours incubation in both whole human

serum samples were:

TABLE XIX.	
Pooled group O	5.5%
Group B serum	3.5%

The control values for macrophages incubated for the same total

time/

time in medium 199 + lactalbumin alone, that is for 24 hours + 4 hours + 2 hours, were 64.5 for the average of two determinations.

These experiments confirm that the depressive effect of serum upon the phagocytosis of human red cells by mouse macrophages is contained in the serum fractions 4 and 5 for human serum, and fraction 4 of mouse serum. The major protein components of each of the active fractions are the two immunoglobulins IgG and IgA. In human serum fraction 4, which is the most active, ultracentrifugational analysis revealed a single major peak, with an S_{20w} value of 7.03. Immuno-electrophoresis and gel diffusion showed only slight traces of IgA, and no other major serum components in this fraction. The apoprotein of alpha I lipoprotein, and a Gc reactive component occur. It was therefore assumed that the serum fractions, from both mouse and human sera, which cause a depression of the natural reactivity of the mouse macrophage towards the human red cell, do so by virtue of the immunoglobulin they contain.

8 - SUMMARY OF RESULTS.

The mouse macrophage cultured in serum protein free medium shows phagocytic reactivity towards the fresh human red cell (Average value phagocytic index 37.4%).

Mouse macrophages cultured in human or mouse serum show no phagocytic reactivity towards the human red cell (average phagocytic index in human serum 4.5% in mouse serum 9.9%).

Fresh mouse red cells are not taken up in serum protein free medium, in mouse serum, or in human serum. (Average phagocytic values, in serum protein free medium 1.2%, in mouse serum, 1.10%, in human serum 6.6%).

Serum from either the mouse or man will depress the phagocytic reaction of the mouse macrophage towards the human red cell, and these sera have no effect upon the reaction of the mouse macrophage with the mouse red cell. The depressive effect of both mouse and human serum upon the phagocytic response towards the human red cell, is contained in the immunoglobulin containing fractions of these sera.

The depressive effect of serum is attributed to a direct action upon the macrophage itself. The depression occurs only after 6 hours incubation in human serum, or after 8 hours incubation in mouse serum. Depression is concentration dependent, exhibiting a threshold effect between absolute concentration of serum of 1% and 0.5% in medium 199. Assuming an immunoglobulin concentration in serum 1 g. per 100 ml., the threshold effect occurs between concentrations of immunoglobulin of 10 mg./ml., and 5 mg./ml.

9 - DISCUSSION.

The demonstration of the reactivity of the mouse macrophage towards the human red cell in the absence of serum is a new finding. The observation that mouse and human immunoglobulins can inhibit this reactivity has also never been previously described.

Rabinovitch (1967) has described the attachment of the red cells treated with glutaraldehyde to the surfaces of mouse macrophages. Mouse, horse, rabbit, and human cells were used, and the reaction occurred in the absence of serum. Attachment of unmodified erythrocytes did not occur. The same author has also described the ingestion of altered red cells attached to macrophages, but only in the presence of antibody. The media used in these two sets of experiments were essentially buffered saline solutions. The results of Rabinovitch indicate some reactivity on the part of the macrophage in the absence of serum. The experiments reported have been all conducted within a few hours of obtaining the peritoneal macrophages. From the demonstration (see section 6, b), that the mouse macrophage remained in a state of phagocytic depression for at least 6 hours after removal from the peritoneal cavity, it is not surprising that Rabinovitch did not observe any phagocytic activity towards fresh human red cells.

Lee & Cooper (1966) demonstrated that the adherence of fresh heterologous red cells to the mouse macrophage surface required the presence of serum. In their system macrophage monolayers were employed after 30 minutes in culture. The ingestion of fresh heterologous/

heterologous red cells did not occur, again because of the short time the macrophages were retained in culture before the addition of the particle. The same authors demonstrated the attachment of effete heterologous erythrocytes to mouse macrophages in the absence of serum, and showed that divalent cations were essential for this reaction to occur.

Vaughan & Boyden (1964) Vaughan (1965 a, b), reported that the ingestion of effete homologous red cells and guinea pig macrophages was independent of serum opsonins. The culture medium us ed however contained serum, and therefore a reaction between fresh heterologous cells and macrophages would be unlikely to occur. The medium surrounding the macrophage has been regarded as important in phagocytosis, especially in causing the attachment of the particle to the phagocyte (Lee & Cooper 1966) or providing antibody, or complement which are thought to stimulate the phagocytic reaction. (Spiegelberg et al 1963; Benecarraf & Miescher 1960; Carpenter 1966). A depressive effect of serum upon macrophage reactivity has not previously been considered. Huber, Douglas, and Fudenberg (1969) have demonstrated the adherence of antibody globulin-coated red cells to the surfaces of human and guinea pig macrophages. Essentially these observations are similar to these of Rabinovitch (1966, 1967., 1967 b). One other feature reported was the inhibition of the adherence of antibody-coated cells to peritoneal macrophages by the presence of free immunoglobulin. Splenic macrophages were not influenced by the presence of free immunoglobulin to the same extent. The numbers of antibody molecules bound to the red cell surfaces were found/

found to average about 700 per red cell in order to cause adherence.
In the presence of free immunoglobulin, the numbers of molecules
necessary to cause adherence was increased five or six fold.

It is doubtful whether the adherence of red cells to macro-
phages, as described by Rabinovitch and others, is a necessary
precursor to the phagocytic act. In the experiments reported here,
adherence was not uncommonly found, in particular between macrophages
grown in serum, and fresh human red cells. This adherence was most
frequently due to the careless preparation of red cells, either by
collecting red cells into inadequate anticoagulant, or by the misuse
of alcohol when collecting blood from a finger prick.

As a rule, even extensive adherence of human red cells to macro-
phage monolayers in the presence of serum did not result in an increased
phagocytic index. Paradoxically, in experiments in serum protein
free medium, where the phagocytic index was often high, adherence
was rarely seen. A similar occurrence has been reported by Stuart,
Davidson & Cummings (1967), to result from the sensitisation of human
red cells with naturally occurring isoagglutinins directed against the
Landsteiner antigens. These antibodies would cause adherence of fresh
human red cells to the monolayer of macrophages, but did not increase
the phagocytic index. The results of Huber, Douglas and Fudenberg,
although of great interest, are therefore difficult to relate directly
to the depressive effects of immunoglobulin upon phagocytosis reported
here.

Three important features of the reaction between the immuno-
globulin-containing fractions of serum, and the mouse macrophage/human
red/

red cell system were a) the time required for the macrophage to reveal its full phagocytic potential in serum protein free medium,

b) the time required for the depressive effect of the immunoglobulin containing fractions to become established, and

c) sharp cut off at $<$ 1% concentrations of serum.

These features indicate that the reaction between the macrophage and the active principle in serum is not one of passive adsorption. The effect is also unlikely to be produced simply by modification of the medium. The most acceptable explanation is that the presence of immunoglobulin modifies the macrophage membrane by causing an alteration in its structure. The half life of the cell membrane of the amoeba has been estimated at about 6 hours. (O'Neill, 1964; Wolpert & O'Neill, 1962; Wolpert, Thompson & O'Neill 1964). Some data suggest similar turnover rates for mammalian cell membranes, based upon the half life of radiotracer material in the membrane, and also from observations of the surfaces of living cells.

If these data can be held to be valid for the mouse macrophage, the effect of the immunoglobulin fractions upon the phagocytic potential of the mouse macrophage can be explained by the actual incorporation of antibody molecules into cell membrane material. In serum protein free medium, the initial reactivity of the macrophage as it left the peritoneal sac, would become altered as membrane was resynthesised in the absence of mouse immunoglobulin. After six hours in culture, it would have lost the depressive effects of the mouse serum proteins carried with it into culture. This loss is interpreted/

interpreted in the experimental system used here an increased reactivity towards the human red cell. If human serum or mouse serum is added to the culture, the newly synthesised membrane will incorporate immunoglobulin molecules into its structure once again. The result is interpreted as a depression of macrophage reactivity towards the human red cell.

The effect of the medium upon the process of phagocytosis, can therefore be interpreted as an effect upon the membranes of the macrophage. The active macromolecules participating in this effect fulfill the requirements of the "ligand" substances proposed theoretically in the introduction. For the interaction of the mouse macrophage and the human red cell in this system, neither opsonic substances, nor complement are required. The most important criteria for the investigation of the role of the medium in phagocytosis are:

1. a serum protein free medium.

2. healthy macrophage cultures, which are both morphologically and functionally satisfactory.

CHAPTER 4

THE MACROPHAGE

CONTENTS.

4./

4. RECOGNITION.

    a. The preparation of antisera and the sensitivity of the technique used.

    b. The reaction of the mouse macrophage towards sensitised human cells in serum containing and serum free media.

    c. The variation of the reaction towards sensitised red cells with time in culture.

1 - INTRODUCTION.

The methods of measurement employed in these experiments produce an index of macrophage reactivity. The phagocytic index for any medium/particle combination illustrates the reactivity of macrophages in that culture towards the test particle. The major results obtained indicate that macrophages appear to have the capacity for three types of reaction, and that these classes of reactivity are distinct. The following definitions have been applied to the classes of reactivity demonstrated in subsequent experiments. These are:

a. Heterogene Discrimination: that is the ability of the macrophage to discriminate between cells from a genetically different source.

b. Homogene Discrimination: that is the ability to discriminate between cells from the same genetic source which have become altered or damaged by some pathological process.

c. Recognition: that is the ability of the macrophage to react to particles coated with specific antibody. Such particles, having already elicited an antibody response, are therefore "recognised" or "known" again" by the macrophage.

The general term "discrimination", used without the prefixes of Homogene or Heterogene, applies to the capacity of the macrophage to react to particles such as carbon or silica which are inorganic, and yet to which a macrophage exhibits differential phagocytic activity. These terms represent a method of classifying the types of reaction observed in macrophage cultures, and cannot be held to apply to real events/

events outside the experimental situation.

a. Heterogene discrimination.

This term is held to signify a reaction on the part of the macrophage to the presence of cells from a genetically different source.

The macrophage itself can be shown to react to "foreign" material. Addison (1920) injected pigeon red cells into a rabbit, and then perfused the spleen and examined it histologically. Large numbers of pigeon red cells were found in the splenic phagocytes. Isologous neutrophils and myelocytes were also found to be phago-cytosed during the reaction to the pigeon red cells. These red cells rapidly disappeared from the circulation, and none could be detected 12 hours after injection.

Halpern, Biozzi, Benacerraf, & Stiffel (1957) accurately quantitated the rates of clearance of cirulating pigeon red cells in the mouse and rat. Clearance was complete within 40 to 60 minutes of injection. In the absence of heparin the injected red cells rapidly clumpsed, and the clearance rate increased. The clearance of pigeon red cells from the circulation of the mouse was not dependent upon the presence of naturally occurring antibody, since none could be demonstrated. The pigeon red cells were removed mainly by the phagocytes of the spleen and the liver.

Perkins & Leonard (1963) studied the in vitro response of mouse macrophages to erythrocytes from the chicken, sheep, rabbit, guinea pig, hampster, rat and mouse. Natural antibodies were carefully excluded/

excluded from system by prior adsorption of the serum used in culture, with the test particle. Low phagocytic indices were obtained. The macrophages showed their greatest reactivity towards the red cells of species genetically far removed from the mouse. The phagocytic index was greater in the presence of specific antiserum than it was in the presence of adsorbed serum. Unadsorbed serum also provoked a slight increase in macrophage reactivity towards all the red cells studied.

Vaughan (1965 a) described the reaction of the rabbit macrophage towards a variety of foreign mammalian red cells, both in the presence and absence of serum. Uptake appeared to be opsonin dependent when serum was present, but in the absence of serum some "heterogene discrimination" still occurred. Little phagocytic activity was observed in the absence of serum, and the experiments were poorly controlled, no reference being made to the reactivity of the rabbit macrophage towards fresh rabbit cells. The opsonic factors characterised by this system all appeared in the gamma-globulin fraction of serum. Stuart & Cummings (1968) failed to show any significant reactivity of the mouse macrophage towards human cells in the presence of human serum. Experimental data presented earlier in this thesis confirm this observation.

North (1966) demonstrated phagocytosis of sheep red cells by guinea pig macrophages in a medium containing guinea pig serum.

Macrophages from invertebrate sources show a capacity for the ingestion of heterologous cells. Stuart (1968) demonstrated that the phagocytic blood corpuscles of the octopus, Eledone Cirrosa, would ingest/

ingest human red cells in the presence of octopus serum.

Cameron (1932) demonstrated the capacity of the coelomic
corpuscles of the earthworm to ingest human red cells injected into
the coelomic cavity. The entire reaction took about 48 hours.
Cameron also studied the uptake of mammalian sperm cells by the
coelomic corpuscles of the earthworm. Spermatozoa from rats and
mice were quite rapidly ingested, while spermatozoa from other species
of worm persisted longer in the coelomic cavity. Neither mammalian
spermatozoa, or spermatozoa from unrelated worms persisted for as
long as spermatozoa from worms of the same species as the experimental
animal. This implies that the macrophage-like cells of invertebrate
species are as good as mammalian macrophages in reacting to foreign
cells, despite the assistance the mammalian cell gains from the
complicated opsonic and natural antibody systems.

These data indicate that the macrophage from a wide variety of
species is capable of heterogene discrimination in the presence of
its own serum or body fluids. Furthermore my own observations have
shown that this reaction can occur in the absence of serum factors.

b. Homogene Discrimination.

A survey of the literature reveals that the macrophage appears
to be capable to a second type of reactivity which differs from that
described under the heading Heterogene discrimination. The term
homogene discrimination is here used to define the capacity of the
macrophage to detect differences between similar particles, and to
respond to those differences by the selective phagocytosis of one
particle/

particle only. The general term "discrimination" was used in this manner by Fenn (1921; 1923) to describe the preferential phagocytosis of carbon particles to quartz particles by the rat macrophage. In a more detailed experiment (1923) Fenn showed that this discriminatory ability applied to such similar particles as manganese dioxide and manganese silicate. Both these particles were phagocytosed more readily than quartz, but the macrophage phagocytosed more manganese dioxide than manganese silicate.

Homogene discrimination was demonstrated by Fauve (1964) who showed that mouse macrophages cultured in the absence of serum could discriminate between rough and smooth forms of Salmonella Typhimurium.

The peripheral blood leukocytes of man exhibit homogene discrimination between periodate-treated red cells, and periodate treated red cells with influenza virus adsorbed to them. (Jerushalmy et al 1957). Antibody reacting against periodated and virus coated cells was excluded from the system by pre-testing sera and adsorbing if necessary.

Rabinovitch (1967 a) showed that erythrocytes treated with glutaraldehyde tannic acid, periodate, polylysine, and colloidal silica would attach to macrophages in the absence of serum, while untreated red cells did not. Several different species of red cell donors were used in this experiment, and the results do not necessarily indicate "homogene discrimination" by the macrophage. In a subsequent experiment, Rabinovitch (1967 b) showed that pre-treatment of the glutaraldehyde treated red cells with specific antibody induced phagocytosis in the absence of serum. Rabinovitch (1969) reported that/

that glutaraldehyde treated horse red cells were phagocytosed by peritoneal macrophages, from the mouse. Partial digestion of the glutaraldehyde treated red cell with a variety of enzymes will also induce uptake, while coating the cells with various proteins reduced phagocytosis. Rabinovitch regards this process as an example of "recognition" (i.e. heterogene discrimination) reaction to a foreign red cell by the macrophage, after treatment of the cell to remove a coating of "anti-phagocytic" substance from its surface. The fact that fresh horse red cells in this system were not taken up leads to the conclusion that this experiment described homogene discrimination by the macrophage between normal and damaged horse red cells.

