# *LEISHMANIA TROPICA*: STUDIES ON THE MECHANISMS OF ATTACHMENT TO AND ENTRY INTO MACROPHAGES

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by

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LEISHMANIA TROPICA: STUDIES ON THE MECHANISMS OF ATTACHMENT TO AND ENTRY INTO MACROPHAGES. ASSADOUR ZENIAN


ABSTRACT

The *in vitro* attachment and entry of promastigotes of *Leishmania tropica* into mouse peritoneal macrophages were studied under defined experimental conditions. The attachment of promastigotes to macrophages was found to be dependent on temperature, macrophage intracellular metabolism, and the normal function of microfilaments and microtubules. Parasite binding was abolished by fixation of macrophages but not of the parasites.

Modification of the surface of macrophages and parasites by various surface reactive agents revealed that surface carbohydrates of parasites and macrophages were important in parasite binding. Periodate and neuraminidase affected both parasites and macrophages in a complementary manner. Treatment of macrophages with trypsin and concanavalin-A also prevented parasite attachment.

In the extracellular medium, parasite binding required the presence of magnesium, glucose and a macromolecular component of foetal calf serum and was inhibited by 2-deoxyglucose. The requirement for extracellular glucose could be replaced by mannose, suggesting that the requirement for glucose is structural rather than for purposes of energy metabolism. The active fraction of serum was nondialyzable, heat labile and precipitable by ammonium sulfate. The extracellular and

surface structural requirements of parasite binding suggest the involvement of a biochemical process which is probably mediated by an enzymatic reaction.

Scanning electron microscopic examination of macrophages infected with parasites revealed that motile promastigotes attached to host cells by their flagellar tips to which macrophages responded by producing closely fitting lamellar sheaths and progressively enveloping first the flagellum and then the body of the parasite. Lamellar advance during engulfment was rapid in the first 10 minutes but much slower later on. Fully engulfed parasites could be seen after one hour, but most parasites associated with host cells remained extracellular even after four hours. On the other hand, parasites immobilized by fixation or heat shock adhered by either their flagellar or somatic ends. Engulfment proceeded at a steady rate and was complete by two hours. No clear distinction between the attachment and engulfment stages of parasite uptake could be established since both were inhibited by low-temperature, cytochalasin and mild fixation of macrophages. The rheological features of the host cells' response to parasite attachment indicate that invasion by parasites is through phagocytosis rather than penetration.

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## INTRODUCTION

Species of the genus *Leishmania* represent a group of parasitic protozoa within the Kinetoplastida that infect man and vertebrate animals. (Garnham, 1971; Zuckerman and Lainson, 1977). They are distinguished by having two developmental stages in their life cycle, a promastigote stage in which parasites are elongate, possess a flagellum and in nature inhabit the alimentary tracts of phlebotomine sandflies, and an amastigote or intracellular stage in which parasites lack a flagellum, and assume a rounded form (Adler, 1964). After entering the vertebrate host through a skin bite promastigotes invade the cells of the mononuclear phagocyte system where they transform into amastigotes and propagate intracellularly (Zuckerman, 1975). The predominant form found in culture is the promastigote whether the culture has been seeded with amastigotes or promastigotes. In nature the parasites survive in various vertebrates many of which act as reservoir hosts for the transmission of the parasites to man as a zoonosis, (Adler, 1964; Garnham, 1971).

Although there are many species within the genus *Leishmania* which are exclusively parasites of lower vertebrates or mammals other than man, such as *Leishmania enriettii* of the guinea pig and *Leishmania* spp of reptiles (Adler, 1964) the genus as a whole is renowned for being disease agents in man

over geographically widespread parts of the world, (Adler, 1964; Lysenko, 1971).

There are three main groups of Leishmania species that are pathogenic to man. They are classified according to the three different patterns of disease they produce, (Manson-Bahr, 1971; Mauel et al., 1975).

1. Leishmania tropica the causative agent of cutaneous leishmaniasis of the Old World which is widespread in North Africa, the Mediterranean basin, the Middle East, and Central Asia. This group of parasites usually causes a self-healing lesion on the exposed parts of the human skin known as Oriental sore. To this group also belongs L. mexicana of the New World which causes a characteristic lesion on the ear (Chiclero's ulcer).

2. Leishmania braziliensis the causative agent of mucocutaneous leishmaniasis in the New World, which after producing a primary skin lesion invades the mucous membranes of the nasopharyngeal and buccal cavities and causes severe permanent disfigurement.

3. Leishmania donovani the causative agent of kala azar or visceral leishmaniasis which invades the phagocytic cells of the liver, spleen and bone marrow causing splenomegaly and anaemia and is usually fatal if untreated.

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Inside the vertebrate host most species of Leishmania live in

the phagocytic vacuoles of mononuclear phagocytes. In natural conditions the infective forms of the parasite are the promastigotes which are introduced into the skin of the vertebrate host by the bite of the sandfly vector (Adler, 1964). The early events after the inoculation of the promastigotes into the skin by infected sandflies are not well known, but it is presumed that the majority of the promastigotes are ingested and killed by heterophil leukocytes of the early inflammatory response. A few promastigotes gain entry into histiocytic macrophages in which they transform into the amastigote form and propagate (Zuckerman, 1953; Bryceson, 1975). Around this primary lesion the formation of a focus of proliferating macrophages usually follows (Manson-Bahr and Winslow, 1971). The multiplying amastigotes eventually destroy the host macrophage and are in turn ingested by adjacent macrophages, a process which ensures the survival of the parasites inside the host and is probably responsible for the pathological manifestations of the infection (Bryceson, 1975).
The early histiocytic response is followed by late invasion of lymphocytes and plasma cells and after a prolonged period the lesion is resolved by a process of self healing as in the case of Old World cutaneous leishmaniasis. The parasites may also spread into other tissues such as cartilage and mucous membranes as in the case of New World cutaneous leishmaniasis or into the phagocytic cells of the liver, spleen and bone

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marrow in the case of visceral leishmaniasis, always carried inside macrophages (Manson-Bahr and Winslow, 1971). In all forms of the disease the underlying cellular elements which harbour the parasites are cells of the mononuclear phagocyte system which constitute the preferred site for Leishmania parasites to proliferate and survive as pathogens.

The susceptibility of various vertebrate hosts to infection with leishmanial parasites is not very well understood. Some laboratory animals like the rabbit are refractory to infection whereas other animals are susceptible to variable degrees, ranging from local skin involvement to complete visceralization (Stauber, 1970). Little is known about the factors that make a given Leishmania species infective to a certain species of host animal. Even among the different strains of a species of host there is considerable variation as to susceptibility to a given species of Leishmania (Schnur et al., 1973).

Several factors are believed to influence the survival and reproduction of parasites inside the vertebrate host. These include the local skin temperature, sex and nutritional state of the host (Stauber, 1970). Species specificity also governs the infectivity of Leishmania to some vertebrate hosts as in the case of cutaneous leishmaniasis of laboratory rodents. Thus, Leishmania enriettii, which causes a cutaneous infection in guinea pigs is not infective to mice while L. tropica which causes a similar condition in mice is not infective to guinea

pigs. This phenomenon was correlated to the ability of parasites to survive inside macrophages of the host animals, (Mauel et al., 1974). Different strains of inbred mice also show a great variability in their susceptibility to infection with a certain species of Leishmania. In the case of L. donovani this phenomenon was found by Bradley (1974) to be genetically determined and controlled by a single gene or a tightly linked group of genes. The susceptibility of a certain strain of host to infection was not apparently related to the ability of the host to mount an immune response against the parasite. A phenomenon of similar nature was reported by Preston, on the infectivity of L. tropica to mice (in Bryceson, 1975). It is interesting to add that despite the presence of natural antibodies and other substances in the sera of various animal hosts, that can agglutinate and lyse various Leishmania parasites (Ulrich et al., 1968; Schmunis and Herman, 1970), the survival of these parasites inside the same hosts is not apparently compromised by those factors.

Recovery from leishmanial infections is in general a slow and often inefficient process. It is thought to be produced mainly by cell mediated immunity (Bryceson, 1975; Preston and Dumonde, 1976) although antibodies are thought to play an enhancing role (Belehu et al., 1976; Preston and Dumonde, 1976). It is usually followed by long lasting immunity to reinfection

with the same parasite (Guirges, 1971; Heyneman, 1971, Manson-Bahr, 1971).

A principal feature of a vertebrate host's protective armoury against invading microorganisms are the cells of the mononuclear phagocyte system. These cells have been recognised since the time of Metchnikoff as highly phagocytic scavenger cells capable of ingesting and killing a wide variety of microbes. Nevertheless, Leishmania and a number of other intracellular microorganisms such as Mycobacterium, Toxoplasma, Besnoitia and Trypanosoma survive and grow inside macrophages which are themselves supposed to rid the host of such parasites, (Jones, 1974; Trager, 1974). Many of these parasites have evolved various mechanisms to survive otherwise hostile environmental conditions inside a phagocyte (Trager, 1974; Bloom, 1979). Toxoplasma parasites have achieved this goal by preventing the fusion of lysosomes with the phagocytic vacuoles in which they live (Jones and Hirsch, 1972). Some trypanosomes escape from phagolysosomal vacuoles into the cytoplasm of the macrophage where they would be safe from the detrimental effects of lysosomal products, (Tanowitz, et al., 1975).

In the case of Leishmania no such definite mechanism has been discovered to explain their ability to survive inside the macrophage. After ingestion they remain inside phagocytic vacuoles where they multiply and survive despite the discharge

of lysosomal granules into those vacuoles (Alexander and Vickerman, 1975; Chang and Dwyer, 1978). Ultrastructural studies of intracellular amastigotes show that they are surrounded by a host membrane which is believed to aid in their nutrition but seems to play no role in their refractoriness to lysosomal injury (Chang and Dwyer, 1978). A recent report by Chang (1978) has shown failure of lysosomal fusion in human skin fibroblasts infected with Leishmania. However, the significance of this phenomenon in overall parasite survival needs further assessment. Another possible mechanism has been suggested by Sharma et al. (1978) who have found that L. tropica promastigotes suppress the in vitro blastogenic response of lymphocytes to mitogen and antigen from the same parasite. They propose that the suppresive action of L. tropica on the immune response can facilitate parasite survival in macrophages.

Since leishmanial amastigotes are capable of withstanding the degradative effect of lysosomal products, an intracellular environment is clearly more favourable for their survival than an extracellular one where they would be exposed to the inhibitory effect of various serum factors. It is not clear why Leishmania and a number of intracellular parasites have chosen the macrophage as their site of propagation. It is possible that the extraordinary ingestive capacity of the macrophages have rendered them a suitable

target for parasite invasion. However, before entry, parasites must be able to interact with the macrophage surface in some biochemical way that would open the door for their invasion into the cell.

A successful parasite is one that neither evades the immune response of its host completely nor is too susceptible to that response. In either case the survival of the parasite could be jeopardized either by the death of the host if its escape from the host defence mechanism is perfect, or by its early destruction if it is too much exposed or susceptible to the host's protective armoury (Bloom, 1979). In this respect many leishmanias seem to have achieved a fine balance between their survival, multiplication and spread from cell to cell on the one hand and their evasion from the immune response of the host on the other (Garnham and Humphrey, 1969).

The various forms of leishmaniasis could therefore be visualized as the balance between two forces. The duration of the infection and its accompanying clinical and pathological features would depend on the capacity of the parasite to survive inside cells of the mononuclear phagocyte system on one hand and the development of the host's immune response that is directed against such invading organisms on the other. An effective approach that aims at the control of leishmanial infections requires a better understanding of the two aspects of the leishmania-host interaction that shape the outcome of

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the infection.

In the past decade or two the principal approach to attain such a goal has mainly been confined to attempts at understanding the mechanism of recovery from leishmaniasis. Less attention has been paid to study the mechanisms by which Leishmania parasites have ensured their perpetuation inside the vertebrate host at various stages of the infection. However, studies aiming at the elucidation of the mechanism of recovery from leishmanial infections have not so far been very fruitful. There is no firm evidence of a protective role for antibodies and the role of lymphocytes cytotoxic to parasites or parasitized macrophages is still uncertain. Immunity to reinfection is likewise very little understood, (Mauel et al., 1974, Bryceson, 1975). In the laboratory model for cutaneous leishmaniasis, it has been found that activated macrophages obtained from animals recovered from a leishmanial infection, destroy parasites in vitro that are not infective to non-immune animals of the donor host species in vivo, whereas they are unable to destroy parasites of the species from which the donor animal had recovered. This applies to L. enriettii which is infective to guinea pigs but not mice and L. tropica which is infective to mice but not guinea pigs, (Mauel et al., 1974 , 1975). This would probably explain the host specificity of the two species of parasites but not the mechanism of immunity to reinfection with Leishmania.

In the face of limited success in the field of leishmanial immunology the obvious alternative would be the study of the mechanisms of parasite survival in the host. Since macrophages are the preferred site for the survival of leishmanial parasites, their interaction with macrophages assumes a special significance in such an area of investigation.

An obvious requirement for the entry and colonization of host cells by parasites is their ability to attach successfully to the host cell's surface. The recognition by parasites of specific receptors on the host cell surface could help to trigger their internalization and thus open the door for them to reach their preferred habitat. After successful entry, however, parasites usually multiply to a point where the infected macrophage is no longer able to support their growth and eventually disentegrates. Parasites from disentegrating macrophages are then taken up by uninfected macrophages that are present at the site of infection or have been mobilized to the area by the host's cellular response, (Zuckerman, 1975; Chang and Dwyer, 1978). Hence, parasite attachment and entry into host cells is not merely an initial step for the establishment of an infection but is an on-going process on which parasite survival is continuously dependent. Therefore, the ability of parasites to adhere successfully to the macrophage surface assumes a key role

in their survival as intracellular parasites.

It is contemplated that understanding the mechanisms of recognition and internalization of parasites by macrophages could provide us with new possibilities in the control of leishmanial infections, in addition to the existing schemes of chemotherapy. It could take the form of a drug that would prevent parasite attachment or entry into macrophages by blocking a necessary step in these processes. Parasites deprived of their privileged sites for survival and reproduction would be less able to propagate and more exposed to the inhibitory action of lethal factors found in the extracellular fluids. A similar mode of action has been suggested for the drug Promethazine hydrochloride that is used in the treatment of erythroblastosis (Gusdon and Witherow, 1973). This drug which inhibits phagocytosis and macrophage oxidative metabolism (DeChatelet et al., 1976) was also found to suppress the phagocytosis of Rh positive red blood cells by fetal macrophages and its ameliorative effect on erythroblastosis was linked to these inhibitory effects, (Gusdon et al., 1974).

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Until recently, studies of host-parasite relationships in leishmaniasis at the cellular level have been confined mainly to observations on the mode of parasite entry into host cells and the subsequent intracellular survival of parasites in

vitro. These consisted of time lapse observations with the phase contrast microscope (Pulvertaft and Hoyle, 1960; Miller and Twohy, 1967; Akiyama and Haight, 1971), or ultrastructural studies with transmission electron microscopy (Akiyama and McQuillen, 1972; Lewis, 1974; Merino et al., 1977; Chang and Dwyer, 1978).

The fact that leishmania parasites invade cells that are highly phagocytic in nature and thus capable of ingesting a variety of foreign particles has raised the question whether parasites penetrate macrophages actively or are passively engulfed by phagocytes. The strong predilection of parasites to macrophages and their attachment by their flagella has suggested the possibility that parasites play an active part in their entry, (Lewis, 1974; Merino et al., 1977; Chang, 1978) whereas the morphological features of the macrophages response to parasite adherence favour engulfment as the mechanism for parasite entry (Miller and Twohy, 1967; Chang and Dwyer, 1978). Although most recent views favour engulfment as the method of parasite entry, (Jones, 1974; Trager, 1974; Chang and Dwyer, 1978), certain aspects of parasite internalization such as flagellar adherence and the role of the parasites' metabolism in the process of entry need further clarification.

In contrast, surface phenomena in Leishmania-macrophage interactions have received much less attention. Very little is known about the surface properties of parasites or the physiological requirements of macrophages that promote parasite attachment and trigger their internalization. In this respect our knowledge is less precise than what has been developed for other intracellular pathogens such as viruses and malaria parasites (Southgate, 1976). A few recent studies have approached this problem and produced some preliminary findings on the surface interactions of Leishmania (Chang, 1977) and Trypanosoma (Baker and Liston, 1978) with macrophages. These studies suggest that parasite attachment is not mediated by the familiar macrophage surface receptors for antibody or complement. The recent advances in cellular physiology and cell surface chemistry make the study of surface recognition phenomena in Leishmania-macrophage interactions an interesting exercise that may also shed some light on phagocyte surface properties.

Since promastigotes are the forms in which parasites make their first encounter with host-cells in natural conditions, they are the obvious choice for a starting point in a study of the interaction of Leishmania with macrophages. Their transformation usually takes place after they have entered the host cells (Akiyama and McQuillen, 1972; Chang, 1978). In parasite attachment studies, the application of rigorous

biochemical conditions and the requirement for unlimited numbers of parasites can only be achieved with the use of promastigotes which can be grown in semi-defined media in large quantities. Furthermore, no surface antigenic difference of any significance has been found between promastigotes and amastigotes (Dwyer et al., 1974; Doyle et al., 1974), which further justifies the use of promastigotes in attachment studies. On the other hand, the size and flagellar motility of promastigotes make them a more interesting object of study in parasite entry whereas the mode of entry of a small round shaped amastigote would not be expected to differ much from the way in which most inert particles are internalized.

To study parasite attachment, the monolayer technique for the quantitative measurement of particles by phagocytes (Michell et al., 1969) was used in all experiments. The estimation of parasite adherence was based on Giemsa stained preparations. This method is considered adequate in studies of attachment phenomena since stained parasites are readily recognizable under the light microscope and statistically reliable estimates can be made by counting a moderate number of infected cells in each preparation. This method also provides the possibility of performing certain experimental procedures such as the application of various surface reagents to monolayers and their removal before the assay. It also permits the study of parasite adherence under altered chemi-

cal conditions.

In a study on L. donovani-macrophage interactions, Chang (1977) has utilized a modification of the monolayer technique in which radiolabelled parasites were used and parasite adherence to macrophages was measured according to the increase in the radioactivity of the monolayers. While this method eliminates the laborious procedure of counting parasites under the light microscope it introduces at the same time a series of disadvantages which outweigh the only advantage that such a technique would give. As a result some aspects of parasite macrophage interaction that can only be monitored by direct microscopic observation are by-passed by this method. These are the percentage of infected macrophages, the topographic distribution of adherent parasites and the viability and morphological integrity of the macrophages as a whole. This method cannot also distinguish nonspecific adherence of parasites to the coverslip surface from specific adherence to macrophages.

The technique of scanning electron microscopy provides us with a new tool in investigating the process of parasite entry. While phase contrast microscopy enables the examination of live preparations and gives a stereological picture of the process of parasite entry, its limited power of resolution is not sufficient to reveal the finer morpholo-

gical features of the phagocyte's activity. On the other hand transmission electron microscopy provides a higher degree of ultrastructural detail but at the same time fails to resolve some of the problems in parasite macrophage interactions which are of topographic or morphological nature. From observations based solely on thin section preparations it is exceedingly difficult to reconstruct the rheological aspects of the macrophages' response nor is it possible to visualize the overall orientation of parasite macrophage contacts.

The use of scanning electron microscopy combines the advantages of the two methods of observation previously used, by providing high ultrastructural detail in images that reflect three dimensional situations.

Armed with these two basic experimental techniques, the initial phases of the in vitro interaction of promastigotes of Leishmania tropica with macrophages were investigated. In the study of parasite attachment the factors to which special attention was given were: a) the macrophage's intracellular metabolism; b) surface structures of parasites and macrophages and c) the extracellular chemical requirements for parasite adhesion.

The role of macrophage intracellular metabolism was studied by means of metabolic inhibitors having well documented

effects on cellular physiology. The role of cell surface structures was tested by means of membrane-probes whose effect is believed to be confined mainly to the cell surface. Extracellular requirements for parasite binding were assessed by the use of chemically defined assay media from which various ingredients could be omitted.

The entry of parasites into macrophages was studied by examination of macrophage monolayers exposed to parasites for various intervals of time. The effect of certain metabolic inhibitors was also studied morphologically in an attempt to define the role of the macrophage in parasite entry.

MATERIALS AND METHODS

1. Culture Media

a) Medium for cultivation of Leishmania tropica

Promastigotes of L. tropica were cultivated in 4N biphasic blood-agar medium, prepared as described by Taylor and Baker (1968). The solid phase of the medium consisted of 4% blood-agar base No. 2 (Oxoid), 2% Bacto-agar (Difco) and 20% defibrinated rabbit blood. 4 gm. of blood-agar and 2 mg of Bacto-agar were dissolved in each 100 ml of distilled water by boiling. The liquid agar was dispensed to 100 ml Erlenmeyer flasks in 25 ml portions or bijoux bottles in 2 ml portions. The flasks were plugged with cotton wool wrapped in cheesecloth and the bottles were screw-capped, before being sterilised in an autoclave at 121° C and 1 kg/cm$^2$ pressure for 20 minutes. They were stored at 4° C until needed.

Whole blood was obtained from lop-eared rabbits. 0.5 ml of Hypnorm (Jansen Pharmaceutica, Beuse, Belgium) was injected i.v. into the rabbit to immobilize the animal and increase its peripheral blood flow. Then, using a 20 ml syringe and 26G needle, about 20 ml of blood was drawn from the artery that runs along the midline on the upper surface of the animal's ear. The blood was transferred into a sterile medical flat bottle containing glass beads and the bottle was shaken gently for 10 minutes. The defibrinated blood

was stored then at 4°C. The solidified agar gels were next melted and allowed to cool down to 45°C before the defibrinated blood was added to the molten agar in the ratio of 1:4 (v/v). The mixture was stirred gently and then transferred to 4°C to solidify. The liquid overlay of phosphate buffered saline (pH 7.3) containing antibiotics (500 u./ml benzyl-penicillin and 250 mg/ml streptomycin sulphate) was added to the solidified blood agar mixture in the ratio of 1:2 (v/v). The culture media, prepared under sterile conditions throughout, were then incubated at 37°C overnight, to check for sterility and allow the diffusion of nutrients from the solid to the liquid phase. If not in use, the media were kept in the refrigerator at 4°C. The incorporation of Bacto-agar which is not an essential ingredient of the growth medium conferred extra firmness to the solid phase and prevented excessive diffusion of erythrocytes to the liquid phase.

The composition of the blood-agar base No. 2, as stated by the manufacturers was:

| | |
|---|---|
| Proteose peptone | 15 g/l |
| Liver digest | 2.5 g/l |
| Yeast extract | 5 g/l |
| Sodium chloride | 5 g/l |
| Agar | 12 g/l |

and its pH approximately 7.4.

b) Medium for cultivation of macrophages

Macrophages were cultivated in vitro in complete tissue culture medium (TCM), consisting of Eagle's minimum essential medium (MEM) and 10% foetal calf serum. Eagle's minimum essential medium, (X10), foetal calf serum and sodium bicarbonate solution (4.4%) were all purchased from Wellcome Reagents Ltd., (Beckenham, Kent, UK). To prepare 100 ml of working strength Eagle's medium, 10 ml of X10 Eagle's (MEM) and 2.5 ml of $NaHCO_3$ solution were added to 87 ml of sterile distilled water. Antibiotics, 500 units/ml of benzylpenicillin BP and 250 mg/ml of streptomycin sulphate were always added to this medium to prevent bacterial growth.Foetal calf serum was added when required.

The composition of a working strength of serum free Eagle's MEM as given by the manufacturers was:

| Inorganic Salts | mg/l | Vitamins | mg/l |
|---|---|---|---|
| Sodium chloride (NaCl) | 6800.0 | Aneurine hydrochloride | 1.0 |
| Potassium chloride (KCl) | 400.0 | Choline chloride | 1.0 |
| Calcium chloride ($CaCl_2$) | 200.0 | Folic acid | 1.0 |
| Magnesium sulphate ($MgSO_4 7H_2O$) | 200.0 | Inositol | 2.0 |
| | | Nicotinamide | 1.0 |
| Sodium phosphate ($NaH_2PO_4 2H_2O$) | 150.0 | Calcium pantothenate | 1.0 |
| | | Pyridoxal hydrochloride | 1.0 |
| | | Riboflavin | 0.1 |

| Amino Acids | mg/l |
|---|---|
| l-arginine hydrochloride | 125.0 |
| l-cystine | 24.0 |
| l-histidine hydro chloride | 40.0 |
| di-isoleucine | 104.0 |
| di-leucine | 104.0 |
| l-lysine hydrochloride | 70.0 |
| dl-methionine | 30.0 |
| dl-phenylaline | 64.0 |
| dl-threonine | 98.0 |
| dl -tryptophane | 20.0 |
| l-tyrosine | 36.0 |
| dl-valine | 92.0 |

| Miscellaneous | mg/l |
|---|---|
| Glucose | 1000.0 |
| l-glutamine | 292.0 |
| Phenol red | 10.0 |
| Sodium bicarbonate ($NaHCO_3$) | 1,100.0 |
| Penicillin | 200,000.0 units |
| Streptomycin | 100,000.0 g |

2. Solutions

a) Earle's balanced salt solution (EBSS)

The ten times concentrated form of this solution was purchased from Wellcome Reagents Ltd. To make a 100ml working strength solution, 10 ml of X10 EBSS solution and 2.5 ml of 4.4% Sodium bicarbonate were added to 87.5 ml sterile distilled water. Its composition, per litre of working strength solution, as given by the manufacturers was·

| | |
|---|---|
| D(+) Glucose | 100 mg |
| $CaCl_2$ | 200 mg |

| | |
|---|---|
| KCl | 400 mg |
| $MgSO_{4.7}H_2O$ | 200 mg |
| $NaHCO_3$ | 1100 mg |
| NaCl | 6800 mg |
| $NaH_2PO_4H_2O$ | 140 mg |
| Phenol Red | 10 mg |

b) Glucose-free Earle's balanced salt solution (GF-EBSS)

This solution was prepared in the laboratory. It had the same composition as Earle's balanced salt solution, the only difference being the absence of glucose from its ingredients.