Stuart & Cummings (1968) commented upon the abilities of the mouse macrophage to distinguish between fresh and injured human red cells. Stuart, Clark, Boulton, & Collee (1969) produced electron microscopic evidence that the mouse histiocyte occurring in micro-granulomata in liver during mouse typhoid, could distinguish between the intact and damaged hepatic parenchyma cells, and between healthy and damaged cytoplasm and mitochondria from the same cell.

The phagocyte can therefore be held to discriminate between different types of inorganic particle (Fenn, 1920, 1922), between different strains of the same bacterium, (Fauve 1964), between fresh and injured red cells (Stuart & Cummings 1968), and perhaps even between injured and healthy cell organelles (Stuart et al 1969(. Most authors regard discriminatory processes as being independent of opsonins or antibody, or in some cases even of serum. (Vaughan 1965; Jerushalmy et al 1957, Fauve 1964).

c./

c. Recognition.

The most spectacular expression of macrophage reactivity is
towards complexes of antigen and antibody. All the experimental data
available report strongly positive phagocytic reactions by macrophages
towards particles coated with antibody, despite the variability in
techniques employed. Recognition is here defined as the ability of
the macrophage to ingest any particle coated with antibody globulin
directed against an antigenic group or groups present on that particle.
Hektoen (1906) described the opsonic power of substances appearing
in the blood following the injection of foreign red cells. Rabbits
were immunised to the red cells of goats, and to bovine red cells,
goats were immunised with sheep red cells, guinea pigs were immunised
to rabbit red cells. Phagocytes from the peripheral blood of the
dog, the guinea pig, the goat, and from human sources were employed.
He demonstrated that the opsonins occurring after immunisation could
be adsorbed out of immune serum by the cells used to prepare it,
and that in some cases immune sera gave rise to a non-specific opsonic
effect. Thus immune rabbit serum produced against goat red cells
also opsonised the red cells of the sheep, dog, rabbit, chicken,
pigeon, guinea pig, and man. In this experiment the leukocytes were
derived from the dog, but when leukocytes from the guinea pig were
employed, the opsonic activity extended only towards the red cells of
the goat and the sheep. In general it was found that immune serum
opsonised red cells for various leukocytes, including the homologous
ones, but the phagocytic activity of the different leukocytes towards
opsonised erythrocytes was shown to vary. Lee, Zandrew, Gernand,
and/

and Davidsohn (1968) in contrast, found that antisera against the erythrocyⁱes of one species gave negative results with the red cells of other species. They also showed the extreme sensitivity of the erythrophagocytosis test when compared with agglutination and lysis tests. It was found to be at least five times more sensitive.

A similar effect was found by Stuart, Davidson, & Cummings (1967) in a system employing the mouse macrophage grown in human serum.

Hess & Luscher (1968) demonstrated the uptake of sheep red cells by mouse macrophages in the absence of serum, but only after the sheep cells had been sensitised with antiserum raised in mice. Fractionation of the antibody into macroglobulin and gammaglobulin fractions revealed the two types of antibody to possess equal powers of opsonisation on a weight for weight basis. (Burton & Mollison 1968). Haemolytic antibodies are generally held to be more potent than agglutinating antibodies in producing phagocytosis. (Hektoen 1906; Vitale et al 1967). Fragments of antibody prepared by papain digestion, although still capable of agglutinating red cells no longer had any opsonising power in vivo. (Vitale et al 1967).

Rabinovitch (1967) demonstrated that the uptake of glutaralde-hyde treated red cells would occur in the absence of serum if these particles had been treated with antibody prior to their ingestion.

In a study of erythrophagocytosis occurring in serous effusions, it was reported that the naturally occurring isoagglutins directed against Landsteiner antigens provoked an intense phagocytic response, (Harmont et al 1958). Phagocytosis was preceded by the attachment of/

of red cells in rosettes around the macrophages. Human material was used throughout in this experiment. The observations of Stuart, (1969) in contrast, show that although the isoagglutinins provoked adherence of incompatible red cells to monolayers of human macrophages, phagocytosis was not increased by these substances.

In vivo studies of the accelerated destruction of red cells sensitised with haemolytic antibodies in general support the findings of in vitro experiments. Swisher & Young (1954) showed accelerated destruction of A1 red cells sensitised with canine anti-A antibody in the dog. This antibody is a potent haemolsin, similar in many respects to the human immune anti-A. A2 erythrocytes, which are agglutinated but not lysed by anti-A antibody, were also cleared at an accelerated rate in the dog, but not as quickly as A1 red cells. Canine anti-B, C, & D, antibodies agglutinate incompatible cells strongly, but do not lyse them. Group C erythrocytes survived for 120 days in the presence of antibody capable of producing firm agglutination. In man, it has been shown that A.B.O. incompatible cells are rapidly sequestered and destroyed in the liver, in the absence of lytic antibody. (Jandl et al 1957). Cells sensitised with lytic antibody are as rapidly destroyed, but primarily in the spleen. This may indicate a difference of reactivity between the hepatic and splenic macrophage towards red cells sensitised with agglutinating and lytic antibodies.

The "primitive" phagocytic cells of invertebrate species are as capable as the mammalian macrophage in their abilities of discriminate in respect of foreign red cells and other particles, and between healthy/

healthy and damaged tissue. (Salt 1956). They appear incapable of phagocytosing particles coated with gammaglobulin. The phagocytes of the octopus will not phagocytose human red cells sensitised with haemolytic antibody in the absence of octopus serum (Stuart 1968). In the presence of octopus serum there is no difference in the uptake of fresh red cells and red cells sensitised with haemolytic antibody. This is not surprising as gammaglobulin molecules are not secreted by this animal, or by any other invertebrate.

The macrophage appears to be capable of three classes of activity. These have been defined as follows:

DISCRIMINATION: in general terms it applies to the preferential phagocytosis of one class of inorganic particulate material in the presence of another. When applied to reactions between cells, Discrimination occurs in two forms:-

a. Homogene discrimination between cells of the same genetic class which differ only in their structural or biochemical integrity.

b. Heterogene discrimination between cells of different genetic class, which are structurally and biochemically whole.

RECOGNITION: this term describes the phagocytic activity expressed towards a particle coated with specific antibody. Since a similar particle must have previously given rise to that antibody, it is considered that the phagocytic reaction represents the "knowing again" of that particle in biological terms.

2 - THE REACTION OF MOUSE MACROPHAGES TOWARDS THE RED CELLS OF VARIOUS
SPECIES: "HETEROGENE DISCRIMINATION".

Mouse macrophages have been demonstrated to show sharp
differences of reactivity towards fresh human red cells in serum
containing and serum free media.   It is stated that this represents
the process of heterogene discrimination.   The reaction of the mouse
macrophage to the healthy red cells of the mouse, rat, rabbit, guinea-
pig, sheep, goose, and man was therefore investigated in serum free
and serum containing media.

a.   The reaction of mouse macrophages towards "foreign"
     red cells in serum free and serum containing media.

Fresh red cells from all species were collected into heparinised
saline.   Red cells from the mouse were obtained by bleeding from the
retroorbital plexus.   Blood from the rabbit and guinea-pig was
obtained by bleeding from the marginal ear vine.   Blood from the
goose and sheep was collected by venepuncture.   (Kindly supplied
by the Animal Research Centre, Moredun).   Blood from the rat was
obtained by cardiac puncture, and human red cells were obtained
from a finger prick.   All samples of red cells were washed four times
in ten times their own volume of sterile saline containing 5 I.U.
heparin per ml.   They were then resuspended in saline at a con-
concentration of 5 by volume.   All red cells were freshly collected,
and all were used within 6 hours of collection.

Macrophage monolayers were grown for 24 hours in either medium
199 + 10% serum protein free medium supplement, or medium 199 + 10%
pooled/

pooled mouse serum.

Serum was obtained by bleeding mice from the retroorbital plexus into polypropylene tubes. From 20 to 30 mice were used for each pool. The serum was sterilised by filtration, and used after storage for 24 to 48 hours. Serum was also tested for its agglutinating and lytic reactivity towards the red cells used. Each sample of red cells was observed in dilutions of mouse serum up to 1/100 for agglutination or lysis. Red cells from the sheep and goose occasionally showed agglutination by neat mouse serum, but this was never demonstrated at titres of greater than 1 in 10; i.e. the concentration at which the serum was used in the experimental cultures. The test red cell suspensions were added to the monolayers in quantities of 0.1 ml. per monolayer. Incubation was continued for 2 hours at 37°C., the coverslips were washed, fixed and stained as before.

Results.

Three experiments were performed with separate samples of red cells for each. Red cells from the goose and the sheep were available only on two of the three occasions.

TABLE XX./

TABLE XX.

See Histogram.

Species	P in lactalbumin			P in mouse serum		
	a	b	c	a	b	c
mouse	2%	0.1%	0.2%	0.6%	0.3%	0.6%
rat	4%	4%	1%	5%	8%	1.5%
rabbit	6%	14%	7%	8%	3%	2%
guinea pig	7%	14%	18%	12%	15%	4%
sheep	-	18%	18%	-	1%	3%
man	40%	38%	35%	5%	5%	7%
goose	-	32%	20%	-	30%	36%

These results indicate that the reaction of the mouse macrophage towards foreign red cells is more complex than suggested by the experiments of Perkins & Leonard (1963). Uptake of cells from the rat rabbit, and guinea pig occurs to the same extent in serum as in serum free medium. The uptake of human and sheep red cells is depressed by serum, and the uptake of goose red cells is probably enhanced by serum possibly due to naturally occurring antibody. The general trend of the results in serum supports the conclusion reached by Perkins and Leonard (1963) that the further removed the species of donor is from the mouse, the greater the uptake. The two exceptions are red cells from the sheep and from man. The mouse serum employed in this experiment was not adsrobed with the test particle before use. In the experiment of Perkins & Leonard (1963) the uptake of sheep red cells in adsorbed mouse serum was given as 5.4%. Data for human red cells was not included.

Adherence/

Adherence was marked in monolayers to which the red cells of the goose and sheep had been added. The adherence of sheep red cells to mouse macrophages also occurred in serum protein free medium. (See footnote). This phenomenon has also been described by Nelson (1969). For some particles, notably with red cells from the rabbit and guinea pig, the range of phagocytic reactivity of the macrophage was large. This occurrence might be due to the selection of separate random bred donors. In the case of the rat and mouse, slight differences in the constitution of the red cell between individuals would almost certainly not be detected by these experiments. Similarly for species widely separated from the mouse, individual variations are likely to be less than the large genetic difference between the mouse and donor expressed by the red cell surface. Thus for these two classes of donor, individual variations are unlikely to be demonstrated in a small experimental series.

b. <u>The reaction of human macrophages towards the red cells of various species in serum containing and serum protein free media.</u>

To support the conclusions reached in experiments with mouse macrophages, which indicated heterogene discrimination ability, the experiments were repeated using monolayers of human macrophages. These/

(Footnote: Sheep red cells also appear to lyse very rapidly within the mouse macrophage, giving rise to the appearance of many vacuoles containing material which stains poorly with eosin. Adherent sheep red cells on the other hand, were usually intact).

These experiments were performed on only two occasions, using macrophages from patients undergoing peritoneal dialysis. (For preparative techniques see Stuart 1968). Although incomplete the results are of sufficient interest to merit their inclusion.

Monolayers of macrophages were 48 hours old when used. Red cells were obtained and prepared as before. Pooled group O human serum, and serum protein free medium supplement, both at concentrations of 10% in medium 199 were used as culture media. supplements.

Results.

TABLE XXI.

Red cells	Serum protein free	Serum
Mouse	16%	16%
Rat	16%	60%+
Rabbit	15%	18%
Guinea-pig	15%	14%
Human	0.8%	0.5%

Each result refers to one experiment only. Despite the odd datum obtained from the combination of rat red cells and pooled O serum, these results would seem to confirm the occurrence of some heterogene discrimination ability in the phagocytic cells of the human and mouse peritoneum. It also suggests the importance of the phylogenetic relationship between macrophage donor, and red cell donor, in determining the reactivity of the macrophage towards the test red cell./

cell. The human macrophage seems to react similarly to cells from a variety of rodents, and the depressive effect of serum in this experiment was not observed.

c. The kinetics of heterogene discrimination.

The reaction of the mouse macrophage towards the red cells of the rat, guinea pig and rabbit does not appear to be influenced by serum. The phagocytic index is too low for any effect to be detected. The reactivity towards sheep and human red cells is depressed by serum. The uptake of goose red cells is enhanced by serum. Thus three different kinds of activity may be expressed by the mouse macrophage, and these could be classified as serum depressed, serum independent, and serum enhanced processes.

In order to investigate these three apparently distinct forms of heterogene discrimination reactivity by the mouse macrophage, the response of the macrophage to "foreign" red cells was studied after prolonged culture in serum free and serum containing media.

Methods.

Mouse macrophages were cultured as before in serum free medium supplemented with lactalbumin, and medium 199 with 10% mouse serum. The cultures were studied at 24 hours and again at 3 days.

Red cells.

Red cells were obtained and prepared as 5% suspension in saline as before. They were added to the cultures in quantities of 0.1 ml. of suspension per culture. Incubation was continued for 2 hours at 37°C./

Histogram of the phagocytic response of mouse macrophages towards Foreign Red Cells in the serum containing and serum Free media after 24 and 27 Hours in culture.

■ Macrophages in serum free media.

▨ Macrophages in 10 % serum containing media.

▦ % adherence of red cells
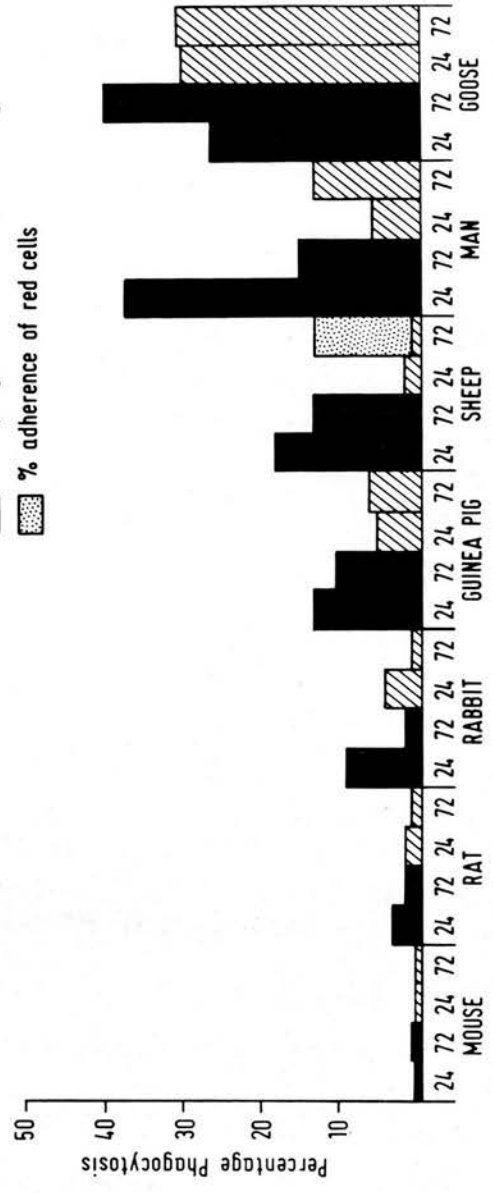
37°C., and the monolayers washed, fixed and stained as before.