All of the salts except $NaHCO_3$ were dissolved in distilled water, dispensed into 150 ml medical flat bottles, sterilized in the autoclave at 121°C and 1 kg/cm$^2$ pressure for 20 minutes, and stored at 4°C. Before use, each 100 ml of solution was supplemented with 2.5 ml of 4.4% $NaHCO_3$ solution, which also contained the phenol red indicator.

c) Saline bicarbonate potassium glucose solution (SBK Gluc.)

This solution was prepared for use in experiments on the role of divalent cations. It is based on the formulae described by Rabinovitch and Destefano (1973) but with the addition of glucose.

The buffer solution had the following composition:

| | |
|---|---|
| $NaHCO_3$ | 1.1 g/l |
| NaCl | 9 g/l |
| KCl | 100 mg/l |
| Glucose | 1000 mg/l |

KCl and NaCl were dissolved in sterile distilled water. The solution was dispensed to 150 ml medical bottles and sterilized in an autoclave. Sodium bicarbonate (2.5% v/v of 4.4% (w/w) stock solution) and glucose were added to the solution after sterilization.

d) Phosphate buffered saline

This buffer solution was used as liquid overlay for 4N blood-agar medium and also as suspending medium for the washing of parasites. It was prepared according to the formula given by Cruickshank et al., (1975) and had the following composition:

| | |
|---|---|
| NaCl | 8.00 g/l |
| $K_2HPO_4$ | 1.21 g/l |
| $KH_2PO_4$ | 0.3 g/l |
| Distilled $H_2O$ | 1 litre |

The salts were dissolved in distilled water and the solution dispensed into 150 ml bottles and sterilized in the autoclave for 20 minutes. Antibiotics, in the proportion of 500 units/ml benzylpenicillin and 250 ug/ml streptomycin were added only after the temperature of the autoclaved solution had come down. This buffer solution usually gave a pH of about 7.2.

e) Phosphate buffer solution for Giemsa stain

The diluent for Giemsa stain was prepared from two solutions of phosphate salts.

Solution A ($\frac{1}{15}$ M solution of $Na_2HPO_4$) prepared by dissolving 9.47 g of anhydrous disodium hydrogen phosphate in 1 litre of distilled water.

Solution B ($\frac{1}{15}$ M solution of $KH_2PO_4$) prepared by dissolving 5.07 g of potassium dihydrogen orthophosphate in 1 litre of distilled water.

A working solution of buffer was prepared by mixing 7 parts of solution A with 3 parts of solution B. This mixture usually gave a pH of 7.4.

f) Sodium cacodylate buffer

This is a buffer of high osmolality, used both as a diluent for Karnovsky's fixative and for washing of specimens to be processed for SEM. 0.2M solution of the buffer was prepared by dissolving 4.2806 g of sodium cacodylate powder (Emscope Laboratories, London) in 100 ml of distilled water, to which 25 mg of $CaCl_2$ was also added.

g) EDTA solution

A 5 mM solution of the metal chelating agent ethylene-dinitrilo-tetracetic acid (Sigma, Chem. Ltd., St. Louis, M.O., USA) in SBK Glucose buffer was prepared by dissolving 0.1681 g in 100-1 of SBK buffer, from which a further dilution of 2 mM EDTA in the same buffer solution was also prepared.

3. Fixatives

a) Glutaraldehyde

A 25% aqueous solution of the fixative was obtained from Serva, Feinbiochemica (Heidleberg, Germany). The stock solution usually had a pH of 5, and it was important that it had remained acidic until dilution. At basic pH the dialdehyde tends to polymerize and lose some of its activity (Bowes and Cater, 1966). In preparing a working solution of glutaraldehyde, care was taken to avoid the introduction with any compound bearing amino groups, since the latter are the chief chemical groups that react with the carbonyl group of glutaraldehyde, the chemically reactive sites of the fixative.

b) Formaldehyde

37-40% solution of formaldehyde (BDH Chemicals Ltd., Poole, England), was diluted in solutions free of any amino groups, (EBSS usually), for use in fixation of parasites. The powder form of the fixative, paraformaldehyde (BDH) was used in the preparation of Karnovsky's fixative.

c) Karnovsky's fixative

A fixative of high osmolality described by Karnovsky (1965) was used for preparation of specimens for SEM. It consisted of 2% formaldehyde, and 2.5% glutaraldehyde, and 0.5 mg/ml $CaCl_2$ in 0.08 M sodium cacodylate. To prepare 50 ml of the

fixative, 1 g of paraformaldehyde powder was added to 25 ml of distilled water and heated gently until the temperature of the water reached 60°C. At this temperature 2-3 drops of 1N NaOH solution were added to dissolve the paraformaldehyde powder. The solution was then cooled and 5 ml of 25% glutaraldehyde was added to it. Finally 20 ml of 0.2 M cacodylate buffer solution, containing 0.5 mg/ml $CaCl_2$ was added.

Osmium tetroxide was obtained as a 2% solution from Emscope Laboratories, (London) and diluted twice with cacodylate buffer before being used.

Acton, "Arklone P", (1,2 trichlorotrifluroethane, was obtained form ICI (Mond Division, Runcorn, Cheshire, England).

4. Staining Solutions

Giemsa's stain

The staining solution was prepared by diluting commercially obtained Giemsa stain from Hopkin and Williams, (Searle Company) in phosphate buffer of pH 7.4 in the ratio of one drop to each ml of buffer.

Neutral red stain

A 0.1% stock solution of neutral red was prepared by dissolving 0.1 g of neutral red powder, obtained from G.T. Gurr (London, UK) in 100 ml of distilled water. A small volume of this solution was diluted 5 times in EBSS, to prepare a

1:5000 (w/v) solution of the stain in which the mouse peritoneal exudates were diluted for staining.

Colloidal carbon solution

A 1% dilution of india ink (Reeves & Sons Ltd., Enfield, Middx., UK) was made in complete TCM and used for labelling of macrophages to determine their proportion in monolayers of peritoneal exudate cells.

5. Chemicals and Reagents

a) Inorganic salts

The following inorganic compounds were obtained from BDH Ltd. (Poole, England) which whenever possible were of analytical reagent grade, 'Analar':

Magnesium ulphate ($MgSO_4 7H_2O$), Ammonium sulphate ($(NH_4)_2SO_4$ 99.5%), Calcium chloride ($CaCl_2$), Magnesium chloride ($MgCl_2$), Sodium azide ($NaN_3$), Sodium fluoride (NaF), Periodic Acid ($HIO_4, 2H_2O$, 50% w/w).

b) Metabolic inhibitors

The following reagents were obtained from Sigma Chemical Co., (St. Louis, M.O., USA):

Iodoacetamide, (anhydrous, crystalline), DNP, (2.4 Dinitrophenol), 90-95%, (Grade II), cycloheximide (3 (2 (3,5-Dimethyl-2-4 Oxocyclohexyl) 2 hydroyethyl) Glutarimide, Cytochalasin B, from Helminthosporum.

dematioideum (mol. wt. 479.6), Cytochalasin D from Metarrhizium anisopliae (mol. wt. 507.6) and Colchicine (mol. wt. 399.4).

c) Carbohydrates

The following sugars and sugar analogues were also obtained from Sigma Chemical Co. which were all of Sigma grade purity:

D (+) Xylose (mol. wt. 150), D (+) Galactose (crystalline, substantially free of glucose, mol. wt. 180.2) D (+) Mannose (mol. wt. 180.2) L (-) Fucose (6 Deoxy-L-Galactose, mol. wt. 164.2), 2 Deoxy-D Glucose (Grade III, mol. wt. 164.2) and D (+) Glucose

d) Macromolecular reagents

The following reagents were also obtained from Sigma Chemical Company.

Concanvalin A, Glucose and Mannose receptor binding lectin, obtained in lyophilized, salt free and highly purified form (Grade IV), substantially free of carbohydrates.

Trypsin (E.C. No 3.4.4.) from bovine pancrease twice crystallized, dialysed and lyophilized and substantially free of salts. Activity in BAEE units 10,000 u/mg of protein.

Neuraminidase (E.C. 3.2.1.18) from Clostridium per-

*fringens* purified Type V, prepared by salt fractionation, dialyzed and lyophilized powder, containing approximately 10% buffer salts NaCl and $KH_2PO_4$. Activity 0.17 units/mg protein.

6. Animals

Partially inbred hairless "shasha" mice were used for *in vivo* maintenance of parasites. Animals of both sexes and aged between 6-10 weeks were used. These animals are particularly suitable for producing infections with *Leishmania tropica*, since the lesions produced on the animals' bare skin can be easily monitored and biopsied. Outbred female TO white mice aged between 8-12 weeks were used as the chief source of peritoneal macrophages.

7. Parasites

a) Isolation and *in vitro* cultivation

The parasites used in this study were promastigotes derived from a stock of *Leishmania tropica* that had been isolated from the skin of a patient with 'Oriental sore' who contracted the disease in the Dead Sea area of Israel, on 8th January, 1971. After one week of *in vitro* cultivation in blood-agar medium, the parasites were frozen in liquid nitrogen at $-196^{\circ}C$ and the stabilates were stored in the cryobank of the Department of Medical Protozoology, designated as LUMP 344. To revitalize the parasites, the frozen

contents of three capillary tubes from the same stabilate were thawed, transferred into 4N blood-agar medium in bijoux bottles and incubated at 28°C. Motile promastigotes would usually become detectable after 4 days of incubation. Thereafter, the parasites were maintained in vitro by weekly subculture. To obtain large numbers of promastigotes needed for experimental studies, 4N culture media prepared in 100 ml conical flasks and each having 15 ml of liquid overlay were each inoculated with $10^6$ organisms. Parasites were harvested from the liquid overlay 4 days later when growth has reached the stationary phase.

b) Harvesting and quantitation

Promastigote-rich liquid overlays from several cultures were pooled, dispensed into 10 ml glass centrifuge tubes, and centrifuged once at 500 g for 10 minutes to precipitate immobile promastigotes and rosettes, and particulate material like agar particles and red blood cells. The supernatants which now contained predominantly single motile promastigotes were pooled and kept while the sediments were discarded. The pooled supernatant was diluted twice with phosphate buffered saline and centrifuged at 12000 g for 20 minutes in an MSE refrigerated high speed centrifuge. The supernatant was discarded and thepellet of promastigotes was resuspended in 1 ml of PBS. In experimental studies washing by centrifugation at

12000 was usually repeated three times. The number of parasites harvested in this way was estimated by hemocytometer counts on diluted samples as described by Lumsden et al., (1973). A drop of the promastigote-rich suspension was diluted 100 times with EBSS. To this a drop of 5% glutaraldehyde was added to immobilize the promastigotes. A small volume of the fixed preparation was introduced into the counting chamber of an improved Neubauer hemocytometer, and the promastigotes in the four corner squares were counted. The number of promastigotes per ml of the original suspension was derived from the following formula:

$$\text{Number of promastigotes/ml} = \frac{\text{Total count in the 4 squares}}{4} \times 10^4 \times \text{dilution factor } (10^2)$$

c) Maintenance of parasites in vivo

When not immediately required, or after 2-3 months of maintenance in vitro, parasites were reinoculated into susceptible mice, from which they were subsequently recovered by skin biopsy, and cultivated again in vitro in 4N medium.

To infect mice with L. tropica, promastigotes were washed once and resuspended in EBSS at a concentration of $10^8$ organisms/ml. Prior to inoculation the mouse was anaesthetized with ether and the area above the tail was disinfected with 70% ethanol. Using a 1 ml syringe and 26G needle, 0.05 ml

containing 5 x $10^6$ parasites was injected into the skin of the animal. A bleb filled with the injected liquid indicated a successful intradermal injection. Within 2 days a nodule would usually appear at the site of injection and ulcerate in two to four weeks. The dermal lesion would usually remain evident for about 3 months, after which it would heal, leaving a smooth area at the site of the lesion. The site of inoculation would be positive for parasites, as long as a lesion in the form of a nodule or an ulcer was present. A subcutaneous biopsy taken subcutaneously from the nodule or from below the periphery of the ulcer with the help of a sterile syringe and needle and inoculated into 4N medium in a bijoux bottle would usually yield promastigotes within 3-6 days.

7. Macrophages

a) Isolation of macrophages

Normal macrophages were obtained from the peritoneal cavity of unstimulated TO mice by the method of Stuart et al., (1973). Each animal was killed rapidly with ether and its abdominal fur damped with 70% ethanol. A small incision was made through the skin of the anterior abdominal wall that was lifted by a pair of forceps, and the fur stripped back. 3 ml of TCM was injected into the exposed peritoneum along the mid-anterior line, taking care to avoid puncture of the gut.

The injected fluid was circulated by gentle prodding of the abdomen. After 2 minutes, the injected fluid was aspirated by inserting the needle into the left flank and applying lateral traction, so that a pocket would be formed from which the fluid could be drawn. The peritoneal fluids from several mice were pooled into a sterile universal bottle immersed in an ice bath to prevent attachment of macrophages to the glass surface. Foetal calf serum was added to the pooled aspirate to a final proportion of 10% (v/v). The total number of macrophages in the collected peritoneal fluid was estimated from hemocytometer counts on a differentially stained preparation. To distinguish macrophages from other cells of the peritoneal fluid, diluted samples were stained with the vital dye neutral red. Within minutes, macrophages avidly take up the stain, whereas lymphocytes remain largely unstained (Genser and Howard, 1967). A small sample taken from the peritoneal aspirate pool was diluted four times with a 1: 10000 (w/w) solution of neutral red in Eagle's medium and neutral red positive cells were counted in the white blood cell area of the hemocytometer. The collected peritoneal cells usually yielded about $3 \times 10^6$ macrophages per mouse.

b) Technique for the <u>in vitro</u> cultivation of macrophages

The tissue culture tubes and coverslips were cleaned and steri-

lized according to the procedure recommended by Nordling et al., (1965). Coverslips were boiled in concentrated hydrochloric acid for 30 minutes and then thoroughly washed in distilled water. The coverslips were immersed for 2 minutes in two changes of absolute ethanol followed by two more immersions in diethyl ether. The clean coverslips were then distributed to tissue culture tubes, which were previously washed with Decon. The culture tubes containing the coverslips were sterilized in a hot-air oven at 175°C for 2 hours. Dry heat sterilization of glassware has been shown by Nordling et al., (1965) to be superior to steam sterilization in promoting cell-to-substratum attachment. Screw caps were replaced by steam sterilized rubber bungs to prevent the escape of $CO_2$ from the culture tube. The technique for the cultivation of macrophages was similar to the one described by Cohn and Benson (1965). The concentration of macrophages in the pooled exudate was lowered to $0.5 \times 10^6$ cells/ml by addition of complete TCM. The peritoneal cells were then seeded into the culture tubes at a proportion of $3 \times 10^5$ macrophages per $cm^2$ of the area of culture well. The culture tubes were gassed with 5% $CO_2$ in air briefly and sealed with rubber bungs. The cell cultures were incubated at 37°C overnight to allow the firm attachment of macrophages to the glass substratum. The following morning, non-adherent

cells were washed out by replacing the supernatant of the cultures with fresh complete TCM. It was found that the longer the supernatant of the original peritoneal cell suspension was left in the culture tubes, up to a maximum of 16 hours, the better the macrophage monolayer obtained. The quality of the monolayers were judged by the per cent of monolayer loss (Stuart et al., 1973) over an ensuing period of six days. Monolayers prepared in this way usually contained 95% macrophages, as judged by the colloidal carbon test (Stuart et al., 1973). Experiments on macrophage monolayers were always carried out within 24 hours of in vitro cultivation.

METHODS

I. Parasite Attachment to Host Cells

1. Binding Assay

a) Basic procedure

The macrophage monolayers were prepared one day before parasite binding assay was performed. After isolation from the peritoneal cavity of mice, the number of macrophages in pooled peritoneal fluid was estimated and the fluid was distributed to Leighton tubes containing 9 x 33 mm coverslips, which cover almost completely the base of the tubes' rectangular chamber. One million macrophages were seeded into each culture tube and the non-adherent cells were removed the following day by changing the medium. In the meantime promastigotes from 4 day old cultures were harvested, washed by centrifugation, and resuspended in complete TCM at various concentrations. The culture medium was removed from the macrophage cultures by a pasteur pipette and the parasite suspension was added to the macrophage monolayers. The Leighton tubes were gassed with 5% $CO_2$ in air, sealed and incubated in a 37$^{o}$C incubator for the appropriate period of time. In experimental studies, 3 macrophage monolayers were assigned to each experimental group. To terminate the parasite-macrophage interaction, the supernatants containing unbound parasites were removed and the macrophage monolayers were washed once with serum free Eagle's MEM

and wet fixed with absolute methanol with the coverslips bearing the macrophage monolayers still inside the Leighton tubes.

b) Calibration of the assay

The effect of several concentrations and periods of incubation on the extent of parasite binding was examined, to decide a standard parasite load and time of incubation to be used in all subsequent experiments. In deciding such a standard experimental dose, three factors were taken into consideration:

1. a parasite adherence value of sufficient magnitude to be compared with any experimentally obtained reduced results at a significant level;
2. an incubation period short enough to forestall any physiological deterioration of macrophages in abnormal conditions;
3. a parasite suspension that would have sufficient volume to be handled with reasonable accuracy, and dilute enough not to cause any damage to the macrophage monolayers.

After testing several parasite concentrations and periods of exposure to parasites, it was found that a parasite suspension having a concentration of $10^7$ organisms/ml and a volume of 0.5 ml per monolayer gave a high parasite adherence value

when incubated with macrophages for 20 minutes. This parasite load corresponded to a parasite-to-host cell ratio of 5:1. The rigorous application of these conditions was observed in most subsequent experiments on parasite-to-host cell binding.

c) Estimation of parasite attachment

The extent of parasite-to-host cell adhesion was estimated by counting the number of promastigotes associated with macrophages in Giemsa stained preparations under the light microscope. Evidence for specific biological adhesion was deduced from the absence of promastigotes from macrophage free areas in the coverslip bearing the monolayers. Whenever an even distribution of promastigotes over the entire area of the coverslip's surface, was seen suggesting non-specific physical adsorption , the result was considered false positive and the samples were discarded.

After fixation of the monolayer with absolute methanol, the coverslips were removed from the Leighton tubes and air dried at room temperature. They were then placed on a staining tray and stained with a 5% solution of Giemsa's stain for 20 minutes. They were next rinsed with tap water to remove excess stain and dried by incubation at 37°C for 1 hour. Finally, the coverslips were mounted on glass slides in Euparal vert with monolayer face down and kept overnight in

the 37°C incubator to allow hardening of the mounting medium.

The stained preparations were examined under the light microscope at 400X magnification. The number of promastigotes attached to 200 macrophages was counted in randomly chosen fields along the length of the coverslip. Within the same population of macrophages, the number of macrophages to which one or more promastigotes were attached was also counted. Parasite adherence was expressed in average number of parasites per macrophage, which was obtained as follows:

$$\text{Av. no. of parasites per macrophage} = \frac{\text{Total number of parasites}}{200}$$

The proportion of infected cells was obtained by dividing the number of infected macrophages by 2:

$$\text{Per cent infected macrophages} = \frac{\text{Number of infected macrophages}}{200} \times 100$$

$$= \frac{\text{Number of infected macrophages}}{2}$$

Counts made on two hundred macrophages in each specimen proved to be sufficient to give consistent results. Counting more than two hundred cells did not result in any improvement in the consistency of the results obtained. Similar satisfactory results were obtained by Allen and Cook (1970) in their study of attachment of bacteria to macrophages.

d) Statistical method for the evaluation of significance of difference

The significance of difference between any pair of samples was

assessed by the Wilcoxon Rank Sum test (Armitage, 1971). The two samples were combined together and values for each parameter were classified according to their overall rank in the combined group. Due to the small size of the samples the difference between any two samples were considered statistically significant only if the overall ranks of all the values in one group did not overlap with any of the values in the other group. In other words, the difference between any pair of samples was considered significant only if the lowest value in one group was higher than the highest value in the other. The degree of overlap in the ranks of values in one pair of samples, however, may be greater than in another pair of samples. For this reason the degree of overlap for each pair of intermingling samples was mentioned in the footnote at the bottom of each table as part of the statistical analysis of results. The degree of overlap is always expressed in intergers greater than zero. A degree of overlap equal to zero represents no overlap and a statistically significant result. The degree of overlap for any pair of small samples can be expressed in the following empirical formula:

$$\text{Degree of overlap} = \Sigma R - \Sigma R_1$$

where R is the observed rank of any value in the sample of higher average value and $R_1$ is the expected rank of any value in the same sample. For a sample with n = 3 $\Sigma R_1$ is equal to six.

2. Kinetic Studies

a) Effect of temperature

The effect of ambient temperature on the attachment of parasites to macrophages was studied by performing the binding assay at three different temperatures: 37°C, 24°C and 10°C. The temperature of the macrophage cultures and parasite suspensions were both equilibrated to the particular temperature by a 10 minutes pre-incubation, before performing the binding assay at that temperature. Binding assay at 37°C was carried out in an incubator. The room temperature in the laboratory was 24°C, the test at this temperature was, therefore, performed on the bench. To carry out tests at 10°C, a refrigerator having the same temperature was used as an incubator.

The effect of heat inactivation of parasites on their attachment to the host cell was also investigated. Parasites were inactivated by incubation at 45°C in a hot water bath for 15 minutes before being tested.

b) Fixation with glutaraldehyde or formaldehyde

Parasites were washed three times in PBS and resuspended in 0.01% solution of the fixative in EBSS, and incubated at room temperature for 30 minutes. The fixed parasites were washed three times with serum-free Eagle's medium to remove any free unreacted fixative from the suspending medium.

Amino acids found in Eagle's medium also provide the necessary amino groups to block any free carbonyl group on the surface of the fixed parasite. This is of particular concern with glutaraldehyde, since each molecule possesses two reactive sites. In the absence of an available amino group nearby, the second carbonyl group in the dialdehyde will remain free to react with an amino group on a protein molecule in the medium, or adsorbed onto the glass surface. The latter may give rise to chemical adsorbtion of the parasite to the entire surface of the coverslip. Hence, it is important too, to avoid the inclusion of any protein during fixation to prevent adsorption to the parasite's surface, due to the crosslinking action of glutaraldehyde.

Fixation of macrophages with glutaraldehyde or formaldehyde was carried out by replacing the macrophage culture medium with the same concentration of fixative in EBSS and incubating the culture for 30 minutes at 37$^{o}$C. The monolayers were then washed free of the fixative with three changes of medium, with serum free Eagle's medium MEM, and finally reincubated in complete TCM for 10 minutes before being tested for parasite uptake.

3. Studies with Metabolic Inhibitors

Treatment of macrophages with the inhibitors of energy metabolism and the cytoskeletal system was carried out at 37$^{o}$C

for 1 hour, prior to binding assay. Since, in most cases, the effect exerted by these metabolic suppressors is reversible, these inhibitors were also introduced into the binding assays, by incorporating them into the test suspension. Accordingly, parasites were resuspended in the same concentration of the inhibitor in complete TCM prior to testing. An exception was cycloheximide, an inhibitor of protein synthesis, which was applied only in pretreatment of macrophages.

a) Cycloheximide

The effect of cycloheximide on parasite adherence was studied by incubating macrophages with two concentrations of cycloheximide for a period of 24 hours prior to testing. Six hours after isolation, the macrophage culture medium was replaced with $10^{-4}$M and $10^{-5}$M solutions of cycloheximide in complete TCM. The macrophages were incubated for 24 hours in the presence of the inhibitors. Parasites were not treated with this inhibitor and the binding assay was performed in the absence of the inhibitor, immediately after the removal of the drug from the macrophage cultures.

b) Inhibitors of energy metabolism and cytoskeleton

The water soluble antimetabolites; sodium fluoride (NaF), sodim azide ($NaN_3$), potassium cyanide (KCN), iodoacetamide

and colchicine, were dissolved directly in complete TCM at an initial concentration of $10^{-2}$M from which further dilutions were prepared.

In the case of 2, 4, dinitrophenol and cytochalasins B and D, which are not water soluble, a different procedure was followed to incorporate them into complete TCM, which is an aqueous solution.