Results - see histogram.

TABLE XXII.

A COMPARISON OF THE EFFECTS OF PROLONGED CULTURE OF MOUSE MACRO-
PHAGES UPON THEIR ABILITY TO PHAGOCYTOSE A VARIETY OF FOREIGN RED
CELLS IN SERUM FREE AND SERUM CONTAINING MEDIA.

Species of red cell donor.	Phagocytic Index in Serum Free Medium At 24 hours/at 72 hours		Phagocytic Index in mouse serum containing medium At 24 hours/at 72 hours	
Mouse	0.7%	0.6%	0.5%	0.5%
Rat	3%	1%	2%	1%
Rabbit	9%	2%	4%	1%
Guinea Pig	13%	10%	5%	6%
Sheep	18%	13%	2%	"1%
Man	37.5%	15%	5.9%	14.6%
Goose	26%	40%	30%	30.4%

"IN ADDITION TO PHAGOCYTOSIS 12%
OF MACROPHAGES SHOWED STRONG
ADHERENCE OF SHEEP RED CELLS.

These results support the original hypothesis of three

classes of heterogene discrimination reaction occurring in cultures

of mouse macrophages.

For red cells of the mouse, rat, rabbit, and guinea pig, the

results obtained in serum containing and serum free medium are of the

same/

same order after 3 days in culture, as at 24 hours.

With sheep red cells, there was a noticeable tendency of these cells to adhere to macrophages cultured for 3 days in mouse serum. This adherence was a constant feature, and was much more pronounced than the tendency to adherence already described as occurring in cultures of macrophages in serum free medium. With human red cells, the heterogene discrimination process appeared to degrease in intensity after 3 days culture in the absence of serum. (from P = 37.5% to P = 15%).

In serum, on the other hand, this process increased in efficiency after 3 days in culture. (P = 5.9% to P = 14.6%).

The efficiency of the phagocytic reaction towards goose red cells in serum free medium increased after 3 days in culture. (from P = 26% to P = 40%), while in serum it remained the same.

d.   Discussion.

The results obtained indicate that in the absence of serum the mouse macrophage is capable of reacting by phagocytosis to "foreign" red cells. For red cells donors closely related phylogenetically to the mouse, phagocytic activity is of a low order, and is not influenced by the presence of serum. For the red cells of unrelated donors, the phagocytic reaction in the absence of serum is vigorous, and phagocytic index is high. In serum the reaction of the macrophage towards foreign red cells may a) be depressed by the presence of serum, or b) be enhanced by its presence.

This suggests that three different types of macrophage reactivity towards/

towards foreign red cells can be distinguished. These types of
reactivity have been termed: a) Serum independent activity.

b) Serum depressed activity.

c) Serum enhanced activity.

With increasing time in culture, macrophages grown in serum
free media show an increase in reactivity towards the red cells of
species in which uptake is enhanced by the presence of serum (Goose).
Activity towards the red cells of these species (sheep, man), where
uptake is depressed by serum decreases with increased time in culture
in serum containing media. Activity towards the red cells of other
rodent species was not thought to change significantly during pro-
longed culture in serum protein free medium. In media containing
serum, reactivity towards related species did not alter, with
increasing time in culture. Reactivity towards the red cells off
that species in which uptake was enhanced by serum did not alter with
increasing time in culture. Reactivity towards the red cells of
that species, initially depressed by serum, increases with prolonged
time in culture. The red cells from the sheep adhered strongly to
monolayers grown for three days in serum, but no increase in phago-
cytosis could be demonstrated. The reaction of the mouse macrophage
towards foreign red cells is summarised in the table below.

SUMMARY OF TABLE XXII./

SUMMARY OF TABLE XXII.

Type of Reactivity.	Red cell donors.	
Serum independent	Mouse, rat, rabbit, guinea pig.	
Serum depressed	Sheep, man.	
Serum enhanced	Goose.	
Type of Reactivity.	In serum free medium @ 3 days.	In serum @ 3 days.
Serum independent	Unaltered	Unaltered.
Serum depressed	Decreased	Increased (with adherence)
Serum enhanced	Increased	Unaltered

These experiments show also that the initial actions of whole serum upon the reactivity of the mouse macrophage towards foreign red cells, can be reproduced by macrophages in the absence of serum providing the time of culture is sufficiently prolonged. The actions of whole serum are rapid, serum depression requiring only 6 to 8 hours to become established. The alteration of the activity of macrophages in the absence of serum requires up to 3 days to develop.

In the case of reactions depressed by the presence of serum at 24 hours, the reactivity of the macrophage monolayer increases during the subsequent 2 days in culture. (this is true if the adherence of sheep red cells is regarded as an increase in macrophage reactivity). The reaction of the macrophage towards the same particles in serum free medium is found to be high at 24 hours, and decreases with increasing time in culture up to 3 days. Where the initial reaction is enhanced by serum, macrophage reactivity towards that particle in serum free medium appears to increase with prolonged culture.✓

culture. This effect occurs without prior contact with the particle.

What is observed to occur in the reactions described is an increase or decrease in the numbers of macrophages reacting to the particle studied. The population of macrophages must initially be regarded as varied in respect of the reactivity of individual cells. As this population ages in culture, the reactivity of the individual cells tends to become "averaged" out over the whole population.

In the case of the reaction to the goose red cell in culture, of the initial population of macrophages only 26% prove capable of phagocytosing this particle in serum free medium. After 3 days in culture, the reactivity has "spread" to 40% of the total number of macrophages present. The initially unreactive culture can also be made to react by adding serum, which enhances uptake, but achieves this effect within 24 hours. It can be concluded that the macrophage system can control its own reactivity towards foreign cells. This reactivity is expressed as the sum of the individual reactivities of all the macrophages composing that system. The process of "averaging out" the individual reactivities of a heterogenous population of macrophages can be shown to occur in cultures of macrophages maintained for up to 3 days. The effects of serum upon macrophage reactivity are rapidly produced, but closed cultures of macrophages can modify the serum induced effects if the cultures are maintained for 3 days. The "averaging out" process can obviously either increase or decrease the reactivity of the whole population when compared with the initial culture expressing the sum of individual reactivities.

3 - HOMOGENE DISCRIMINATION.

Homogene discrimination has been defined as the ability of the macrophage to distinguish between cells of the same genetic class which differ only in their structural or biochemical integrity. The example from previous experiments is the ability of the macro- phage to discriminate between the fresh human red cell and the saline injured human red cell in the presence of serum.

a.   Homogene discrimination in serum.

Two models illustrate the capacity of the macrophage for homo- gene discrimination, the first is the reaction towards fresh and injured human cells in human serum.   The data for this reaction have already been fully described (See Chapter 2 - THE RED CELL).   The second model is the reaction of the mouse macrophage towards fresh and injured mouse red cells in mouse serum and serum free medium.

Methods.

Mouse macrophages were obtained as before, and cultured for 24 hours in medium 199 supplemented with 10% mouse serum.

Red cells were obtained by bleeding mice from the retroorbital plexus into saline containing 5 units of heparin per/ml., in poly- propylene centrifuge tubes.   Cells were obtained from this mixture by centrifugation, washed four times in ten times their own volume of saline, and reconstituted as a 5% suspension in saline.

Mouse red cells were injured by incubation as a 5% suspension

for/

for 24 hours at either 22°C., or 37°C. It was found that mouse
red cells incubated at 37°C. in glass, lysed within 24 hours. Lysis
did not occur if the incubation temperature was reduced to 22°C.,
although the cells still became injured. Lysis was minimal for red
cells incubated in polypropylene tubes at either 22°C. or at 37°C.

Fresh and injured human red cells, and fresh mouse red cells were
used as control suspensions.

Each red cell sample was added in 0.1 ml. quantities to 24 hour
old cultures of mouse macrophages grown in mouse serum. Incubation
was continued for a further 2 hours at 37°C. and the monolayers
washed, fixed and stained as before.

Results.

The figures given are average results for a series of separate
experiments. Macrophages grown in 10% mouse serum.

TABLE XXIII. - Mean Values. THE ABILITY OF THE MOUSE MACROPHAGE TO
SELECTIVELY PHAGOCYTOSE INJURED RED CELLS
IN MEDIA CONTAININGMOUSE SERUM.

Particle	Phagocytic index	Number of experiments.
Fresh mouse red cell.	0.8%	18
Injured mouse red cells.	28%	5
Fresh human red cells.	6.6%	18
Injured human red cells.	14%	5

These results indicate that the mouse macrophage in mouse serum
can discriminate between fresh and injured mouse red cells.

Discrimination/

Discrimination between fresh and injured human red cells is markedly less than in human serum. About 60% of mouse macrophages are reactive towards the injured human red cell in human serum, while only 14% are reactive towards the same particle in mouse serum. It was thought that this could be accounted for by invoking the presence of an opsonin for human injured cells in human serum, but adsorption of human serum repeatedly with the injured human red cell did not decrease the uptake in human serum.

d. Homogene discrimination in serum free medium.

Mouse macrophage cultures were prepared in medium 199 supplemented with lactalbumin solution 10%. Cultures 24 hours old were used. Fresh and injured human red cells, and fresh and injured mouse red cells were prepared as before. 0.1 ml. of the appropriate red cell suspension was added to each monolayer culture. Incubation was continued for a further 2 hours, and the cultures washed, fixed and stained as before.

Results.

TABLE XXIV.

THE ABILITY OF THE MOUSE MACROPHAGE TO SELECTIVELY PHAGOCYTOSE INJURED RED CELLS IN SERUM FREE MEDIUM.

Test Particle	Mean Phagocytic Index	Number of Experiments
Fresh mouse red cell	0.2%	18
Injured mouse red cell	31%	5
Fresh Human red cell	47.5%	18
Injured human red cell	56.5%	5

These/

These results indicate that the mouse macrophage can discriminate
between fresh and injured mouse red cells in serum free medium. The
reaction towards human/red cells is slightly greater than towards
injured
fresh human red cells in serum free medium. It is not clear from
these results if the discriminatory ability of the mouse macrophage
also extends to the human red cell system in the absence of serum,
although this ability is clearly shown in the presence of serum.
The variation of heterogene and homogene discrimination reactions by
the mouse macrophage in respect of time of contact of these macro-
phages with human serum was therefore studied.

c. <u>The effects of human serum upon the homogene
   discrimination and heterogene discrimination
   capacities of the mouse macrophage.</u>

Mouse macrophages were prepared as before in medium 199
supplemented with lactalbumin solution 10%. After 24 hours culture
the medium was aspirated and replaced with prewarmed medium 199 con-
taining 10% human serum. This was allowed to interact with the
monolayers for periods of 10 minutes, 1 hour, 3 hours, 5 hours,
12 hours, and 24 hours. After the appropriate interval, the serum
containing medium was removed and replaced with medium 199 containing
10% lactalbumin. Incubation was then continued for a further period
of 1 hour to allow the cultures to recover from the effects of
changing the medium. Fresh human, and injured human red cells were
added as 0.1 ml. of a 5% suspension, and incubation was continued
for a further 2 hours. Cultures were washed, fixed, and stained as
before.

Results ✓

Graph showing the depression of the phagocytic response to fresh / injured human red cells, as a result of contact with serum.
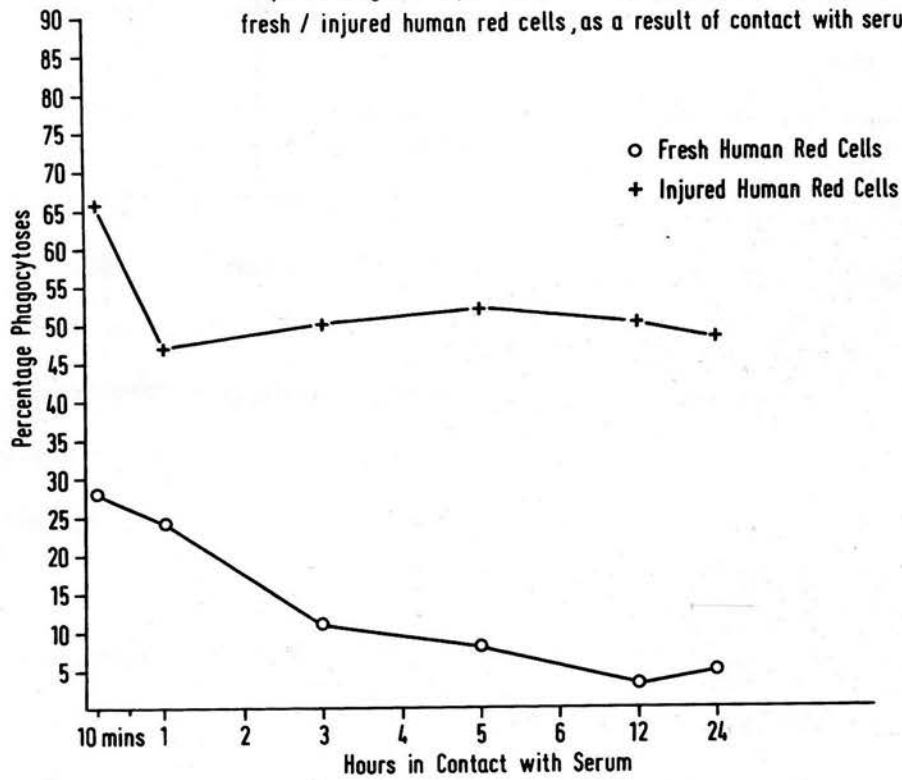
Results.

TABLE XXV.

THE INDEPENDENCE OF THE PROCESSES OF HOMOGENE DISCRIMINATION AND
HETEROGENE DISCRIMINATION ILLUSTRATED BY THE VARIATION OF HETEROGENE
DISCRIMINATION CAPACITY OF MACROPHAGES WITH TIME IN CONTACT WITH
SERUM.

Particle	10 min.	1 hour	3 hours	5 hours	12 hours	24 hours.
Fresh human	28	24	11	8	3	5
Injured human	66	47	50	52	50	48

These results clearly demonstrate the independence of the
processes of heterogene and homogene discrimination.   The presence
of serum does not, in this case, depress the response of the mouse
macrophage towards the injured human red cell.   Serum does depress
the response to the fresh human red cell, and achieves this effect
in 5 hours.   Injured human red cells always provoke a vigorous
reaction on the part of the mouse macrophage.   This reaction is
independent of the presence of serum, and is always in excess of the
reaction to fresh human red cells in the medium studied.

 d.   The effect of prolonged culture upon the reactivity
       of the macrophage towards fresh and injured human red
       cells in serum free and serum containing medium.

Macrophages obtained as before were cultured in medium 199 with
lactalbumin supplement, or medium 199 with human serum for periods
of 1 to 8 days inclusive.   The medium was changed after 24 hours in
culture./

culture, and again on days 3 and 6.   Fresh human and saline
injured human red cells were prepared as a 5% suspension in saline.
0.1 ml. was added to each monolayer culture, on the appropriate day.
Incubation was continued for a further 2 hours, and the coverslips
washed, fixed, and stained as before.