2, 4, Dinitrophenol was first dissolved in absolute ethanol to make a concentration of O.1M. From this stock solution, further dilutions in complete TCM were prepared.

Cytochalasin B or D were first dissolved in Dimethylsufoxide (DMSO) at a concentration of 1 mg/ml. From this solution, further dilutions of the drugs in complete TCM were made.

The use of non-aqueous solvents to solubilize these drugs in aqueous solutions necessitated the preparation of control solutions. These were blank solutions of complete TCM, containing the concentration of the non-aqueous solvent (ethanol or DMSO) that was found in the test solution containing the highest concentration of the drug.

4. Treatment of Parasites and Macrophages with Surface Reactive Agents

a) Sodium metaperiodate

The conditions of treatment of parasites and macrophages with

periodate were similar to those described by McClain et al., (1975). Parasites were washed 3 times with PBS, resuspended in 2 x $10^{-3}$M $NaIO_4$ in PBS, and incubated for 10 minutes at room temperature. They were washed again 3 times in PBS and resuspended in complete TCM to be tested for uptake by macrophages. Likewise, macrophage monolayers were washed 3 times with PBS, incubated in 2 x $10^{-3}$M $NaIO_4$ in PBS for 10 minutes at room temperature, washed again 3 times with PBS, and reincubated in complete TCM for about 30 minutes, before being tested for parasite binding in complete TCM. Using the same concentration of $NaIO_4$ McClain et al., (1975) found no adverse effect of this chemical on the physiology of in vitro-cultivated lymphocytes.

b) Trypsin

Parasites were washed 3 times with EBSS and resuspended in serum-free Eagle's MEM, containing 500 ug/ml trypsin, at a density of $10^7$ organisms $ml^{-1}$. The suspension was incubated at 37°C for 30 minutes and the parasites were then washed 3 times with Eagle's MEM and resuspended in complete TCM at a concentration of $10^7$/ml.

Macrophages were trypsinized by replacing the culture medium with 1 ml of Eagle's MEM, containing 500 ug/ml trypsin and incubating for 30 minutes at 37°C. The enzyme was removed by three changes of the medium with complete TCM. The

macrophages were tested immediately for parasite binding.

c) Neuraminidase

Washed parasites were resuspended in a solution of Neuraminidase in Eagle's MEM, containing the equivalent of 0.2 $\mu g/ml^{-1}$ activity, at a concentration of $10^7$ $ml^{-1}$. The parasites were incubated with the enzyme for 45 minutes at 37°C, washed three times with EBSS and resuspended in complete TCM at a concentration of $10^7$/ml and tested immediately for uptake by macrophages. In a similar manner, macrophage monolayers were incubated in Eagle's MEM, containing the same concentration of enzyme and incubated for 45 minutes at 37°C. The enzyme was then removed by changes of the medium with complete TCM. Tsan and McIntyre (1977) have reported 20% removal of total leukocyte sialic acid by a similar treatment with neuraminidase.

d) Concanvalin A

Washed promastigotes were resuspended in 10 $\mu g/ml^{-1}$ Con A in Eagle's MEM and incubated for 30 minutes at room temperature, then washed once with Eagle's MEM and resuspended in complete TCM. Macrophage monolayers were treated with 2 concentrations of Con A in Eagle's MEM 50 ug and 10 $\mu g$ $ml^{-1}$. The monolayers were incubated with the solution of lectin for 30 minutes, washed twice with Eagle's MEM, and reincubated in complete TCM. In one experimental group the assay, in addition to

preincubation, was carried out in the presence of 10 µg/ml Con A.

5. Biochemical Experiments

These experiments were aimed at studying parasite binding under defined chemical conditions. The basic experimental approach was the creation of chemically defined situations during the parasite-to-host cell interaction. In general, macrophage cultures were not preincubated in these experimental conditions for any length of time, to avert any physiological deterioration due to deficiencies in the composition of the modified media.

a) Media with different divalent cation contents

Parasites were washed twice in SBK glucose buffer solution, resuspended in the same buffer containing 5 mM of EDTA, and incubated for 10 minutes at room temperature, to eliminate traces of extra-cellular divalent cations.

The buffer itself is reasonably free of any divalent cations like calcium and magnesium, which can be added separately at the desired concentrations. The use of sodium bicarbonate as buffering agent in this case is important, since other organic buffers, like immidazole or Tris, can also act as chelators of divalent cations and affect their concentrations in the solution, (Rabinovitch and Destefano, 1973).

The parasites were then washed 3 times in SBK Glucose buffer and resuspended in 4 types of SBK Glucose buffer solutions, having the following compositions:

1. SBK Glucose + 2mM EDTA
2: SBK Glucose + 1mM $CaCl_2$
2. SBK Glucose + 1mM $MgCl_2$
3. SBK Glucose + 1mM + 1mM $MgCl_2$

Macrophage monolayers were washed twice with SBK glucose buffer then incubated with 5mM EDTA in SBK glucose buffer for 10 minutes at room temperature. The EDTA solution was removed by three washes of SBK Glucose before addition of the parasite suspension.

b) Media with different hexoses

The role of glucose and other hexoses in parasite uptake by macrophages was studied by performing the binding assay in various solutions of the hexose in glucose-free EBSS supplemented with 10% dialyzed foetal calf serum.

Parasites were washed three times with PBS and resuspended in five glucose-free EBSS solutions, containing 10% dialyzed FCS and each of the following hexoses: glucose, xylose, galactose, mannose and fucose at 5mM concentration. Glucose-free EBSS, containing only dialyzed serum served at control. Before addition of parasite suspensions the macrophage monolayers were washed three times with glucose-free EBSS.

c) Studies on the effect of serum

Parasites were washed three times with EBSS and resuspended either in Eagle's medium supplemented with 10% calf serum, or serum-free Eagle's medium. Macrophage monolayers were washed three times with serum-free Eagle's medium before being reincubated in serum-free medium or being tested for parasite uptake in serum-free conditions.

i) Dialysis of serum

Dialysis of non-inactivated foetal calf serum was performed in Visking dialysis tubing under conditions described by McPhie (1971). About 30 cm of cellophane dialysis tubing (Visking dialysis tubing, diameter, 8/32) was soaked in distilled water. A knot was made at one end of the tubing which was filled with distilled water and gently squeezed from the top to make sure that no leakage could take place from the bag. The bag was emptied and 5 ml of foetal calf serum was introduced, and the bag sealed with a knot 2 cm above the surface of the serum. The bag was immersed in dialysis liquid inside a 100 ml medical flat bottle. The liquid used for dialysis of serum was phosphate-buffered saline to which antibiotics (500 u/ml benzylpenicillin and 250 µg/ml streptomycin) were added to prevent bacterial contamination. Dialysis was allowed to proceed for three days at 4°C. During this period the dialysis liquid was changed by transfer-

ring the dialysis bag to a new bottle containing fresh liquid. On the third day of dialysis, the bag was removed from the bottle and opened by making an incision below the upper knot. The retentate was aspirated by a pasteur pipette and stored at $4^{o}C$.

ii) Heat denaturation of serum

For heat inactivation, small samples of foetal calf serum were distributed into sterile screw capped tubes and incubated in a hot water bath for 30 minutes at various degrees of temperature.

iii) Salt fractionation of serum

The fractionation of the proteins in foetal calf serum was accomplished by precipitation with ammonium sulfate (AS). The conditions for fractionation by this method were similar to those described by Heide and Schwick (1978).

To prepare a saturated solution of ammonium sulfate, 100 g of the salt was heated with stirring in 100 ml of distilled water at $50^{o}C$ until most of the salt was dissolved. It was then allowed to stand overnight at room temperature, until the undissolved salt turned crystalline. This preparation having excess solid AS represented a 100% saturation. A graded series of partially saturated solutions having 30%, 40%, 50%, 60% and 70% saturations were also prepared to be used in washing precipitated fractions of the serum.

To precipitate a serum sample at 50%, one volume of saturated AS solution was added to one volume of serum and the mixture was allowed to stand for 30 minutes and centrifuged, then for 30 minutes at 10,000 g to pack the precipitated proteins. The supernatant was decanted to another container and designated solution A. To precipitate the serum proteins at other saturations, the fraction obtained by 50% saturation was dissolved in 30% saturated AS solution and centrifuged at 10,000 g. The resulting precipitate was termed the 30% fraction and the supernatant solution B. To both solutions A and B, further volumes of 100% saturated AS solutions were added to raise their saturation to 60% and 40% respectively. The volume of saturated solution added to the supernatants to achieve a higher degree of saturation can be calculated using the following formulae:

Volume (in ml) of saturated AS solution required

$$= V \frac{(S_2 - S_1)}{1 - S_2}$$

Where V is the volume of the AS solution of the lower saturation expressed in millilitres, $S_1$ is the saturation of the initial AS solution expressed in fractions of one, and $S_2$ is the saturation aimed at, also expressed in fractions of one.

The precipitates obtained from 40% and 60% saturation of the supernatants from solution A and B were raised further to 70% and 50% respectively by addition of more 100% saturated AS solution. Once more the precipitates obtained by these higher saturations were separated by centrifugation, whereas

the supernatants were discarded at this stage. All precipitates were washed once by centrifugation in the corresponding concentrations of saturated solutions. Each of the obtained fractions was redissolved in 5 ml of distilled water, which was the volume of the whole serum at the start of fractionation. The solutions of the different fractions were extensively dialyzed against distilled water under conditions similar to those described in the section on dialysis of serum, to get rid of residual ammonium sulphate in the solutions of the serum fractions. The different fractions were then incorporated in Eagle's medium in the ratio of 10% to compare their activity with whole serum.

II The Study of Parasite Entry

1. Preparation of Specimens for Scanning Electron Microscopy

The entry of parasites into macrophages *in vitro* was studied by scanning electron microscopy. After specified periods of interaction, the macrophage monolayers were washed 3 times with serum-free Eagle's medium to remove serum proteins that might adsorb to monolayers during fixation, and result in some loss of clarity in the surface features of the cells (P. Rowles, personal communication). The monolayers were then fixed with Karnovksy's fluid for 30 minutes. After fixation, the monolayers were washed twice with sodium cacodylate

buffer, while inside the culture tubes, and fixed again in 1% osmium tetroxide for five minutes. The monolayers were washed again with cacodylate buffer and then dehydrated through a graded series of ethanol concentrations, starting from 50% with 10% steps to absolute ethanol. The coverslips bearing the monolayers were transferred from one solution to the next using a pair of forceps with fine tips. The coverslips were left in each ethanol solution for 3 minutes, a period of time which is sufficient to allow for phase transition to take place in these rather thin tissues. Special care was taken to transfer the coverslips quickly, to prevent drying of the monolayers. Next, the monolayers were impregnated with Arcton in two steps: first by two changes of 50;50 mixture of ethanol/Arcton, followed after 20 minutes, by two changes in pure Arcton. Changes of the ethanol-Arcton solvents were made with a pasteur pipette, leaving the coverslips inside the tubes. The coverslips were kept in pure Arcton until being dried at the critical point in a Polaron critical point drying apparatus with liquid $CO_2$. Finally, the dried specimens were mounted on aluminium stubs and shadowed with gold in an argon atmosphere using a Polaron diode sputter E 5000. The gold embedded specimens were ready for examination with the scanning electron microscope. Scanning of the specimens was made in a Jeol JSM 35 model scan-

ning electron microscope, operating at 18-20 KV and at a tilt angle of 20°.

2. Experimental Studies of Parasite Entry

The entry of normal and fixed parasites into macrophages was studied in monolayers exposed to parasites for various periods of time. Monolayers were prepared on 12 x 12 mm coverslips in flat bottomed culture tubes. Macrophages were given a pulse of parasites at 10:1 parasite to macrophage ratio for 2 minutes. The unbound parasites were then removed by washing with complete TCM. The monolayers were reincubated in parasite-free complete TCM for various periods of time before fixation. With normal parasites the periods of interaction studied were 2, 4, 6, 8 and 10 minutes and ½. 1, 2 and 4 hours. At the end of these periods, the monolayers were fixed and processed for scanning electron microscopy. With fixed parasites the periods of interaction studied were ½, 1, 2 and 4 hours.

The effect of temperature and cytochalasin D on parasite entry was also studied. The experimental conditions were, up to fixation, similar to those described in experimental procedures in parasite binding studies.

RESULTS

## I Physical and Kinetic Aspects of Parasite Binding

### 1. Relationship of Parasite Binding by Macrophages to the Time of Exposure to Parasites

A first step in choosing standard conditions for the assay of parasite uptake would be to determine the period of time needed by macrophages to take up a substantial number of parasites.