Results.

TABLE XXVI (a).

THE DECREASE IN THE HOMOGENE DISCRIMINATION CAPACITY OF MOUSE
MACROPHAGE MONOLAYERS WITH PROLONGED CULTURE:

a)   WITH HUMAN SERUM AND HUMAN RED CELLS.

Phagocytic Index

Days in Culture	Fresh Human red cells.	Injured Human red cells.
1	5%	75%
2	2%	68%
3	2%	52%
4	1%	20%
5	-	3%
6	-	-

The values for the phagocytic index indicated by a dash - were
all below 0.5%.   In some cases no erythrophagocytosis was observed
in over 3000 cells counted.

TABLE XXVI (b) ✓

TABLE XXVI (b).

VARIATION WITH TIME IN CULTURE OF HOMOGENE DISCRIMINATION BETWEEN FRESH AND INJURED HUMAN RED CELLS IN SERUM FREE MEDIUM.

(b) WITH HUMAN RED CELLS IN SERUM FREE MEDIUM.

Day	Fresh Human	Injured Human
1	26%	46%
2	12.5%	21.3%
3	5.2%	15.6%
4	2%	9%
5	-	1.5%
6	-	-
7	-	-
8	-	-

The results given are the average values for two determinations. These experiments were repeated using concentrations of serum of 20% and 40% and lactalbumin concentrations of 20% and 40% in medium 199. There was no change in the final result at these concentrations.

e. The effects of prolonged culture upon the reation of the mouse macrophage towards fresh and injured mouse cells in serum free medium.

Macrophage cultures were prepared as before in medium 199 supplemented with lactalbumin solution. Fresh and injured mouse cells were added to each monolayer as 0.1 ml. of a 5% suspension. An attempt was also made to repeat these observations employing mouse serum, but cultures showed morphological and functional changes after 4 or 5 days in culture pronounced enough to preclude the use of/

of any data obtained from them. Red cells were added to cultures from 24 hours to 8 days old. Incubation was continued for 2 hours, and cultures washed, fixed and stained as before.

Results.

TABLE XXVI (c).

VARIATION WITH TIME IN CULTURE OF HOMOGENE DISCRIMINATION BETWEEN FRESH AND INJURED HUMAN RED CELLS IN SERUM FREE MEDIUM.

(c)   WITH MOUSE RED CELLS IN SERUM FREE MEDIUM.

Days	Fresh mouse R.B.C.	Injured mouse R.B.C.
1	0.3%	36%
2	0.2%	17%
3	▪	10.2%
4	▪	3.5%
5	▪	1.0%
6	▪	▪
7	▪	▪
8	▪	▪

f.   Summary of Results.

The mouse macrophage can discriminate between fresh and injured red cells from either man or mouse in both serum and serum containing media. Further studies of homogene discrimination show

a) That the process is independent of serum concentration.

b) That the ability for homogene discrimination decreases with increasing time in culture.

c)/

c) That the processes of homogene discrimination and heterogene discrimination are distinct.

Homogene discrimination occurs in the absence of serum, and is not influenced by its presence. In this respect it differs radically from the heterogene discrimination process, which in the case of the human red cell is depressed by serum.

g. Discussion.

Three different types of heterogene discrimination by the mouse macrophage have been described:- serum depressed, serum independent, and serum enhanced. Only one type of selection is apparent in the process of homogene discrimination, and this is entirely independent of the presence of serum. In the case of discrimination between an injured and a fresh foreign red cell in serum free medium, the heterogene and the homogene discrimination are additive, with the result that more macrophages react to foreign red cells that are also injured, than react to fresh foreign red cells. This reaction is upheld in serum containing and serum free media, differing merely in the "degree" of discrimination, and in the final level of the phago-cytic index.

The discriminatory abilities of the macrophage are retained in culture for up to 4 days. Cultures beyond this age are unreactive towards both foreign red cells, and to injured red cells. Associated with this change in reactivity, a marked change in the morphology of the cultured macrophages occurs. Macrophages cultured for periods in excess of 4 days in serum containing or serum free media lose their cytoplasmic/

cytoplasmic veils, and become elongated and stellate. They show
increasing nuclear hyperchromatism with increasing age after 4 days
in culture. In addition, many cells show fine peripheral vacuol-
isation, and those grown in serum free medium occasionally show large
vacuoles, which remain unstained by haematoxylin and eosin, or Giemsa
stains. (See Figs. 4 & 5). Associated with these changes, is a
loss of reactivity towards fresh human, injured human, and injured
mouse red cells. It is thought that this change represents an
artefact of prolonged culture. Although it decreases the reactivity
of the macrophage towards foreign and injured red cells, some other
aspects of macrophage function remain undisturbed. They still
retain the capacity to phagocytose yeast particles and antibody coated
red cells and in these two respects are functionally normal.

4 - RECOGNITION.

Previous studies in this and other laboratories have demonstrated that particles coated with antibody are readily phagocytosed. The mouse macrophage will react to specific antigen antibody complexes by phagocytosis even when the antibody is derived from sources other than the mouse. Thus thyroid colloid complexed with human anti-thyroid auto-antibody is rapidly phagocytosed in comparison with thyroid colloid alone (Stuart 1966). Human red cells coated with haemolysin from the rabbit, or by human immune haemolysins directed against Rh or I antigens, are extensively phagocytosed. (Stuart, Davidson & Cummings 1967); (Dewar, personal communication).

The studies of recognition undertaken here were directed at exploring the effects of serum and the absence of serum upon the reactivity of the mouse macrophage towards red cells coated with haemolysin, and the effects of prolonged culture upon this reaction.

a. Preparation of antisera, and the sensitivity of technique used.

Two antibody systems were used, in the first the sensitised particle was the human red cell, and the antiserum was derived from the immunisation of a rabbit. In the second, mouse red cells were sensitised with a similarly prepared rabbit antiserum directed against mouse red cells. The fortuitous discovery of a naturally occurring haemolysin for mouse red cells in serum from one human donor provided a third antibody system. These three antisera are hereafter referred to as antisera A, B, and C.

Antiserum/

Antiserum A was produced by the injection of two rabbits with 20 mg. (Dry weight) of pooled human red cell ghosts. Each rabbit received 7 injections in saline in the first 21 days, and thereafter a single booster dose 3 days before bleeding to obtain antiserum. Antiserum A was therefore Rabbit anti-human-red-cell serum. Antiserum B (Rabbit anti-mouse-red-cell serum) was prepared in rabbits using 0.5 ml. of a 20% suspension of washed mouse red cells in saline as the antigen. The immunisation schedule was similar to that employed for the preparation of antiserum A.

Antiserum C (Human anti-mouse-red-cell serum) was discovered while testing for lytic and agglutinating activity in human serum with mouse red cells (See Chapter 3, section 5 c). A quantity of this serum was retained for study.

The agglutinating and lytic titres of these antisera for the appropriate particle were determined by standard techniques, and are shown below. Fresh guinea pig serum was used as a source of complement in the determination of lysis.

TABLE XXVII.

THE AGGLUTINATING AND LYTIC TITRES OF THREE ANTI-RED CELL ANTISERA.

Antiserum	Lysis titre	Agglutination titre
A	1/350	1/600
B	1/30	1/350
C	1/4	1/16

The sensitivity of erythrophagocytosis in respect of red cells coated with these antisera was determined thoroughly for antiserum C. The/

The other antisera were found to give comparable results.

Method.

Macrophage cultures were prepared as before in human serum. Mouse red cells were incubated for 30 minutes at 37°C. in dilutions of antiserum C in neutral human serum. Dilutions ranged for 1/1 up to 1/1000. The sensitised mouse red cells were washed twice in saline, and added as 0.1 ml. of a 5% suspension to each monolayer. Incubation was continued for 2 hours, and the cultures washed, fixed and stained as before.

This antiserum produced macroscopic lysis at a dilution of 1/4 and agglutination at a titre of 1/16. The titres effective in producing erythrophagocytosis are shown below. All dilutions greater than 1/10 produced maximal phagocytosis.

Results.

TABLE XXVIII.

THE PHAGOCYTIC INDEX FOR MOUSE RED CELLS SENSITISED WITH VARIOUS DILUTIONS OF HUMAN ANTI-MOUSE RED CELL SERUM (SERUM C).

Titre	1/10	1/15	1/20	1/30	1/50	control
	98%	48%	35%	15%	5%	0.6%

For the other sera tested, maximal phagocytosis of sensitised cells occurred in 1/10th of the minimal serum concentration necessary to cause agglutination, and from 1/20th to 1/40th of the concentration necessary to produce lysis. Thus it appears that the macrophage/red cell system can detect lytic antibody in concentrations up to 1/40th of/

of the level detected by the more common in vitro tests.

b.   The reaction of the mouse macrophage towards
sensitised human red cells in serum containing,
and serum free media.

Methods.

From antiserum A a gammaglobulin containing fraction was prepared
by salt fractionation, using ammonium sulphate, followed by gel
filtration on a Sephadex G 200 column, in Borax/phosphate buffer
(pH 8.6).   Haemolytic activity was contained in the single major
peak, corresponding to the IgG component of rabbit serum.   This
preparation was standardised after concentration, and the same con-
centration of haemolysin was used to sensitise red cells in each of
the following experiments.   The standard concentration used represented
1/10th of the minimal lytic concentration.   For the standard prep-
aration this represented a dilution of 1 in 800.   Sensitisation was
accomplished by incubating fresh washed human red cells in this con-
centration of haemolysin for 30 minutes at 37°C.   The red cells were
then washed once in saline, and reconstituted as a 5% suspension.
Mouse red cells treated in the same manner, and fresh human red cells
were used as controls.

Macrophages were obtained and cultured as before in medium con-
taining human serum, mouse serum, or lactalbumin supplement at a 10%
concentration.   After 24 hours in culture 0.1 ml. of a 5% suspension
of sensitised red cells was added to each tube.   Incubation was
continued for 2 hours, and the cultures washed, fixed and stained as
before.

Results.

Results.

TABLE XXIX.

THE REACTION OF MOUSE MACROPHAGES TO ANTIBODY COATED RED CELLS IN
SERUM CONTAINING AND SERUM FREE MEDIUM.

Medium containing	Sensitised red cells	Fresh human	Mouse
Mouse serum	96%	8%	6%
Human serum	98%	4%	6%
Lactalbumin	97%	34%	5.8%

These results indicate that antibody coated red cells are
phagocytosed in either the presence or absence of serum, and that
mouse macrophages are highly reactive towards this type of particle.
Control mouse red cells incubated in rabbit gammaglobulin fraction
for 30 minutes were slightly more susceptible to phagocytosis than
fresh mouse red cells. Specific antibody therefore is a much more
potent "opsonin" than antibody globulin not directed against a
specific antigenic determinant. The reactivity of the macrophage
towards an antibody coated particle within the limits specified is
not dependent upon the source of either antibody or particle.

The rabbit haemolysin sensitised red cells were used throughout
the experimental series as a test particle for the standardisation of
cultures. (See appendix). Macrophages showing fewer than 90% of
the population reactive to this particle after 2 hours incubation
were discarded as being functionally unsatisfactory.

    c.   <u>The variation in the reaction towards sensitised red
cells with time in culture.</u>

Macrophages/

Macrophages were obtained as before, and cultured from 1 to 8 days in medium 199 with 10% human serum, or 10% lactalbumin solution. Washed human red cells were sensitised by 30 minutes incubation at 37°C. in a dilution of 1 in 800 rabbit haemolysin. Red cells were washed once, before being reconstituted as a 5% suspension in saline. 0.1 ml. was added to each culture. After 2 hours incubation, the cultures were washed, fixed and stained as before.

Results. Reaction to sensitised human red cells.

THE REACTION OF MACROPHAGES IN LONG TERM CULTURE, TOWARDS ANTIBODY SENSITISED RED CELLS.

Day	In Human Serum			In Serum Free Medium
	a	b	c	
1	96%	96%	92%	98%
2	95%	95%	98%	95.6%
3	96%	97%	97%	97%
4	89%	83%	60%	94.5%
5	76%	40%	75%	95%
6	58%	53%	65%	92%
7	42%	46%	44%	92%
8	34%	28%	34%	90%

The data for three experiments employing mouse macrophages grown in human serum are shown in full. The data for cultures grown in serum free medium are expressed as average values.

The macrophage grown in human serum shows a gradual decline in its reactivity towards the haemolysin coated human red cell after 4 days in culture. This decline occurs at the same time as the decline of/

of the heterogene and homogene discrimination reactions. It is never complete, as in the other two cases. Macrophages grown in serum free media show no decrease in reactivity towards haemolysin coated cells, although the two "discrimination" reactions decrease as rapidly in serum free medium as in serum.

This evidence therefore supports the hypothesis that the macrophage is capable of three types of reaction towards the red cell. These have been termed heterogene discrimination, homogene discrimination and recognition. Evidence is presented to show that these three types of reactivity are not identical.

CHAPTER 5

DISCUSSION.

CONTENTS.

1. THE ACT OF PHAGOCYTOSIS.

2. THE THEORETICAL ASPECTS OF MACROPHAGE REACTIONS TOWARDS RED
   CELLS.

   a. Recognition.

   b. Heterogene discrimination.

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3. THE REACTIONS OF INDIVIDUAL MACROPHAGES AND THE MECHANISMS OF
   REACTION.

   a. Interpretation of the results of the basis of a receptor
      site theory.

   b. The nature of possible receptors.

4. THE POSSIBLE MECHANISMS OF PHAGOCYTOSIS BY MACROPHAGES.

   a. Objections to the receptor site theory.

   b. The application of the Field Theory of Membranes to the
      experimental data.

5. SUMMARY.

1 - THE ACT OF PHAGOCYTOSIS.

Observations upon the reaction between mouse macrophages and red
cells in-vitro, by phase contrast microscopy, reveals that the act
of ingestion occupies at the most a few minutes. Antibody coated
cells are ingested rather more rapidly than injured or foreign red
cells. For whole cultures, the phagocytic index obtained after.
fifteen minutes incubation represents nearly 50% of the index after
2 hours incubation. This demonstrates that phagocytosis occurs
rapidly, if it is going to occur at all. During the act of ingestion
it has been repeatedly observed that red cells become deformed. This
deformation usually involves the red cell assuming an elongated pear
shape. (See fig. 8). Occasionally red cells will fragment into
multiple small globules during ingestion. Ingested red cells often
shrink into irregular refracticle remnants shortly after ingestion.
Similar changes in red cells during the act of the ingestion by "fibro-
blasts" and other cell types have been described by Gropp (1963).
The process described by this author took much longer than the
ingestion of red cells by mouse macrophages, but otherwise the act
of phagocytosis appeared similar in both cases. Red cells adherent
to macrophages can also appear deformed, assuming an elongated shape.
The occurrence of this deformation illustrates that a force of forces
are acting upon the red cell in the vicinity of the macrophage membrane.

Red cells not susceptible to phagocytosis may approach the
macrophage membrane but they rarely show any evidence of deformation.
Occasionally these red cells will induce the formation of a pseudopod

by/

by the macrophage. Red cells not susceptible to phagocytosis are
pushed away by the pseudopod, sometimes appearing to _"bounce off"
it. Susceptible red cells are held to the membrane and "drawn into"
the cytoplasm. These observations are essentially similar to those
made by Hirsch (1965) and Gropp (1963). These observations on the
act of phagocytosis indicate the importance to the process of forces
acting between the particle and the macrophage during ingestion.

Macrophage reactions are usually said to occur following "contact"
between the particle and the macrophage membrane. For colloidal
substances such as carbon, the individual particles do seem to approach
very close to the membrane. This may be because of their small
diameter. (See Pethica 1961). For larger particles, such as
mammalian cells, true "contact" between the membranes does not appear
to occur, a gap of dimensions in the region of 150 A can be clearly
shown (Habeshaw personal observations;) (see Figs. 10 and 11). The
gap varies in width, but at the points of closest approach of the
opposing membranes, it measures between 100 and 150 A. At these
points of close approach, changes in the electron density of the
macrophage membrane occur, and similar changes can be observed in
the opposing membrane of the red cell. Changes of this type have
been described by Karrer (1963) and Lo Buglio, Cotran, and Jandl
(1967). In this macrophage/red cell system, true contact between
the membranes of macrophage and red cell has never been observed.
This applies to the separation even between the phagosome membrane,
and the membrane of the phagocytosed red cell.

The forces acting between the red cell and the macrophage during
phagocytosis/

phagocytosis must therefore act over a distance. The source of these forces must be the two interacting membranes.

2 - THEORETICAL ASPECTS OF MACROPHAGE REACTIONS TOWARDS RED CELLS.

Theoretical studies have indicated two principal ways in which the membrane of the macrophage can detect and react to a particle. These are a) by direct alteration in the field at the macrophage surface,

b) by the incorporation into, or adsorption onto a membrane of a "ligand" which directly modified the field properties of that membrane. Supportive evidence for the existance of biophysical forces in the process of phagocytosis, and their importance in relation to the act of phagocytosis stems from the observations:

a. That a gap visible by electron microscopy exists between the membrane of the macrophage and the membrane of the red cell during phagocytosis, and this gap is of the order of 150 A in width.

b. Studies of the phagocytic process in living cultures reveals deformation of red cells during the process, and evidence of strong attractive and repulsive forces acting between macrophages and red cells.

a. Recognition.

The macrophage membrane will react vigorously to particles coated with antibody. In this system lytic antibodies directed against red cells caused a reaction in almost all the macrophages present in the culture. The phagocytic reaction between the antibody coated red cell and the macrophage is independent of the constitution/

constitution of the medium in which this reaction takes place.
The constancy of this reaction in media differing in their composition
suggests that bound specific antibody spontaneously triggers a
phagocytic reaction irrespective of the environment or the state
of the macrophage membrane. The antibody molecule seems to be unique
in this respect. Most mammalian antibodies have similar chemical
and molecular structures, although antigenically they are quite
distinct. Their activity in respect of phagocytosis must lie in
the similarities in their molecular weight and molecular shape.
The variability described in the light chains of mammalian antibody
leads to the conclusion that their activity in respect of phago-
cytosis may reside in the less variable heavy chain.

It has often been observed that gammaglobulin stimulates
pinocytosis, particularly by macrophages (Cohn & Parkes 1967;
Holtzer & Holtzer 1960). Gammaglobulin is also capable of
modifying the response of the macrophage membrane as is shown by the
depressive effect of this protein upon the phagocytosis of sheep and
human red cells. The kinetics of this process indicate that this
effect may be achieved by the incorporation of gammaglobulin into the
macrophage membrane. It is therefore suggested that the incorporation
of gammaglobulin into the macrophage membrane is a physiological
process. This process of incorporation is continuous, and the
reactivity of the macrophage membrane from one instant to another is
dependent in part upon the concentration and type of gammaglobulin in
the macrophage environment.

In this respect, the macrophages of mammalian species differ
from those of invertebrate species (Stuart 1968). The macrophages
of/

of these species do not react to gammaglobulin but still seem to be
dependent upon the presence of opsonic substances in their body fluids
for efficient phagocytosis. (Prowse & Tait 1969; Stuart 1968).
These observations suggest the possible evolution of the antibody
forming system through the specialisation of opsonin forming tissues.
The lymphoid tissue in the mammal may represent the ultimate develop-
ment of a system originally concerned with the production of serum
and tissue proteins which, by a biological accident, happened to
have the two properties of combining with foreign material, and the
ability to modify the reactivity of the primitive phagocytic cell.

b.   Heterogene discrimination.

The phagocytes of primitive animals are capable of ingesting
"foreign" material, and removing damaged tissue. (Cameron 1932).
The necessity for opsonic substances in the heterogene discrimination
process has already been noted. Primitive animals, including the
invertebrates, are also capable of altering their reactivity towards
foreign material, that is are capable of producing an immune reaction.
Some workers have commented upon the rapidity with which an immune
state arises in primitive animals. Immunity to pathogenic bacteria
can arise within 24 hours, in both insects and crustacae. (Paillot
1920; Metalnikov & Gaschen 1921; Cangacuzene 1922; McKay et al
1969). This immune state arises primiarily through the increased
phagocytic and digestive powers of the invertebrate phagocyte.
(Huff 1940; Nelstrop et al 1968). The rapidity with which immunity
occurs within the invertebrate, and the dependence of this effect upon
the/

the phagocytic cell may indicate an analagous situation with respect
to the mammalian phagocyte in its reaction to foreign red cells.
In the section concerning heterogene discrimination (See Chapter 4,
Section 2c), it was demonstrated that the mouse macrophage population
in vitro can alter its phagocytic potential towards a foreign red
cell with prolonged time in culture. One significant difference
between the experiments described here, and the conclusions derived
from work on invertebrate immunity, is that in the former case no
prior contact with the foreign red cell was required to provoke an
altered state of reactivity. The difficulties posed by this problem
can be largely overcome by investigating the state of the whole
invertebrate, and comparing it with the cultur of macrophages as an
enclosed system. Immunity in invertebrates is rapidly acquired,
and depends primarily upon the increase capacity of the phagocytic
cell to react to the antigen employed. In the experiments described
here, an increased or decreased capacity of the macrophage to react
with foreign red cells occurs spontaneously with increasing time in
culture, even in the absence of serum. The change in the reactivity
of the macrophage towards the foreign red cell was thought to occur
by a process of "averaging out" the reactivity of the individual
macrophages throughout the system. In any population of macrophages,
three classes of cellular reactivity towards foreign red cells can
exist:

1. Those macrophages reactive towards the foreign particle.

2. Those macrophages potentially reactive towards that particle.

3. Those macrophages inactive towards that particle.

The/

The initial reactivity of the culture is an expression of the numbers of macrophages in Class 1. The activity at three days is an expression of the numbers of macrophages in Classes 1 and 2. A recruitment of potentially reactive macrophages in Class 2 to Class 1 has occurred. This situation could arise because macrophages in these experiments were derived from outbred mice, and might therefore be expected to be dissimilar in their original reactivity towards foreign red cells. It has recently been shown that macrophages from a single strain of inbred mice are capable of spontaneously enhancing their reactivity towards foreign red cells (sheep red cells) in culture at three days. (Perkins et al 1968). It must therefore be concluded that the macrophages from the mouse peritoneum are innately heterogenous in respect of their reactivity towards the foreign red cell. After three days in culture it has been found that the reactivity of the macrophage population towards a foreign red cell has increased or decreased. Macrophages have therefore been recruited to react, or not to react, to that particle by the presence in culture of other macrophages inherently different in their reactivity towards that particle. The change in reactivity applies only to the class of reaction termed heterogene discrimination, and no effect is observed upon homogene discriminatory reactions, or the ability to ingest red cells coated with specific antibody.

It can also be shown that in a given population of macrophages in serum free medium the numbers of macrophages initially reactive towards a foreign red cell increases as the "foreignness" of the red cell increases. With prolongation of the time in culture the reactivity/

reactivity of the whole population tends to a limit which may be either greater or less than the initial reactivity expressed by the whole population of macrophages. The limit obtained is obviously dependent upon the state of the system in which the population as a whole exists. In vivo, with increasing time, the system will react in a different manner to that observed in vitro - the sensitivity of the reaction being increased by the secretion of specific antibody. Since almost 100% of the macrophage population will react to antibody coated cells, the limit obtained in vivo will always tend to a maximum, that is to complete phagocytic activity of the Reticulo Endothelial System to the foreign red cell. In the system in vitro, the limit obtained is dependent upon:

a. The proportion of macrophages inherently reactive towards that particle.

b. The proportion of recruitable macrophages in the original population.

c. The dynamic equilibrium between loss of reactivity which occurs with increasing time in culture, and the rate of recruitment of potentially reactive macrophages.

d. The particle used. This determines the numbers of macrophages in classes 1 and 2.

In the invertebrate, the injection of killed pathogenic bacteria introduces a variable into the system not present in the above scheme. As already shown for the in vitro system, changes in reactivity tend to a limit which is defined by, essentially, the system used to detect the change. The immune state of an invertebrate depends upon the increased/

increased capacity of its phagocytic cells to ingest and destroy
bacteria which have already at one time been present in the system.
The immune state therefore arises because prior contact with the
organism has altered the original system, and invertebrate immunity
represents the new limit to which macrophage reactivity will tend in
that altered system.   On purely theoretical grounds, the immunity
acquired by invertebrates is likely to be of a broad specificity, and
this can be shown to be the case.   (MacKay et al 1969).

In the invertebrate, the priming injection of a bacterium is
significant only in the sense that it alters the internal environment
of the whole animal, and hence the environment of the macrophage.
In vitro, this state arises by the removal of the mouse macrophage
from the mouse peritoneum, and its establishment in culture.   The
central feature common to both processes is the capacity of a
population of macrophages, considered as a whole, to alter their
reactivity as a consequence of environmental change.   The macrophage
therefore can be said to possess the two qualities of:

1.   detection of environmental change and

2.   the ability to alter the reactivity of the total population of
macrophages in any given system, as a consequence of environmental
change.

This concept differs from the established concepts of immunity
in that there appears to be no requirement for a prior contact with
specific antigen.   The role of the antigen is limited to its effects
upon the whole internal environment of the system studied.   It has
no direct affect, by way of inducing altered reactivity, upon the
macrophage./

macrophage.

There are two requirements for this concept of the primitive
state of immunity:

1. that the macrophage system as a whole can detect environmental
change, and,

2. that the individual macrophages can alter their reactivity in
response to an altered environement.

The mechanisms by which macrophages achieve these effects are
of central importance to the understanding of the heterogene dis-
crimination process.

c. Homogene discrimination.

In vivo, the capacity to mount an immune response may depend
upon the detection of alterations in the environment of the macrophage,
by the Reticuloendothelial system as a whole. The evidence that
gammaglobulin is incorporated into the macrophage membrane, and that
this incorporation alters the reactivity of the macrophage, suggests
that one mechanism by which the macrophage "samples" its environment
is by the incorporation of ligands such as gamma globulin into its
membrane from the environment. Perhaps the most common alterations
occurring in the environment of the macrophage in the whole animals
are injury to cells and the ageing of cells. The capacity of the
macrophage to detect an injured cells, and to remove it is termed
"homogene discrimination". The efficiency of this process seems to
be independent of the composition of the medium, and in this sense
is quite unlike the heterogene discrimination reaction. Two aspects
of/

of the homogene discrimination process are of interest, firstly that
the greater the degree of injury of the test particle the more reactive
the macrophage population is to that particle, and secondly, the
spontaneous decrease in the reactivity of the whole population with
increasing time in culture.

It has been shown that the bulk of a population of macrophages
are spontaneously reactive towards one kind of particle, while they
remain less reactive to a very similar particle. They will react
strongly to an injured human red cell, but fewer macrophages will
react towards an uninjured human red cell. The more severe the
injury to the red cell, the greater the number of macrophages reactive
to it. Red cells incubated in saline in anaerobic conditions were
reacted to by 70 to 80% of macrophages (See Chapter 2, section 3f),
while those incubated in saline containing calcium pantothenate were
reacted to by only 30% of macrophages. (See Chapter 2, section 3g).
These data imply that the discriminatory activity of the whole macro-
phage population is determined primarily by the state of the membrane
of the injured cell, and is independent of the conditions of culture.
Although most, if not all, macrophages in a given population are
potential reactors to the injured red cell membrane, the proportion
that do so react is determined by the distribution of the individual
sensitivities of the macrophages within the population studied. This
can be demonstrated by an analysis of the events occurring in respect
of homogene discrimination in long term culture. In all cases the
total numbers and hence the proportion of macrophages in a culture
which reacted towards an injured particle decreased with increasing
time/

time in culture. The ability of the culture to discriminate as a whole was retained. It was merely the sensitivity of the discriminatory activity that decreased. After 4 days in culture the homogene discrimination ability of that culture was determined by the 4 - 5% of cells still reacting to the presence of an injured red cell. In these particular experiments, the injury to the red cell was carefully controlled, so that a constant degree of injury was produced.

There is no evidence from the experiments described here that the capacity of homogene discrimination can be acquired by unreactive macrophages, from reactive ones. The ability for homogene discrimination must therefore be inherent in the macrophage membrane. In this respect, it may be mentioned that the incorporation of gamma-globulin into the macrophage membrane also seems to be an inherent characteristic of the macrophage membrane.

In order to react to the environment, the macrophage population must detect environmental changes. The properties of the membrane which enable the macrophage to do this must be common to the whole macrophage population. At this point it may be noted that homogene discrimination reactions, and reactions to antibody coated cells are not dependent upon the composition of the medium. The conclusion from these speculations is that:

a. The macrophage "measures" two parameters in its environment; the level of one (or more) specific "ligand" substances, and alterations in cell membranes in the environment.

b. The resultant of this "measuring" or "sampling" process is a change/

change in the reactivity of the macrophage system as a whole. This change can be interpreted as a primitive immune state.

This part of the discussion has been mainly concerned with the activity of the macrophage population as a whole. In the next sections the reactivity of the individual macrophage will be discussed.

3 - THE REACTION OF INDIVIDUAL MACROPHAGES AND THE MECHANISMS OF
REACTION.

The macrophage population in culture is composed of individual
cells which differ markedly in respect of their sensitivity towards
detecting injured membranes, and their reactivity towards foreign
red cells. Although marked differences in the individual
reactivities of these cells occur, the whole macrophage population
is capable of reacting to cells coated with antibody. The reactions
of individual macrophages can be examined by interpreting the
experimental results on the basis of a series of "receptor sites"
upon the macrophage membrane.

a. Interpretation of the results on the basis of a
"receptor site" theory.

The receptor site theory considers that one particular class of
macrophage activity is determined by the presence of a chemical group-
ing present on the macrophage membrane which reacts specifically with
one class of particle. Receptor sites have been described for a
number of classes of cellular activity. Fawcett (1965) described
and illustrated receptor sites upon the membrane of the erythroblast
for ferritin. Receptor sites are thought to be responsible for the
transport of sodium and potassium ion through cell membranes.
(Glynn 1961). The transport of glucose through the red cell membrane
is thought to involve receptor substances classed as "phosphoproteins".
Nelson (1968) lists seven separate receptor sites on the macrophage
membrane thought to be responsible for the phagocytic reaction of the
macrophage/

macrophage towards different types of particle.