To investigate the relationship between the period of exposure to parasites and their binding by macrophages, parasites were incubated with macrophages for increasing intervals of time up to a period of 1 hour. One ml of parasite suspension containing $5 \times 10^6$ promastigotes was added to each macrophage monolayer consisting of approximately $10^6$ cells. After incubation at $37^{\circ}C$ for 5, 10, 20, 30 and 60 minutes excess parasites were removed from duplicate cultures, and the washed monolayers were fixed and stained with Giemsa.

In initial studies on the kinetic aspects of the interaction of parasites with macrophages the percentage of infected cells was used as a measure of parasite uptake. Cells having one or more parasites attached were counted and their proportion in a random population of macrophages was calculated.

Figure 1 depicts the increase in the proportion of infected

cells as a function of the period of exposure to parasites. It was noticed that the rate of this increase was very high in the initial phase of the interaction period especially in the first 5 minutes, when the biggest increase in the proportion of infected cells took place. The rate of this increase declined in the ensuing periods until at 30 minutes, when it nearly levelled out.

## 2. Relationship of Parasite Binding to the Volume of the Assay Medium

Before determining the volume of parasite suspension to be used in the assay of parasite binding it was decided to find out whether changes in the volume of parasite suspension might affect the binding of parasites by macrophages *in vitro*, at the same parasite to macrophage ratio.

Figure 2 shows the results obtained by exposing macrophages to parasite suspensions in three different volumes for three different durations of time. It was found that the proportion of infected cells after ten minutes of exposure to parasites was inversely proportional to the volume of parasite suspension. After twenty minutes of exposure however, this relation was somewhat reversed where the highest proportion of infected cells was obtained with a volume of 0.5 ml, followed by 0.25 ml and 1 ml. After thirty minutes, this relationship was even more reversed in that although a volume of 0.5 ml gave the highest proportion of infected cells, this was followed by 1 ml, the largest volume instead of 0.25 ml.

If volumes of 0.5 ml and 1 ml were taken as a pair it could be seen that the smaller volume would always produce a higher proportion of infected cells at all periods of exposure. This suggests an inverse relationship between parasite uptake and volume of parasite suspension. The smallest volume of 0.25 ml, however, did not obey this relationship at twenty and thirty minutes of exposure since no appreciable increase took place at those periods of exposure. Two reasons can explain this anomaly. First, a volume of 0.25 ml can produce such a high concentration of parasites that it could be detrimental to macrophages. Secondly, in such a small volume of suspension the availability of parasites to the macrophage monolayer situated on one surface of the coverslip might be seriously affected.

From these two experiments it appears that the kinetics of parasite uptake is affected both by the period of time during which the macrophages are exposed to parasites and the volume of medium in which parasites are suspended. Such a relationship indicates the need for uniformity of conditions for an assay for parasite binding in experimental studies.

It was decided that among the three volumes tested 0.5 ml would be the optimum volume for a standard assay since it resulted in the highest level of parasitization attained earlier than in the two other cases. A period of twenty

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minutes for exposure to parasites was thought to be appropriate since exposure for longer periods did not result in any real increase in macrophage parasitization. Such a relatively short period of exposure has an obvious advantage when a certain experimental design does not fulfil the normal culture requirements of macrophages.

2. Effect of Temperature on Parasite Uptake

The influence of ambient temperature on parasite binding was studied by the exposure of macrophages to parasites at three different temperatures. Macrophages were challenged with parasites at 37°, 24° and 10°C, in otherwise standard assay conditions. Results summarized in Table 1 show that parasite binding is strongly affected by temperature. The number of parasites attached at 24°C was equal to about 50% of the number attached at 37°C. At the lower temperature of 10°C the number of attached parasites had further dropped by about 80%. There was also a concomitant but less sharp decrease in the percentage of macrophages infected with parasites as a result of challenge at lower temperatures. At 24°C macrophage parasitization was reduced by 20% whereas at 10°C it was reduced by 60%. Since cells are considered infected irrespective of the number of parasites associated with them, an overall reduction in parasite binding will, therefore, be only partially reflected in the proportion of infected cells. If

the average number of adherent parasites is plotted as a function of temperature (see Figure 3), the distribution of the values obtained at the three temperatures can be most accurately represented by a straight line. This indicates that the rate of increase in parasite binding as a function of suboptimal temperature is constant and suggests that the nature of interaction between parasites and macrophages is probably biochemical.

It could be argued that the decrease in parasite uptake by low temperature is caused by an increase in the viscosity of the assay medium. Such a possibility was investigated by incorporating Ficoll, a substance of high viscosity into the assay medium. It was found that parasite binding by macrophages at 37$^{o}$C was hardly affected by the presence of 10% Ficoll.

The influence of low temperature on parasite motility was also investigated by examining a drop of cold parasite suspension on a slide freshly removed from an ice bath. It was assumed that such a procedure could maintain a temperature of 10$^{o}$C on the surface of the slide for at least one minute during which parasites could be examined microscopically. Unless maintained below 10$^{o}$C for a long period parasites showed no apparent loss of motility.

3. Effect of Chemical Fixation on the Attachment of Parasites to Macrophages

a) Effect of glutaraldehyde

The temperature sensitive aspect of the binding of parasites by macrophages was further investigated by means of separate prefixation of parasites and macrophages, to identify the temperature sensitive partner in the parasite host cell interaction.

The bifunctional cross-linking reagent glutaraldehyde was thought to be a suitable tool for such a purpose. It is active at low concentrations and usually preserves the surface antigenic structure of the cells to a great extent. Being a more stable reactant than other aldehyde fixatives its action is irreversible which makes possible the removal of the fixative after separate pretreatment of parasites and macrophages. Results shown in Table 2 describe the effect of fixation of macrophages and parasites with glutaraldehyde on parasite uptake. Fixation of parasites with 0.01% glutaraldehyde did not affect significantly their uptake by untreated macrophages. Both the average number of parasites per macrophage and the percentage of macrophages infected with fixed parasites were similar to the corresponding values obtained from untreated parasites. Fixation of macrophages with the same concentration of the chemical however, almost

completely abolished their ability to bind parasites. The number of parasites attached to fixed macrophages was reduced to a mere 8% of the control while the percentage of infected macrophages dropped to 15%.

The effect of various concentrations of glutaraldehyde was studied next, to find out the minimum concentration of the fixative that could still substantially inhibit parasite binding. Macrophages were pretreated with four different concentrations of the fixative, ($10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$M) and then challenged with normal parasites. As shown in Table 3 the inhibitory effect of the fixative was strong down to a concentration of $10^{-5}$M. At the next lower concentration of $10^{-6}$M, however, the effect of the fixative was almost negligible and parasite binding was restored to near control levels.

b) The effect of other aldehyde fixatives on parasite binding

Two other aldehyde fixatives, acetaldehyde and formaldehyde, were also tested for their effect on parasite binding. In contrast to glutaraldehyde which has two aldehydic groups, each of these two compounds possesses only one such group.

Upon pretreatment of macrophages with two concentrations of acetaldehyde it was found that a very high concentration of this fixative was needed to obtain any substantial inhibition

of parasite binding (see Table 4). Treatment of macrophages with $10^{-2}$M acetaldehyde caused a 72% reduction in the number of parasites attached to macrophages and a 45% inhibition of macrophage parasitization. Treatment with a $10^{-3}$M concentration of the fixative had very little effect on the parasite binding activity of the macrophages. Values for both parameters of parasite binding, in this case, did not differ significantly from control values.

Unlike acetaldehyde, pretreatment of macrophages with various concentrations of formaldehyde showed that this fixative could inhibit parasite binding throughout a wide range of concentrations (see Table 5). A strong inhibition was obtained at the relatively high concentrations $10^{-3}$M and $10^{-4}$M. Parasite binding was reduced by 79% and 72% respectively, and macrophage parasitization by 75% and 34% respectively. At the next two lower concentrations of $10^{-5}$M and $10^{-6}$M however, the inhibition shown by the fixative was less drastic, although it retained a substantial part of its activity. Parasite binding was reduced by 40% and 28% respectively, and macrophage parasitization by 18% in both cases.

Figure 4 compares the effects of different molar concentrations of glutaraldehyde and formaldehyde on parasite binding. The two fixatives showed similar inhibitory effects at concentrations of $10^{-3}$M and $10^{-4}$M. At the next higher concen-

tration of $10^{-5}$M however, the action of the two fixatives was markedly different. Glutaraldehyde was almost twice as effective as formaldehyde in causing inhibition of parasite binding. This situation was reversed at the next lower concentration of $10^{-6}$ where formaldehyde retained more of its activity and actually caused more inhibition than glutaraldehyde.

The principal characteristic of the effect of glutaraldehyde was the strong inhibition shown at a wide range of concentrations and the sudden abatement of this inhibition with the ten-fold dilution from $10^{-5}$M to $10^{-6}$M. Inhibition by formaldehyde, while being as strong as glutaraldehyde, diminished through dilution earlier than glutaraldehyde. However, this decline in activity was less sharp so that at the highest dilution of $10^{-6}$M formaldehyde retained more inhibitory effect than glutaraldehyde. On the other hand, as far as potency of inhibition is concerned, glutaraldehyde seems to be ten times more effective than formaldehyde.

## II Action of Metabolic Inhibitors on Parasite Binding

### 1. Inhibitors of Energy Metabolism

The role of energy metabolism in parasite binding was studied by means of inhibitors of various intracellular metabolic pathways. After preincubation for one hour macrophages were challenged with parasites in the presence of such inhibitors.

Table 6 shows the effect of Sodium Azide ($NaN_3$) and 2,4 Dinitrophenol (DNP) on parasite binding. Incubation of macrophages in the presence $10^{-2}$M $NaN_3$ reduced the number of parasites attached to macrophages by 50%. There was also a parallel reduction of 25% in the number of parasitized macrophages. It is generally known that this inhibitor is effective only at high concentrations. Incubation of macrophages with such a high concentration of azide did not produce any adverse effect on macrophages as judged by the appearance of the latter under the light microscope.

2,4 Dinitrophenol, an inhibitor of aerobic respiration showed only a slight inhibitory effect on parasite binding (see also Table 6). Since DNP was first dissolved in absolute ethanol to make it soluble in the aqueous culture medium, the same concentration of the ethanol solvent in culture medium had to be used as a control for DNP. In the presence of a $10^{-3}$M concentration of the inhibitor only 20% less parasites attached to macrophages whereas macrophage parasitization was only 6% less than the ethanol control. Furthermore, values for both parameters of parasite binding in DNP were not significantly different from control values.

Potassium cyanide (KCN), another inhibitor of aerobic respiration which acts by blocking electron transport was also ineffective in preventing the binding of parasites by macro-

phages (see Table 7). Results obtained from incubation of macrophages in $10^{-3}$M and $10^{-4}$M concentrations of the inhibitor did not differ significantly from control values.

A substantial inhibition of parasite binding was however, produced by sodium fluoride (NaF), an inhibitor of anaerobic respiration (see Table 7). This compound which curtails the availability of magnesium ions in the medium reduced parasite attachment by more than 40%, at concentrations of $10^{-2}$M and $10^{-3}$M. Macrophage parasitization though as usual was reduced to a lesser extent.

Table 8 shows parasite binding in the presence of DNP and NaF each at a concentration of $5 \times 10^{-4}$M. Together, these two inhibitors produced less than 50% inhibition of parasite attachment based on the ethanol control. Compared to the cumulative effect of the two inhibitors used alone, the simultaneous application of these inhibitors did not produce any substantially increased effect on parasite binding (compare results in Table 8 with those in Tables 6 and 7). Moreover, the greater part of the inhibition produced by the combination of the two drugs seems to stem from NaF. Higher concentrations of the two inhibitors could not be tested together since they caused a gross deterioration of the macrophage monolayers.

The sulfhydryl blocking reagent Iodoacetamide caused a drastic

inhibition of parasite binding (see Table 8). When applied at a concentration of $1.35 \times 10^{-5}$M it reduced parasite attachment by 74% and macrophage parasitization by 57%.

Figure 5 summarized the effects of various metabolic inhibitors on parasite binding. Of the five metabolic poisons used, the inhibitors of aerobic respiration, 2,4 Dinitrophenol and Potassium cyanide, had very little effect on parasite binding. On the other hand, Sodium azide and the inhibitors of anaerobic respiration Sodium fluoride and Iodoacetamide all showed significant effects. Of these three inhibitors the sulfhydryl blocking reagent Iodoacetamide had the deepest inhibitory effect, while the inhibition produced by Sodium azide and sodium fluoride was moderate.