In this system, three types of reactivity by the macrophage have been demonstrated. It can therefore be assumed that three receptors are present upon the macrophage membrane, receptors A, B, and C., each receptor being responsible for one class of macrophage activity. Let it be assumed that receptor A is responsible for heterogene discrimination reactions, receptor B is responsible for the reaction towards antibody coated cells, and that receptor C is responsible for homogene discrimination.

The reaction between the mouse macrophage and the fresh human red cell in the presence of serum can be explained in the following manner: Receptor A for the "foreignness" of the human red cell is blocked by the presence of gammaglobulin in the serum, and since no antibody is attached to the red cell, and the red cell is not injured, receptors B and C are inoperative, and no phagocytosis occurs.

If the red cell has been injured, phagocytosis in the presence of serum is determined by receptor C, receptor A as before being blocked by serum. In serum free medium, receptor A is not blocked by gammaglobulin and the injured red cell will be phagocytosed. Phagocytosis in this case is determined by receptors A and C together. The effects should be additive, and this has been shown to be the case.

If the red cell is coated by antibody, phagocytosis is determined by receptor B. In this case receptor A will be blocked by any free antibody (or by the gammaglobulin in serum) and receptor C does not operate since the cell is not "injured".

The results obtained by experimentation can therefore be explained/

explained with reference to a system of receptors upon the macrophage surface.

b. The nature of possible receptors.

Certain requirements must obtain in respect of the receptor sites or substances assumed to be present upon the macrophage membrane.

1. These receptors must be able to initiate a phagocytic response while the membranes of the macrophage and red cell are separated by a gap of at least 100 to 150 A.

2. By an examination of the phagocytic index obtained for each class of macrophage/medium/particle interaction, it is clear that not all macrophages have a full complement of receptor sites upon their surface.

A small proportion (Less than 5%) have all three, since a phagocytic index of zero is never obtained with any combination of particle medium and macrophage. Most macrophages possess only two receptors, and these must usually be the receptors for damaged red cells, and for gammaglobulin. A few macrophages again have only one receptor, B., upon their membrane, since there remains a substantial difference between the phagocytic index from maximally damaged red cells (80%) and for red cells coated with gammaglobulin antibody (nearly 100%).

3. Receptor A is modified by long term culture.

4. Receptors A and C are lost in culture beyond four days, while receptor B is retained.

5. Receptor A is blocked by gammaglobulin.

6.

6. Receptor B is present upon most macrophages and is neither labile nor blockable.

From these observations it is possible to draw the following conclusions: Receptor B is a property inherent in the macrophage membrane which may be related to the physiological necessity of the mmembrane to incorporate gammaglobulin.

Receptor A is labile and blockable. It might therefore be described as a superficial membrane site, probably produced by the binding of a serum or tissue fluid component. This component may be transferrable by diffusion to other cells, and this property could result in the spreading of receptor A activity; it being acquired by non-reactive macrophages, and also lost from reactive macrophages.

Receptor C detects injured membranes, and is present upon the bulk of macrophages. Since macrophages show a wide spectrum of sensitivity towards particles with different degrees of injury, it can be assumed that some have more receptor C upon their membranes than others.

There is no evidence that receptors A, B, and C show any similarity in type, reactivity, or chemical constitution.

4 - THE POSSIBLE MECHANISMS OF PHAGOCYTOSIS BY MACROPHAGES.

The observed phagocytic reactions can be easily explained with
reference to a receptor site theory. It is pertinant to ask "how
valid is a receptor site theory in relation to the likely structure
of cell membranes, and the biophysical forces thought to be involved
in the phagocytic process?".

a. Objections to a receptor site theory.

There are many cellular reactions in which receptor sites are
thought to initiate or influence cellular activity. Among these may
be mentioned ion transport, pinocytosis, sugar transport, phagocytic
reactions of various kinds, the role of chemical mediators in nerve
transmission, cellular differentiation and organogenesis. In fact,
almost any theory of transport mechanisms, or cell to cell adhesion
can be explained, superficially at least, by invoking a specific cell
membrane bound receptor as the active agent in the process. Many
thousands of separate and distinct chemical groupings upon the cell
membrane would be required to explain its in vivo existance. An
additional objection is that the cell membrane is probably being
synthesised and catabolised quite rapidly, and unless the receptor is
an actual chemical constituent of a cell membrane, or is present in
large amounts in the environment, it is likely to be lost from the
membrane. Finally the concept of an ordered environment in the
vicinity of the cell membrane dependent basically upon the arrangement
of molecules within that membrane, renders the whole concept of
receptor/

receptor sites superfluous.

It must be mentioned here that the concept of a ligand substance, and a receptor site or substance are not similar. The action of a receptor substance is envisaged as being dependent upon its chemical composition and independent of the state of the membrane considered as a whole. On the other hand, the action of a ligand is dependent solely upon its ability to alter the structure and physical characteristics of the membrane as a whole. In practical terms, a "receptor site" can be isolated from a membrane and can still retain its capacity to react with the substance it "receives". A ligand is almost certainly unreactive in this respect, and cannot produce its effect other than in the presence of an intact cell membrane. It is doubtful whether "receptor sites" could influence the reactivity between one cell and another since opposing cell membranes always appear to be separated by a gap. The "receptor site" would therefore be limited to the interactions of cells with particles sufficiently small to approach the cell membrnae closely.

For these reasons, the explanation of the phagocytic reaction towards red cells by receptor site theory cannot be upheld. It has already been theoretically demonstrated that the reaction of a phagocytic cell with a particle can occur through modifications in the field at the membrane surface. (See Chapter 1, section 7d.) Changes in the field can be induced directly, or through the mediation of a ligand. The use of this concept has several advantages:

a. It can account for interactions between particle and phagocyte at a distance.

b. It is dependent upon the packing and internal molecular arrangements of/

of the molecules composing the membrane. This in turn is determined
by the action of Van der Waals forces between the molecules of
membrane material synthesised by the cell. The field is therefore
renewed as new membrane is formed, the generation of that membrane
being dependent only upon the supply of the correct amounts of
membrane material by the cell, and the local environment of the cell.
c. It can accountfor the reactions of the cell membrane to all
classes of material from ions to other cell membrane.
d. The specificity of membrane reactions is retained, since the
field structure of any surface is unique, and the reaction of that
surface with any class of substance is also unique.

b. <u>The application of the field theory of membranes to
the experimental data.</u>

The test of any good hypothesis is that is explains in a simple
manner all the observed facts. Conclusive proof of the role of field
forces in determining phagocytosis is not, at the moment, technically
feasible. The concept can, however, be used to explain the data
already presented, and to account for the observed features of
phagocytosis described by other authors. The experiments described
here seem to indicate three classes of macrophage reactivity,
homogene discrimination, heterogene discrimination and recognition.
Homogene discrimination has been defined as the capacity of the
macrophage to phagocytose one particle while showing less phagocytic
towards another particle of similar nature or chemical composition.
Fenn (1923) described the ability of the macrophage to discriminate
between particles of manganese dioxide and manganese silicate. The
presence/

presence of silica in the particles of the latter substance rendered it less susceptible to phagocytosis than the former. The ability of the macrophage to detect and react to such fine differences in the chemical composition of particles cannot be easily explained unless the discriminatory process is effected through modifications in the electrical field structure of the macrophage membrane.

Similarly it has been shown that the macrophage can detect and react to injured red cells which have lost as little as 10 to 20% of their membrane material. From earlier discussions it seems likely that the loss of this amount of membrane material will affect the electrical field produced by the red cell membrane. It has also been noted that the more usual parameters of red cell injury, that is haemolysis, sphering, and increasing osmotic fragility, are not significantly affected by incubation in saline. This suggests that the membranes capacity to act as an osmotic or mechanical barrier is not affected by saline injury. The capacity of the macrophage to discriminate between the healthy and the injured red cell is more likely to depend upon the electrical field differencies between the two types of particle, than upon any gross alteration in the chemical structure or mechanical integrity of the injured cell membrane. The homogene discrimination reaction might therefore be said to represent the direct effect upon the macrophage membrane of an opposing field generated by a dissimilar surface. The electrical field generated by an injured cell membrane must differ from that produced at the surface of the healthy cell. The electrical field structure of the healthy cell membrane must therefore possess some general characteristic (for example the presence of a fixed anionic surface charge)/

charge) which is both common to many types of healthy cell irrespect-ive of the species of origin, and which acts to decrease the susceptibility of the healthy cell to phagocytosis. The loss of this characteristic quality of the normal electrical field at the cell membrane could be said to represent the biological (i.e. In Vivo) definition of the injured cell. The concept of homogene discrimination can be described in general terms by an experimental law stating that damaged cells are always phagocytosed to a greater degree than their healthy counterparts irrespective of the origin of the cells or of the environmental conditions under which the reaction is studied.

Heterogene Discrimination.

There is good evidence that the macrophage can react by phagocytosis to healthy cells derived from a genetically different source. This reactivity represents the process defined as heterogene discrimination. Further evidence suggests that the greater the phylogenetic difference between the macrophage donor and the red cell donor, the greater is the phagocytic response to the red cell. (Perkins & Leonard 1963, Habeshaw Personal Observations).

The compositions of the red cells of various species are known to differ both chemically and antigenically. In man, and in some other species, individual differences in the antigenic structures of red cell membranes has been shown. The unique qualities of the cells of one individual are known only because the cells of another individual can be shown to react to them in a unique manner, for example by the formation of antobidies, or the rejection of a homog-graft.

graft. The qualities which are said to represent the "foreigness" of a cell are therefore always defined as either chemical or antigenic differences between the cells of donor and recipient. In other words, the foreigness of a cell depends upon the system used to detect this quality. A homograft in a tolerant animal or chimeria is, in the biological sense, not "foreign" at all. Antigenic differences between cells may not be the factors which initiate a response to them; it is presumed that they are since the systems employed to detect those responses usually measure antigenic differences. In the system employed in these experiments, it has been shown that the reaction of the macrophage to a "foreign" cell depends upon:

a. The species of red cell donor.

b. The environment of the phagocytic cell - particularly in respect of the immunoglobulin or specific antibody content.

c. The species of the macrophage donor.

It was further concluded that the heterogene discrimination reaction represented the resultant of all these variables applied to an enclosed system of cells. The process of heterogene discrimination in such a system represents the limit of reactivity to which the cells composing that system tend when the environment of those cells is changed as by the addition of a "foreign" particle, or by prolonged culture. It is a moot point whether heterogene discrimination at the level of the individual cell is a meaningful concept. In order to explain the effects of serum and the effects of long term culturres upon the heterogene discrimination reactions of macrophage monolayers it was postulated that the macrophage could:

a. ✓

a. detect differences in the reactivity of the cell membrane of other macrophages in its environment and

b. incorporate a specific "ligand" substance into its membrane from the environment.

The first of these abilities explains the recruitment of potentially reactive macrophages to the reactive population within the system, thereby accounting for the changes in phagocytic index observed with increasing time in culture. The mechanisms by which macrophages can alter their activity when placed in culture with macrophages of inherently different reactivity could occur as follows.

For the purposes of this thesis, it has been assumed that differences in phagocytic activity represent differences in the electrical field produced by the macrophage membrane. As already shown, homogene discrimination reactions can be said to occur because the membrane of a damaged cell maintains an inadequate electrical field in its immediate vicinity. The interaction of this field with the field produced by the normal macrophage membrane results in an alteration of the molecular arrangement within that membrane. The resulting organisational changes in the macrophage membrane are expressed as the act of phagocytosis. Thus phagocytosis represents the capacity of one cell membrane to influence the structure of another cell membrane in close proximity.

In the cell system represented by the macrophage monolayer, "contact" between macrophages must occur with reasonable frequency, since these cells are highly motile under tissue culture conditions (Figs 4 and 5). Contacts of this type could result in temporary or/

or permanent changes in the molecular architecture of the contacting membranes under the combined influence of the membrane fields.
(an analogous situation would be the induction of polarity in an electret under the influence of a strong electrostatic field).
Many such contacts between macrophages in the monolayer could result in the averaging out of the individual reactivities of macrophages in long term culture. The experimental evidence that the phagocytic response to the human red cell increases with increasing time in culture up to three days in the presence of serum, supports the concept that altered reactivity may be transferred in this manner. That the close approach of dissimilar membranes can result in such molecular changes is shown by the homogene discrimination reaction. In general terms this concept can be stated as follows:

Contacts between the membranes of living cells can result in temporary or permanent alterations in the reactive capacities of each of the contacting membranes. In an enclosed system of cells, the resultant of many such contacts leads to a general alteration in the reactivity of the system, which, at equilibrium, could then be said to possess the reactivity of the algebraic sum of all the individual reactivities of the cells composing that system AB Initio.

This concept of the biological averaging of membrane reactivities explains how the macrophage in culture detects changes in its cellular environment. The additional sensitivity of the macrophage to the presence of a "ligand" substance, ensures that the cell can also detect certain types of change occurring in its fluid environment, i.e. those which alter the quantity or quality of the ligand presented

to/

to the cell.

It is known that the immunoglobulin molecules which comprise "specific antibody" are variable in terms of their molecular structure. It is this variability which accounts for the observed specificity of antigen/antibody reactions. On the other hand, "free" or uncommitted immunoglobulin molecules in the system studied appear to act as "ligands" for the macrophage membrane, profoundly altering the reactivity of that membrane in a non-specific manner. Free immunoglobulins in the environment of the mouse macrophage will depress the phagocytic reaction towards sheep, human, and, to a lesser extent, guinea-pig red cells. This depressive effect is presumably independent of the "antibody-like" activity of the immunoglobulins involved, and, since human, mouse, calf and other immunoglobulins produce similar effects, is also independent of the finer molecular structure of immunoglobulin molecules. In other words, the "ligand" like activity of an immunoglobulin molecule differs from, and cannot be predicted by its "antibody-like" activity. However, if a preponderance of one type of antibody molecule occurred in the macrophage environment, it is possible that the "ligand" determined reactivity of the macrophage membrane would then differ from its "ligand" determined reactivity in the presence of indeterminate immunoglobulins. Thus it should be possible to demonstrate that although various mammalian sera depress heterogene discrimination in the same general manner, individual variations in the depressive effects of those sera could occur if a wide enough range of test particles was employed.

The/

The process of heterogene discrimination therefore can be seen to depend upon the state of the macrophage membrane in relation to its environment at the time of test. The state of the macrophage membrane, is, in turn, dependent upon the reactive states of the other cells membranes present in that system, and upon the "ligand"-like activity of the individual immunoglobulin classes present in the surrounding fluid.

In terms of the whole animal, the nature and type of "ligand" substances circulating in the blood or body fluids determines the reactivity expressed by the phagocytic cell towards "foreign" cells, that is the "ligand" substances are "self-marker" proteins. The capacity of the macrophage to detect damaged "self" cells is independent of the self marker protein, and is the direct result of the discrimination by the field at the macrophage surface. Particulate material, of any kind, which fails to produce an alteration in the macrophage membrane will be treated as "self". Material which will both alter the macrophage membrane, and which, in its interactions with that membrane, will affect, or be affected by "ligand" determined reactivity, is treated as "foreign". This concept can perhaps best be explained by the analogy of the macrophage to a person with but two senses, he can feel and he can smell. If objects could be presented to such a person which can be neither smelt, nor felt, that person would be unable to detect the presence of those objects even if they were placed in his immediate environment. He therefore could not be expected to exhibit any kind of reactivity towards such objects.

CHAPTER 6

ILLUSTRATIONS

CONTENTS.
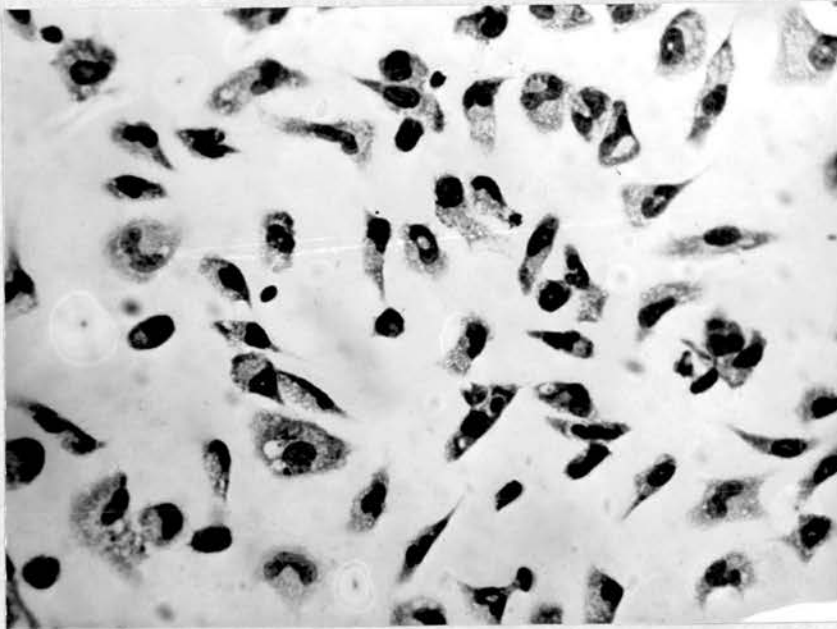
Figure 1. The morphology of a mouse macrophage monolayer after 6
hours in culture. The macrophages are adherent to
glass and have spread out. The cells are phagocytically
active at this time, although they require up to 24 hours
in culture to achieve optimal phagocytic activity.

Giemsa   Magnification 350

Figure 2. The appearance of a healthy macrophage culture after 24
hours incubation in medium 199 with 10% lactalbumin.
supplement. The monolayer shows an even distribution of
cells, and the space between the cells is free of debris.
The majority of macrophages are elongated or stellate in
form. These cultures are optimally active as judged by
the measurement of the phagocytic index.

Haematoxylin and eosin   Magnification 100
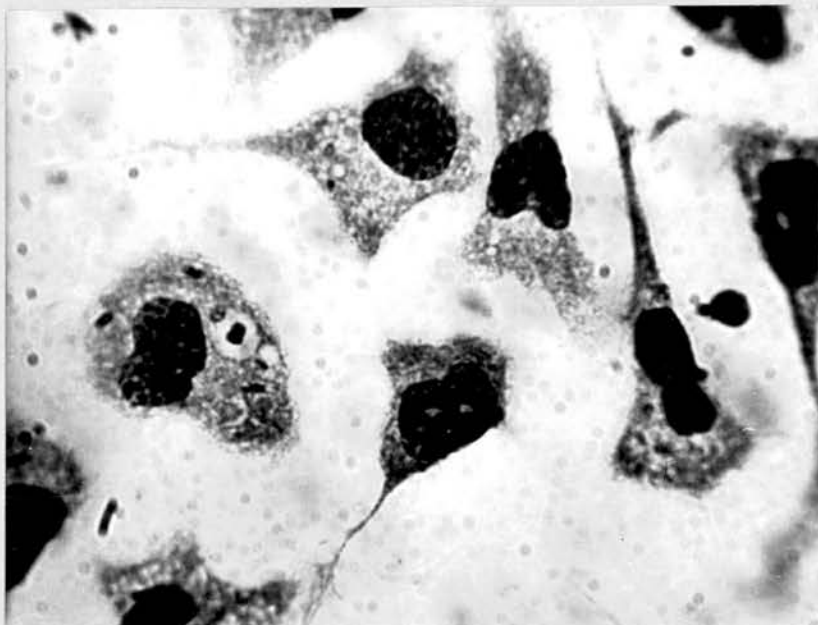
Figure 3.   The appearance of an active macrophage monolayer after

24 hours in culture.   At this magnification the macro-

phages can be seen to have reniform nuclei with finely

dispersed chromatin.   The cytoplasm is rich in organelles

and inclusions, and its border is finely ruffled.   One

of the macrophages shows a well defined phagocytic vacuole

containing two mast cell granules.

Giemsa   Oil Immersion   Magnification 800
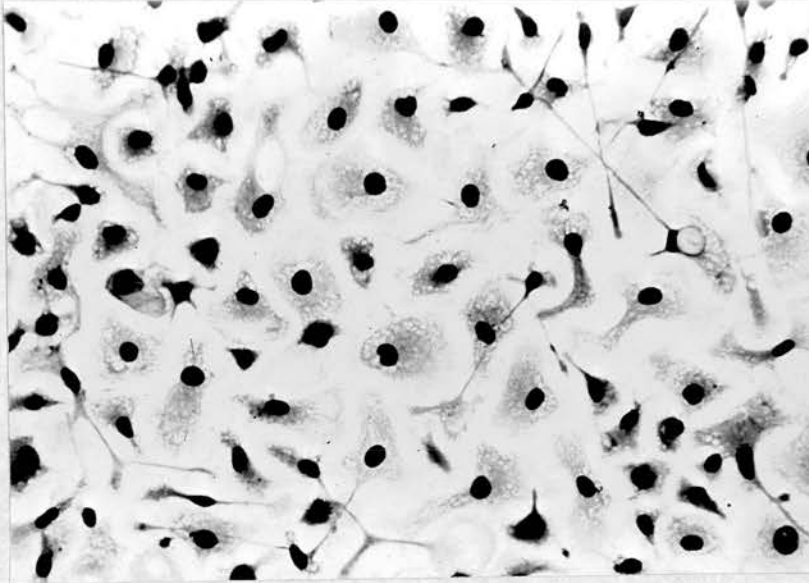
Figure 4. The appearance of a macrophage monolayer after 4 days in culture. The macrophages present show two morphological types. The first is elongated or stellate with fine cytoplasmic processes. The second is flattened and round with voluminous cytoplasm. The nuclei of both cell types are round or oval, and nuclear hyperchromatism is apparent. Cultures of this age will readily ingest antibody coated cells, but show less activity towards damaged red cells or "foreign" red cells. This culture was grown in medium 199 supplemented with 10% lactalbumin.

Giemsa   Magnification 350
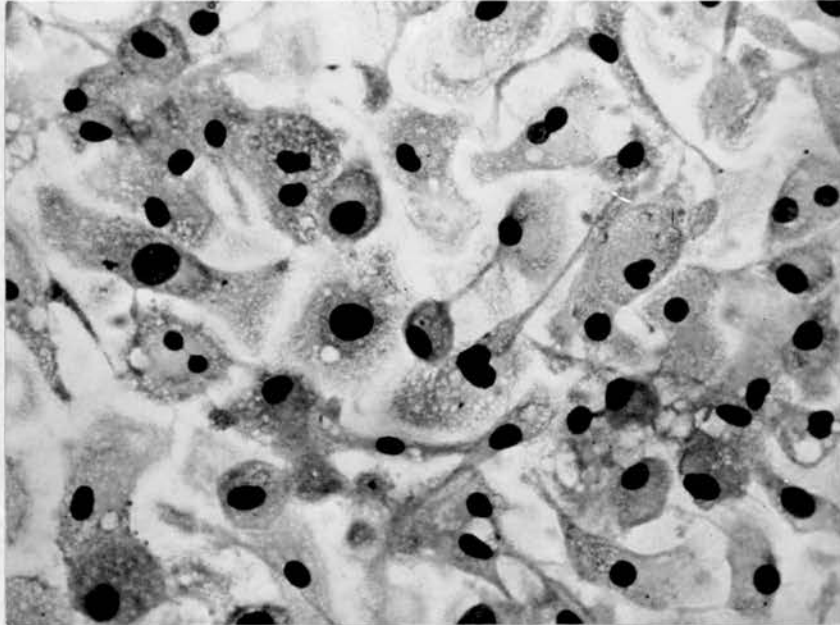
Figure 5.   The appearance of a macrophage monolayer after 8 days in
            culture.   Macrophages cultured for this time in either
            lactalbumin or serum containing media show similar
            appearances.   Pronounced nuclear hyperchromatism, and
            some nuclear pleomorphism is apparent.   The cells are in
            close contact one with another, and several cells contain
            large clear vacuoles.   Cultures of this age will readily
            ingest cells coated with antibody, but do not react to
            foreign or injured red cells.

                    Giemsa stain   Magnification 500

Figure 6. The appearance of a macrophage monolayer to which red
cells sensitised with rabbit haemolysin at a dilution of
1 in 800 have been added. Nearly all the macrophages
present have ingested the sensitised red cells, which
readily lyse inside the phagocytic vacuoles giving this
characteristic appearance.

Giemsa   Magnification 350
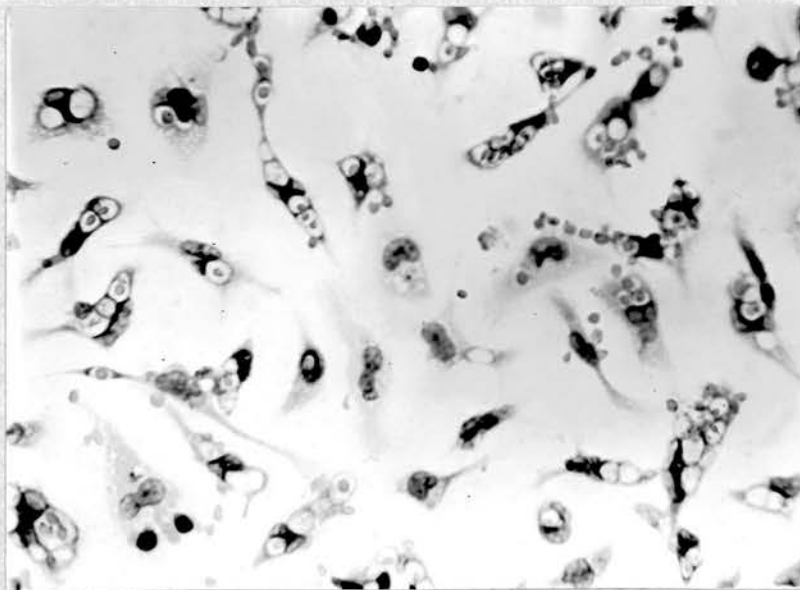
Figure 7. The appearances of a macrophage monolayer to which
saline injured red cells have been added. Many
macrophages have ingested red cells, but in this case
the cells remain intact though shrunken, in the phagocytic
vacuoles.

Giemsa   Magnification 200
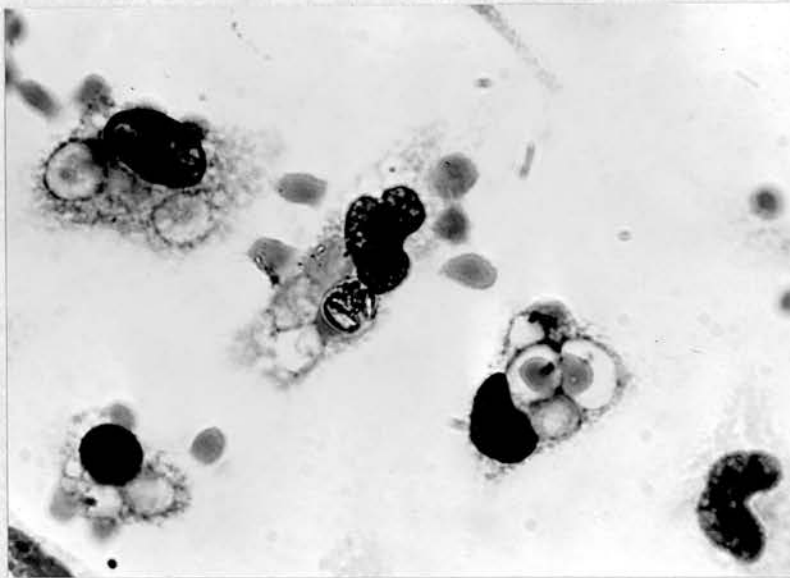
Figure 8.   The appearance of erythrophagocytosis in a macrophage

monolayer to which saline injured red cells have been

added.   The refractile body occupying the central

macrophage is a "myelin figure" which represents the

membrane remnants of ingested red cells.   Adherent red

cells also show deformation at their sites of attachment

to the macrophage surface.

Giemsa   Oil Immersion   Magnification 800
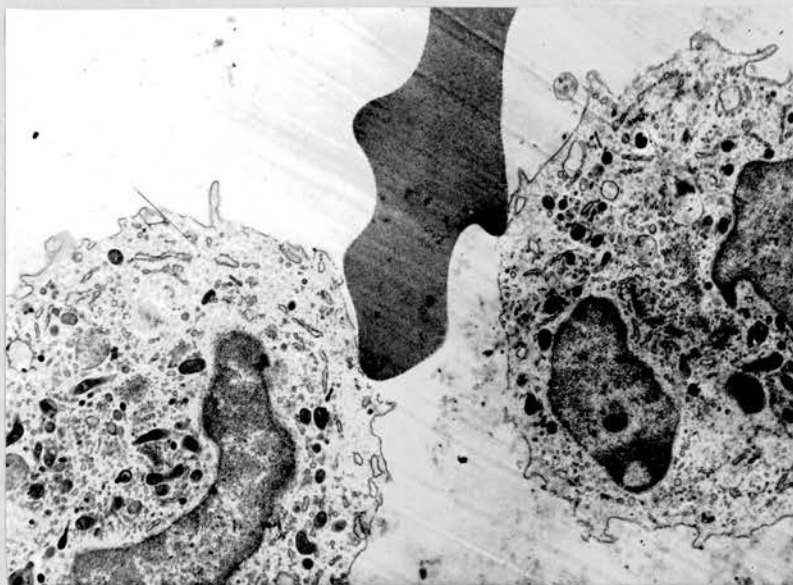
Figure 9.   This electron micrograph shows the deformation of a red

cell in close proximity to two macrophages.   At this

magnification the opposing cell membranes appear to be

in close contact.

Mouse macrophage monolayer grown on araldite in medium

199 supplemented with lactalbumin.   Fixed in osmic acid.

Magnification 3,000
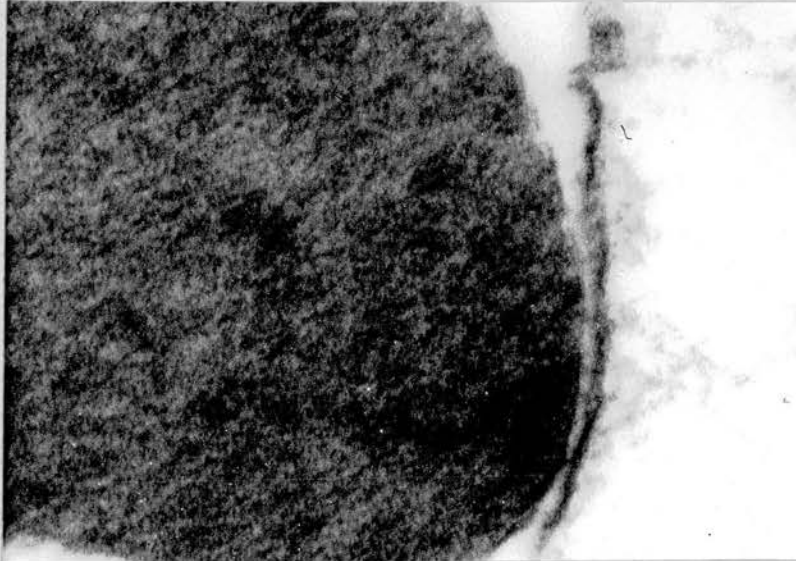
Figure 10. A high power view of the cell contact in Figure 9.