2. Effect of Cycloheximide

Treatment of macrophages with cycloheximide, an inhibitor of protein synthesis did not cause any inhibition of parasite binding. Results shown in Table 9 demonstrate that as a result of a 24 hour incubation of macrophages with cycloheximide the average number of parasites attached per macrophage actually increased. Cells treated with $10^{-5}$M concentration of the inhibitor bound 50% more parasites while those treated with a $10^{-6}$M concentration of the drug bound only 17% more parasites than untreated cells. Macrophage parasitization was much less elevated and to only 4-8% above control levels

by the two concentrations of the drug. Incubation of macrophages with higher concentrations of cycloheximide produced a gross deterioration of the majority of the cells. Even at the lower concentrations of $10^{-5}$M and $10^{-6}$M there was a noticeable loss of cells from the monolayers which reduced their number to an extent that it could have substantially increased the parasite to macrophage ratio of the assay mixture. Although the possibility of cycloheximide causing an enhancement of parasite binding cannot be excluded at present, the increase in parasite to macrophage ratio in the assay due to monolayer loss seems to be a more likely reason to explain the observed increase in parasite binding.

3. The Effect of the Inhibitors of the Cytoskeletal System

The role of the cytoskeletal structures of the host cell in parasite binding was investigated with the aid of drugs that are believed to interfere with the function of such elements.

The fungal metabolites Cytochalasins B and D were used in the investigation into the role of microfilaments of the macrophages in parasite binding. Macrophages were preincubated in the various concentrations of the drugs for one hour and challenged with parasites in the presence of the same concentrations of the drugs. The reversibility of the action of the two drugs was tested by incubating macrophages in the presence of 10 µg/ml of the inhibitor for one hour and

reincubating the same cultures in its absence for another hour before challenge with parasites.

Results in Table 10 describe the effect of different concentrations of cytochalasin B on parasite binding. At concentrations of 10 and 5 µg/ml the drug caused a drastic inhibition of parasite binding which resulted in 74% reduction in parasite attachment and 64% reduction in the number of infected cells. At a concentration of 1 µg/ml the inhibition of parasite binding was somewhat less although it still amounted to a substantial reduction of 56-58% in both parameters of parasite binding. A concentration of 0.1 µg/ml of the drug produced no significant reduction in parasite binding (data obtained from a separate experiment but not shown here). Removal of the drug from the macrophage cultures one hour before the addition of parasites restored 97% of the parasite binding activity of the macrophages (shown in Table 9). It was also found that the presence of 1% (v/v) DMSO alone, a concentration at which it is also present in the highest dose of cytochalasin, did not affect the attachment of parasites to macrophages. This indicates that the inhibition in parasite binding was specifically due to cytochalasin B.

Table 11 describes the effect of cytochalasin D on parasite binding. At a concentration of 10 ug/ml the drug reduced parasite attachment by 77% and macrophage parasitization by

70%. At 1 µg/ml concentration the inhibition was less pronounced. Parasite attachment was reduced by 60% and macrophage parasitization by 44%. When applied at a concentration of 0.1 µg/ml the drug caused only a slight inhibition of parasite uptake. Removal of the drug one hour before challenge with parasites restored 98% of the capacity of macrophages to bind parasites.

The involvement of the microtubular structures of the host cells in parasite binding was studied using colchicine, a drug known to react with microtubules and interfere with their normal function.

Results in Table 12 show the effect of various concentrations of colchicine on parasite binding. All the concentrations of the drug tested had a significant inhibitory effect. However, up to a concentration of $10^{-3}$M the effect of this drug was not very substantial. Inhibition of parasite attachment averaged about 30% while inhibition of macrophage parasitization ranged from 10 to 23%. Only at the highest concentration $10^{-2}$M did the drug produce a sharp reduction in parasite binding. At this concentration parasite attachment was reduced by 66% and macrophage parasitization by 54%.

Figure 6 compares the effect of the various inhibitors of the cytoskeletal system on parasite binding. At comparable concentrations cytochalasins B and D, which are believed to

affect microfilamental function produced a similar and drastic effect which amounted to about 75% inhibition of parasite attachment. On the other hand, colchicine, the drug which interferes with microtubular function was much less effective. At a comparable concentration shown in the same figure it produced only a 30% inhibition of parasite attachment.

III Effect of Surface Reactive Agents on the Interaction of Parasites with Macrophages

The nature of surface receptors involved in the attachment of parasites to macrophages was studied by means of reagents that are known to modify the structure of cellular surfaces. Parasites and macrophages were usually pretreated with these reagents and later allowed to interact with each other in otherwise normal circumstances. The cell surface probes used in these experiments were Sodium metaperiodate ($NaIO_4$), neuraminidase, trypsin and concanavalin A.

1. Periodate

Results shown in Table 13 describe the binding of parasites by macrophages following treatment of either parasites or macrophages with $2 \times 10^{-3}$ M Sodium metaperiodate. Treatment of parasites with metaperiodate actually increased their binding by macrophages. This increase compared to control values was equal to about 25% in the number of parasites attached to macrophages. It is interesting to point out that

this increase in parasite attachment was not accompanied by any increase in macrophage parasitization which was almost identical to control values. This increase in parasite binding was, therefore, confined to the same population of infected macrophages. On the other hand, treatment of macrophages with periodate reduced the number of macrophages that bound parasites by about 32%. This resulted in an overall decrease of about 33% in the number of parasites attached to macrophages. The average number of parasites per infected macrophage though did not differ from control values. When pretreated parasites were added to pretreated macrophages, the number of parasites attached to macrophages was 17% and the proportion of infected macrophages 28% lower than control values. In this case, the average number of parasites per macrophage was nearer to control levels than when either pretreated parasites or macrophages were used alone. The reduction in parasite binding due to treatment of macrophages with periodate was partially compensated by the increase that resulted from treatment of parasites with this reagent. Thus, the number of parasites per infected macrophage was 16% higher than the number attached to treated macrophages alone.

2. Neuraminidase

Table 14 describes the effect of neuraminidase on parasite binding by macrophages. Treatment of parasites with this

enzyme decreased their binding significantly. The number of enzyme treated parasites per macrophage was reduced by 28% while the proportion of infected macrophages was down by 20%. Treatment of macrophages on the other hand, increased their capácity to bind parasites. The average number of parasites attached per macrophage was increased by 38% and the proportion of infected macrophages by 14%. It should be noted that the overall increase in parasite binding as a result of neuraminidase treatment of macrophages was not only due to the increase in the proportion of macrophages becoming infected but also due to the increased parasite binding activity of each infected macrophage.

3. Trypsin

Results in Table 15 describe the binding of parasites after treatment of parasites and macrophages with trypsin. Treatment of parasites with this enzyme caused only a marginal decrease in their binding by normal macrophages. The number of trypsinized parasites that attached to macrophages was 12% less and the proportion of infected macrophages 14% less compared to the control. Treatment of macrophages with trypsin on the other hand, greatly reduced their capacity to bind parasites. The number of parasites attached to trypsinized macrophages fell by 64% while the proportion of macrophages infected with parasites decreased by 58%.

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4. Concanavalin A

The effect of concanavalin A on parasite binding was investigated by treatment of parasites and macrophages with 10 µg/ml of the lectin. Results summarised in Table 16 show that treatment of macrophages with this lectin caused a drastic inhibition of parasite binding. The number of parasites attached to concanavalin A treated macrophages was reduced by 74% and the number of infected macrophages by 52%. Treatment of parasites with the same concentration of the lectin produced a much smaller inhibitory effect on their binding by untreated macrophages. The number of parasites attached to macrophages fell by 39% while the number of infected cells fell by 45%. It was found that treatment of parasites with 10 µg/ml concanavalin A caused aggregation of parasites into clumps. This could indirectly affect parasite binding in two ways. Firstly, it becomes more difficult for macrophages to bind large particles of agglutinated parasites and secondly, as a result of reduced particle count in the parasite suspension through agglutination, the proportion of infected macrophages is reduced due to a change in the distribution of parasites among the macrophage. The latter will be reflected in a lower proportion of cells becoming infected with lectin treated parasites. The binding of concanavalin A treated parasites by concanavalin A treated

macrophages in the presence of the same concentration of the lectin was found to be even lower compared to the binding of normal parasites by concanavalin A treated macrophages. The number of attached parasites was equal to 13% of the control and the proportion of infected macrophages to only 26%. These values reflect the cumulative effect of the separate pre-treatment of parasites and macrophages on parasite binding. The four surface reactive agents can be classified into two groups according to their effect on parasite binding (see Table 17 and Fig. 7). Sodium metaperiodate and neuraminidase are similar to each otherin having a bidirectional effect on parasite binding. In other words, these two reagents produce opposite effects on parasites and macrophages. They produce enhancement on inhibition depending on whether they are applied to parasites or macrophages. However, in its direction of effect metaperiodate was the inverse of neuraminidase.

Trypsin and concanavalin A, on the other hand, are similar in having a unidirectional effect on parasite binding. Both produce inhibition whether applied to parasites or macrophages. Their effect however, is more pronounced if applied to macrophages instead of parasites.

## IV Extracellular Requirements for Parasite Binding

The attachment of parasites to host cells proceeds successfully in normal conditions of tissue culture, without the need for an additional opsonizing factor such an antibody or complement. The culture medium in which macrophages were maintained and challenged with parasites is a semi-defined medium consisting of Eagle's medium and foetal calf serum. Identification of the role of the various ingredients of the assay medium facilitates chemical characterization of the process of parasite binding and is likely to lead to a better understanding of the phenomenon.

### 1. The Role of the Various Components of the Assay Medium

#### a) The role of serum and Eagle's medium in parasite binding

To define the minimal extracellular conditions required for successful parasite binding, the involvement of foetal calf serum and Eagle's medium during the *in vitro* maintenance and subsequent interaction of macrophages with parasites was investigated.

Three hours after isolation and *in vitro* cultivation, macrophage monolayers were reincubated in Eagle's MEM or Earle's BSS in the presence or absence of serum and for a period of 24 hours. The macrophages were then challenged with parasites in Eagle's MEM or Earle's BSS with or without serum.

Preincubation of macrophages in serum free medium for 24 hours resulted in their deterioration as evidenced by their pycnotic appearance at the end of the 24 hour period of incubation. It was, therefore, decided to include serum in all preincubation media. However, due to the short duration of the assay it was possible to omit serum from some assay media without causing any damage to macrophages. The various combinations of preincubation and assay conditions with the corresponding results are outlined in Table 18.

The presence or absence of serum in the assay medium was found to have a significant effect on parasite binding if macrophages were maintained in normal tissue culture conditions. This effect manifested itself in a 37% reduction in parasite attachment and a 25% reduction in macrophage parasitization. The presence of serum also showed a significant effect on parasite binding in macrophages maintained in Earle's BSS. The absence of serum caused a 30% reduction in parasite attachment and a 35% reduction in macrophage parasitization, (compare e) with c) and d) ). However, in one pair of groups (b) and c) ), no difference in parasite binding could be seen despite the absence of serum from the assay medium in one group and its presence in the other. The lack of difference in this particular case should be attributed to the fact that cultures lacking serum in the assay medium

were maintained in Eagle's medium while those having serum were maintained in Earle's BSS a relatively deficient medium.

Macrophages bound parasites to a different extent depending on whether they were maintained in Eagle's medium or Earle's BSS. Thus, with serum in both the maintenance and assay medium, the use of Eagle's medium instead of Earle's BSS made a significant difference in parasite binding. This difference amounted to 25% in parasite attachment and 21% in macrophage parasitization (compare a) and d) ). The reduced ability of macrophages maintained in Earle's BSS could not be restored to normal levels by reintroducing Eagle's medium into the assay medium (compare a) and c) ).