This shows the opposing membranes to be separated by a

gap measuring approximately 100 A in width.   True

"contact" between red cell membranes, and the membrane

of the macrophage has never been observed.

Mouse macrophage monolayer grown on araldite in medium

199 supplemented with lactalbumin.   Osmic acid fixation.

Magnification 80,000

Figure 11. Examination of the membrane of a macrophage, and the
membrane of a red cell "ghost" in close proximity shows
the existance of a gap measuring approximately 150 A.
This plate also illustrates the changes of electron
density of cell membranes at the points of closest
approach.   Mouse macrophage monolayer on araldite in
medium 199 supplemented with lactalbumin.   Fixed in
osmic acid.

Magnification 80,000

CHAPTER 7

APPENDIX.

CONTENTS.

1 - PREPARATION OF CULTURES.

Young, healthy, outbred mice of Tuck's TO strain were used throughout. (A.R. Tuck & Son, Rayleigh, Essex.). Mice weighing between 17 and 22 grams were used. The mice were killed by cervical fracture. The fur over the anterior abdominal wall was then swabbed with 70% alcohol. A small longitudinal incision in the skin of the anterior abdominal wall was made, and the pelt retracted to expose the abdominal muscles. 2 to 5 ml. of medium 199 containing penicillin, 200 i.u. and streptomycin 100 micrograms ml. (Glaxo) was then injected in the mid-line, into the peritoneal cavity. The medium contained 0.5 units of heparin per ml. The fluid injected into the abdominal cavity was circulated by gentle prodding of the peritoneal wall. After two to three minutes, a fresh needle was inserted into the left flank, and advanced behind the spleen. Lateral traction upon the needle produced a pocket of fluid behind the spleen, from which site the medium could be aspirated with ease, and without blockage of the needle with intra-abdominal fat. The fluid was aspirated gently, and placed in a sterile measuring cylinder. The whole procedure was repeated upon batches of six mice, and the aspirates pooled until the required volume of macrophage suspension was obtained. The final cell count in the aspirate varied considerably with the source and age of the mice used. The cell count was found to be between 2×10^6 cells/ml., and 6×10^6 cells/ml. After counting, the cell concentration was adjusted to give a final concentration of 1.5×10^6 cells/ml, and a final supplement concentration of 10% by volume. The cell suspension was then agitated to ensure/

ensure an even distribution of cells and seeded into tissue culture tubes, at 1 ml. containing 1×10^6 cells per tube. Cultures were seeded onto coverslips (Chance No. 1, 19 x 6 mm.) contained in Pyrex glass rimless test tubes (16 x 125 mm.). The cultures were then stoppered with white rubber stobbers (Esco Rubber Ltd.) and incubated in racks at 5 degrees to the horizontal at $37^{\circ}C$. Cultures so prepared were ready for use after 18 to 24 hours incubation.

2 - THE PREPARATION OF MEDIUM SUPPLEMENTS.

In order to obtain satisfactory cultures of macrophages,
supplementary media must be added to medium 199. Those commonly
employed are calf serum and horse serum, but macrophages prepared in
the manner described above will produce satisfactory cultures in
sera from the mouse, rat, guinea pig, rabbit, calf, horse, and man,
and in synthetic, serum protein free media. For satisfactory
cultures to be obtained, certain criteria in the preparation of the
macrophage cultures and the media supplements must be observed.

a. Sterility.

The supplements added to the culture medium must be sterile.
Some commercial preparations of sera contain preservatives such as
Orthocresol, and merthiolate which are harmful to cultured cells.
Sera are best sterilised by filtration. Either asbestos filters,
or "Millipore" filters can be used, but for a viscous medium such
as serum, the former type of filter is preferable. The filtration
process is made easier if the medium is first centrifuged at 20,000
x g. for 2 hours before filtration to remove contaminating particulate
material, such as denatured protein. Bacterial contamination of
cultures was found to be due most frequently to contaminated medium
supplements, especially those stored for long periods.

b. Storage.

Sera can be stored for long periods (2 to 6 months) in the
frozen/

frozen state without effecting their properties in tissue culture.
Frozen supplements, once thawed, should never be re-frozen, since
this leads to denaturation, and loss of their desirable properties
in tissue culture.   Supplements were therefore stored after
sterilisation, in small aliquots, at -20°C. under deep freeze
conditions.

After setting up cultures, any remaining medium supplement
was discarded, or stored for up to one week at 4°C. before discarding.

Some sera are cytotoxic for mouse macrophage monolayers when
fresh.   Examples are mouse serum itself, and some samples of human
serum.   Cytotoxicity usually decreases after storage from 2 days
to one week in the frozen state.   Very occasionally sera do not
show any decrease in cytotoxicity on storage, and these sera cannot
be used in culture.

c.   Haemoglobin.

Sera from the mouse, rat and rabbit are frequently contaminated
with haemoglobin if prepared without care.   Sera heavily con-
taminated with haemoglobin produce vacuolisation of macrophages in
culture, and decrease the phagocytic potential of these cells.   In
the course of these experiments it has been observed that much of
the haemolysis occurring in mouse serum can be prevented by a) taking
the blood into polypropylene, or siliconised glass containers, and
b) allowing the blood to clot at 4°C., rather than at 37°C. or at
room temperature.

Even with these precautions, occasional batches of serum were
heavily/

heavily contaminated with haemoglobin. These sera cannot be used for successful cultures. If it becomes necessary to use such a serum, it is possible to remove a proportion of the contaminating haemoglobin by filtration through activated charcoal which has been thoroughly prewashed with phosphate buffered saline at pH 7.4. This procedure is effective, but has rarely been found to be worthwhile.

d. Compatibility.

In experiments involving erythrophagocytosis, it is essential to test the sera used as a supplement with the test particle before use. This is especially true for human sera, as the presence of a haemagglutinin directed against an imcompatible red cell will result in extensive adherence of the test red cell to the monolayer, and render accurate assessment of erythrophagocytosis difficult. Mouse and human sera may also show agglutinating and lytic activity towards heterologous red cells. These sera frequently promote massive phagocytosis of the test red cells, and cannot be used in culture systems containing these red cells. Agglutinating and lytic activity can be adsorbed out of the serum with the test red cell before employing that serum in culture - but since the adsorption process invariably results in some degree of haemolysis it is preferable instead to seek a serum devoid of agglutinating or lytic activity. Haemagglutination and haemolysis titres for all red cell/serum combinations were determined by incubating washed red cells with dilutions of serum in perspex trays. The end point was read out after/

after 30 minutes incubation at 37°C. Sera which showed haemagglutination titres of less than 1 in 10 were generally acceptible for culture.

3 - STANDARDISATION OF CULTURES.

All the experiments described were conducted using batch cultures
of peritoneal macrophages not less than 18 hours old. Each batch
of cultures was standardised prior to use. The following criteria
were used to standardise the experimental cultures.

a. Mice.

The mice used as peritoneal macrophage donors must be healthy.
Stock mice frequently carry occult infections, and macrophages from
such mice provide unreliable culture material. Such mice cannot
be used. Throughout these experiments, mice were obtained in
batches of 100 animals from a reliable laboratory supplier.
At intervals, randomly selected mice were killed and bacterial cultures
prepared in broth and on blood agar from spleen, lymph nodes, and
peritoneal cavity. The mice were also subjected to an autopsy, in
which the spleen and liver in particular were examined for signs of
bacterial infection. The most reliable index of infection was found
to be the presence of an enlarged spleen, or enlarged mesenteric
lymph nodes. In the strain of mice used in these experiments, the
upper limit of normal spleen weight was found to be 0.13 g. for mice
up to 25 g. in weight. Stock mice which showed more than one in
twelve mice with signs of infection were used to provide mouse serum,
and their macrophages were not used to prepare cultures.

Mice were never kept beyond a total age of 12 weeks. After
this time, an increasing proportion of the mice developed signs of
infection,/

infection, and cultures of macrophages from old mice, even if no signs of infection are present, carry a large proportion of mast cells. Degranulation of mast cells under culture conditions leads to excessive red cell adherence, and uneven patterns of erythrophagocytosis.

b. The effects of bacterial contamination upon erythrophagocytosis.

By its nature, the macrophage from an infected mouse is likely to transfer its organisms into culture. Phagocytosed organisms are protected from the effects of the antibiotics in the medium, and occasionally multiply within the infected macrophages. With the death of infected macrophages, the organisms become liberated into the medium, and by the process of rephagocytosis, can spread to contaminate a whole culture. For this reason it is essential to ensure, as far as possible, that infected mice are never used as a source of macrophages.

The presence of bacteria in a culture profoundly disturbs the process of erythrophagocytosis, whether the organisms present are transferred from infected mice, or with contaminated medium supplements. Some examples are given below.

Gram positive bacteria thought to belong to the bacteroides group, when present even in small numbers, will cause a decrease in erythrophagocytosis. In examples of infection with this organism, the cultures may at first appear normal. Upon close examination, single bacilli may be found within macrophages and affected macrophages show increased vacuolation of their cytoplasm. The staphylococcus/

lococcus and streptococcus produce morphological changes within the
tissue culture population of macrophages, and many strains of these
organisms produce haemolysis. Red cells damaged by these haemo-
lysins are readily phagocytosed by mouse macrophages. These toxins
also appear to be freely diffusible, and can produce their effects
even when a small number only of the macrophage population contain
these organisms.

Occasionally lethal agents can be transferred from infected
mice into the cultured population, and these cause widespread death
of the macrophage in vitro. One particular organism was found to
infect the stock mice used in these experiments in the spring and
autumn. It was never identified.

c. Standardisation of macrophage cultures by
morphology.

The most straightforward method of assessing macrophage cultures
is by the microscopic observation of the cells composing it.
(See Bennet 1967). Healthy cultures show an even distribution of
macrophages over the coverslip surface. In infected cultures, the
macrophages tend to clump together in some areas, and may die off
in others, giving an uneven cell distribution. Under the experimen-
tal conditions described here, the bulk of the macrophages present
conform to two standard morphological types. The most frequent
appearances are those of a cell with an oval or reniform nucleus
with no prominent nucleoli, surrounded by veils of cytoplasm which
stain faintly pink, with eosin. The cytoplasm is evenly distributed
around the cell, and contains only small vacuoles and cytoplasmic
inclusions/

inclusions (See figs. 1,2,3). The second type predominates in older cultures. This cell is extended, and stellate in appearance, usually with an oval or round nucleus. Cytoplasmic veils occur only at one point in the cell, usually on the cell "body" in the vicinity of the nucleus (See figs. 4,5). In infected cultures the cells tend to be found, and may occur in clumps or sheets. Bacteria may be visible within the cytoplasm of infected cells. Macrophages in infected cultures may also show excessive vacuolation, nuclear pleomorphism, and nuclear hyperchromatism. Dead macrophages can frequently be found, and since they adhere to glass, be mistaken for living cells unless closely examined. In infected cultures, the monolayer always appears "dirty" with cell debris, adherent to the glass coverslip in between the surviving cells.

Macrophages cultured in spoiled or deficient media, are frequently rounded, and show loss of cytoplasm. Such cells adhere poorly, and are readily washed off the coverslip surface. The monolayer therefore appears thin, and is composed of cells resembling small lymphocytes in appearance.

d. Standardisation by P (Max).

Morphological assessment only is inadequate for the standardisation of cultures in quantitative work. The most effective method found to achieve this was the use of red cells sensitised with a standard preparation of rabbit haemolysin. Washed red cells (usually human) were sensitised by incubation for 30 minutes at 37°C. in anti-red-cell serum diluted to one tenth the minimal lytic concentration. The/

The use of excessive amounts of haemolysin to sensitise red cells will:

a. cause lysis and clumping of the red cell suspension

b. may enable poor macrophages to phagocytose sensitised red cells, and hence give an inadequate culture a satisfactory phagocytic index. The P (Max) was assessed by incubating sensitised red cells which had been washed once in saline, with the macrophages culture to be tested for 2 hours at $37^{o}C$. The P(Max) was then expressed as the

$$\frac{\text{number of macrophages containing one or more red cells}}{\text{total number of macrophages}} \times 100$$

Cultures which showed a P (Max) or less than 90% were not used for experimental purposes.

The advantage of this method of standardising cultures lies in its reproducability. The P (Max) is independent of the medium in which the macrophages are cultured, and practically, it has been found that the P (Max) appears to reflect the phagocytic capacity of macrophage cultures towards particles other than the antibody coated red cell.

The standardisation of cultures for the quantitative assessment of the erythrophagocytosis was accomplished employing the two criteria of morphological appearance and the p (Max). Cultures which were either morphologically or functionally unsatisfactory were never used for the quantitative assessment of erythrophagocytosis.

e. <u>Ageing of cultures.</u>

Morphological/

Morphological and functional changes are observed in healthy cultures of macrophages if the period of culture is prolonged beyond four days. The cultured cells became elongated and stellate or epitheloid in appearance with pronounced nuclear hyperchromatism. The P (Max) was not affected by prolonged culture in serum free media, but steadily decreased in cultured maintained in serum. In assessing these cultures, morphological criteria only could be used.

f. Washing, Fixing, Staining, and Counting.

After allowing phagocytosis to continue for 2 hours at $37^{\circ}C.$, the culture medium was removed from each tube, and the coverslips washed vigorously with saline to remove unattached red cells. The saline was then removed and the cultures were fixed in a mixture of methanol/acetone 50/50 by volume. Fixed cultures were then stained with Giemsa or Haematoxylin and Eosin stains, in the conventional manner.

A standard counting procedure was employed. Firstly a minimum of four cultures monolayers was required for each single experiment, and each experiment was repeated a minimum of four times unless otherwise stated. The final expression of the phagocytic index was the resultant of at least sixteen separate cultures. This number of experiments was required because of the natural variation in phagocytic ability from one batch of macrophages to the next. For most types of red cell macrophage interaction, this variation was within narrow limits (less than 10%), but the final assessment of phagocytic/

phagocytic index was made much more positive by the use of large numbers of cultures.

Counting was performed by estimating the numbers of macrophages containing red cells as a percentage of the total number of macrophages present in a given microscopic field. Each monolayer was counted in three areas, one at each end of the coverslip and one in the centre. The cells growing near the edges of the coverslip were not counted. A minimum of 1000 cells for each coverslip were counted, and the phagocytic index expressed as an average of at least sixteen coverslips for each experiment. The reproducability of the counting procedure was assessed by recounts done after an elapse of some months by the original observer. Average values were found to vary by not more than 5.

In conclusion, the validity of this method of quantitation of erythrophagocytosis lies in ensuring a healthy and functionally active macrophage culture, and in employing standard methods of preparation and control of these cultures.

CHAPTER 8

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CHAPTER 9

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