The partial dependence of parasite binding on the type of medium used for maintenance already mentioned explains the lack of difference between groups (b) and (c) which differ by the presence or absence of serum in the assay medium but which also differ by the type of medium used for long term mairtenance of macrophages. It also explains the appreciable though not statistically significant difference between groups (b) and (e) which were assayed in similar conditions free of serum but maintained differently in Eagle's medium or Earle's BSS. These results show that the activity of serum in parasite binding depends in part on the biochemical

conditions of long term macrophage culture. Nevertheless, as shown earlier, there is a certain degree of activity always attributable to serum in otherwise similar set of circumstances.

b) Dependence of parasite binding on divalent cations

Macrophages required divalent cations, calcium and magnesium in the extracellular medium in order to bind parasites (see Table 19 and Figure 8). In the absence of both cations very little parasite binding was observed. Parasite attachment was only 21% and macrophage parasitization 39% of the values obtained in the presence calcium and magnesium. Addition of 2mM $Ca^{++}$ to the divalent cation free assay medium produced only a marginal improvement in parasite binding. Parasite attachment was increased by a mere 9% and macrophage parasitization by 17% compared to control values. However, the addition of 2mM magnesium to the assay medium caused a dramatic increase in parasite binding. Attachment increased by 70% to make up 91% of the amount seen in the presence of both cations whereas parasitization even slightly exceeded the control level. These results show that parasite binding is predominantly affected by magnesium ions. The requirement for calcium ions in parasite binding, although of little consequence, could not be obviated by magnesium. The increase in parasite binding produced by calcium and magnesium used together was equal

to the sum of the increases produced by each cation alone.

c) Influence of extracellular glucose on parasite binding

Table 20 shows the effect of glucose starvation of macrophages on parasite binding. The absence of extracellular glucose from the assay medium caused a drastic reduction in parasite binding. Parasite attachment was 60% less and macrophage parasitization 40% than control values. Incubation of macrophages in glucose free medium up to two hours prior to the assay did not produce any additional significant effect on parasite binding. Parasite attachment was reduced further by about 8-11% and macrophage parasitization by 2-8%.

d) Influence of serum deprivation on parasite binding

Results in Table 21 describe parasite binding by macrophages preincubated and/or assayed in the absence of serum. Challenge of macrophages with parasites in the absence of serum without any preincubation produced substantially lower levels of parasite binding. Parasite attachment was 45% and macrophage parasitization 34% less than control values. Prior incubation of macrophages in serum free medium for a period of up to six hours did not produce a further decrease in parasite binding. Macrophages that were incubated in serum free medium for two and six hours before challenge with parasites in the same medium showed essentially similar numbers of bound parasites compared to macrophages challenged

without preincubation in serum free medium. This shows that the effect of serum like that of glucose is expressed primarily during the parasite to host cell interaction.

Preliminary experiments on the effect of serum showed that three washes of monolayers with serum free medium was necessary before challenge with parasites to demonstrate a consistent and significant effect for serum. To prove that the effect obtained by repeated washing of the macrophage monolayers with serum free medium was due to removal of serum by increasing dilutions macrophages were washed three times, preincubated and challenged with parasites with 1% serum. Parasite binding in such conditions was found to be essentially similar to the control.

e) Cooperative effect of glucose and serum on parasite binding

The relationship between the effects of glucose and serum on parasite binding was studied in an experiment the results of which are described in Table 22 and Figure 9. The presence of either glucose or serum alone produced very little improvement on the residual levels of parasite binding that occurred in the absence of both ingredients. Thus, glucose produced only a 6% increase in parasite attachment while serum produced only a 12% increase in the same parameter. However, when both glucose and serum were applied at the same time a dramatic increase in parasite attachment took place. This

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increase amounted to 72% in parasite attachment and brought the level of attachment to almost 90% of the control value. The increase in macrophage parasitization followed a similar pattern. These results show that glucose and serum support parasite binding synergistically.

2. Involvement of Carbohydrates in Parasite Binding

a) Effect of 2 deoxy D-glucose

The glucose dependence of parasite binding was further investigated by using 2 deoxy-D-glucose an analogue of glucose which is metabolically inert. Results in Table 23 and Figure 10 describe the effect of 2 deoxy glucose on parasite binding in the presence and absence of glucose. In the presence of 5.5mM 2 deoxy glucose alone parasite attachment was reduced by 64% and macrophage parasitization by 45%, roughly the same amount of inhibition as produced by the elimination of glucose alone. Addition of pyruvate at equimolar concentrations of deoxy glucose did not improve parasite binding appreciably. However, the presence of glucose at equimolar amounts together with deoxy glucose increased parasite binding more appreciably, yet parasite attachment remained 44% and macrophage parasitization, 30% lower than control values, an improvement of only 20% in the number of parasites attached. The improvement in parasite binding in the presence of 2 deoxy

glucose due to addition of glucose amounted to one third of the activity shown by glucose alone whereas the improvement due to pyruvate was no more than 10% of the activity of glucose. Even the complete removal of deoxy glucose and its replacement with glucose one hour before the assay did not fully restore binding to normal levels. Parasite attachment remained 33% and macrophage parasitization 24% lower than control values.

b) The effect of various monosaccharides on parasite binding

Four other naturally occurring monosaccharides were compared with glucose for their activity in parasite binding. The four sugars tested were (D) xylose, (D) galactose, (L) fucose and (D) mannose. Table 24 and Figure 11 show the activity of these four sugars in promoting parasite binding compared to levels attained in the presence or absence of extracellular glucose. Of these four sugars, xylose, galactose and fucose showed no detectable activity in inducing parasite binding. Among these three inert sugars the highest level of parasite binding was found in the presence of xylose. It amounted to 40% of the glucose control and just about the same level found in the absence of glucose. Parasite binding in the presence of galactose was not also significantly different from values found in the glucose free conditions.

In the presence of fucose, however, parasite binding was so low that values were even significantly lower than those obtained in the absence of glucose. Relative to the glucose free control this difference amounted to 68% inhibition in parasite attachment and 37% inhibition in parasitization. The fourth sugar, mannose, unlike the other three supported parasite binding to a degree which almost equalled the activity of glucose itself. In its presence parasite attachment was restored to 94% and macrophage parasitization to 80% of the glucose control level.

### 3. Characterization of the Active Factor in Foetal Calf Serum

#### a) Activities of the dialyzable and non-dialyzable fractions of serum

In order to characterize the component of serum involved in parasite binding, whole uninactivated foetal calf serum was dialyzed extensively against phosphate buffered saline. Table 25 and Figure 12 describe the activities of the two fractions of serum obtained by dialysis. The retentate, i.e. the non-dialyzable fraction was found to retain virtually all the enhancing activity of serum whereas the diffusate or the dialyzable fraction showed no activity in parasite binding. On a percentage basis, the diffusate showed as much activity as serum free Eagle's medium that was measured in other

experiments. Serum that was reconstituted by mixing the retentate and diffusate produced results similar to undialyzed serum.

b) Effect of heat denaturation on the activity of serum

The heat sensitivity of the active fraction of serum was tested by exposure of whole serum to various temperatures for 30 minutes. Table 26 shows the activities of various heat inactivated sera in parasite binding. Boiling of serum at 100°C abolished the activity of serum completely. Parasite binding was reduced to 39% and macrophage parasitization to 63% of control in its presence. Exposure of serum to 56°C reduced its activity to two fifths of the amount lost upon boiling. Exposure to 80°C resulted in almost total loss of activity since values for binding were similar to the values obtained by boiled serum.

In order to determine the minimum temperature required for complete abrogation of the activity of serum, two more temperatures of inactivation between 56°C and 80°C were tried. Table 27 shows parasite binding in the presence of sera inactivated at 60°, 70° and 80°C. Upon exposure to 60°C serum lost 72% of the activity in parasite attachment lost by inactivation at 80°C. A further loss of 6% based on the same parameter was observed after inactivation of serum at 70°C. This leaves the remaining 22% of its total activity

retained after exposure to 70°C which was abolished by exposure to 80°C.

A partially purified preparation of albumin was also tested for its activity in parasite binding. Parasite attachment in the presence 0.2% albumin (a concentration normally found in 10% foetal calf serum) was equal to only 45% of control. In terms of total heat labile activity of serum this corresponds to a proportion of 18% which suggests that albumin may have some activity in parasite binding.

c) Activities of various fractions of serum precipitated by ammonium sulphate

Table 28 and Figure 13 show the activities of the five fractions of foetal calf serum obtained by precipitation with graded saturations of ammonium sulphate. No single fraction exhibited an activity equal to whole serum and all fractions gave significantly lower activities. However, two fractions (40% and 70%) were prominent in their activity. Of these two the 40% fraction gave the higher values in both parameters of parasite binding. In its presence attachment was 77% and parasitization was 90% of the control. Slightly but not significantly lower values were obtained by the 70% fraction. An intermediate activity was shown by the 50% fraction which produced 13% less parasite binding than the 40% fraction. This difference though was not statistically significant.

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The two other fractions obtained at saturations of 60% and 30% showed negligible activity in parasite binding.

Considering the fact that no single fraction equalled whole serum in its activity and a concentration of 10% v/v is above the minimum required for whole serum to express its activity it seems plausible to suggest tentatively that two components rather than one are involved in the enhancing activity of serum in parasite binding.

Table 29 summarized the properties defined so far of the active fraction(s) of serum involved in parasite binding.

## V Scanning Electron Microscope Study of Parasite Entry into Macrophages

### 1. Entry of Normal Parasites

Motile promastigotes attached to macrophages predominantly by the flagellar tips (see Figures 14 and 15). A close look at the area of flagellum to macrophage contact revealed that attachment could occur at a rather smooth area of the macrophage surface with no preference for any specialized membrane structure or projection (Figs. 16-18). Initial attachment was followed by extensive ruffling of the macrophage membrane at the point of flagellar contact (Fig. 19). This was followed within 2 minutes by the formation of a small lamellar sheath which extended from the macrophage and fitted quite tightly around the flagellum (Figs. 20 and 21). Note also

the undulating appearance of the nascent sheaths in Fig. 22 that suggests the intense activity of the lamellar tubes holding the flagella. The activity of the membrane at the point of flagellar contact resulted in a smooth surface in the immediate area surrounding the area of contact as opposed to the rest of the macrophage surface,(see especially Figs. 20 and 21).

A rare form of flagellar engulfment can be seen in Fig. 23 where a segment of the flagellum lying parallel to the macrophage surface has been covered by the macrophage membrane without the protrusion of a sheath (seen for instance in Fig. 24). In more usual situations the lamellar sheath continued its advance along the flagellum of the parasite (see Figs. 24 and 25) and covered half of the flagellum in 2-4 minutes. The outer surface of the lamellar sheath had in general a smooth but undulating appearance (Fig. 25). In some cases it could be seen tightly applied to the flagellar surface as in Figs. 24 and 25 or have an open mouth as in Fig. 26 which gives the appearance of a rolled shirtsleeve. The lamellar sheath was sometimes seen to be preceded by one or more microspikes extending from the leading edge, see Figs. 27-29. As seen in all three pictures these microspikes do not anchor on the parasite surface but rather float freely in the extracellular space.

After six minutes of exposure to parasites the entire flagellum could be seen covered by the lamellar sheath in full extension from the macrophage body (Figs. 30, 31). Before the onset of the second stage of membrane activity culminating in the engulfment of the parasite body the lamellar sheath often retracted with the result that the flagellum became embedded in an invagination deep into the macrophage body. (See for example, Fig. 32). However, in some cases the lamellar sheath may continue its advance to engulf the parasite body without the full retraction (Fig. 33). With or without retraction the lamellar envelope was sometimes able to cover half the parasite body as soon as 8 minutes after challenge with parasites (Fig. 34). However, between 10 and 30 minutes of exposure to parasites the general picture remained largely unchanged insofar as the advance of lamellar processes were concerned. Throughout this period parasites were seen partially engulfed to variable degrees. Nevertheless, engulfment was mainly confined to the flagella of the parasites while their bodies remained extracellular in general (see Fig. 35).

Engulfment of the parasite body could be seen more frequently at about 1 hour of exposure to parasites; Fig. 33-40 illustrate the various stages of engulfment. In Fig. 33 half of the parasite body can be seen covered by the lamellar envelope while the basal part of the lamella that covers the flagellum has a wrinkled appearance. Judging from its profile the

lamellar envelope does not seem to have undergone much retraction contrary to the general pattern, seen in Figs. 37-40 where the part of the lamellar sheath enclosing the flagellum has retracted to the macrophage body. Another feature of the engulfment of the parasite body is the close opposition of the lamellar sheath to the parasite's surface. The outer surface of the lamellar envelope seen in Fig. 37 is smooth while the leading edge of the lamellar envelope in Fig. 38 has two microspikes. A membraneous fold at the flagellar junction can be seen in Fig. 36. A very late stage of engulfment can be seen in Fig. 39 where only a small part of the parasite body is visible. The covering membrane has a longitudinal fold on each side of the parasite body while a cross sectional fold can be seen two thirds of the way towards the lamellar edge. Fig. 40 represents what seems to be the final stage of engulfment where the parasite body is completely shrouded in the lamellar sheath. The surface of the sheath bears a few microvilli but in general is less rich in such structures than the rest of the macrophage surface.

The general decline in macrophage membrane flow which started as soon as 10 minutes after challenge with parasites resulted in the eventual interruption of parasite engulfment in the majority of cases. Thus, between 1 and 4 hours of interaction many parasites remained largely extracellular with their fla-

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gella anchored into the macrophages. Fig. 41 shows parasites that have remained extracellular for 4 hours after their interaction with macrophages. All lamellar extensions have presumably retracted while the bodies of the parasites have assumed a more rounded shape.

2. Entry of Fixed Parasites

In contrast to unfixed parasites, which attach predominantly by their flagellar tips, fixed parasites attached in a random fashion, whith almost any part of their surface capable of adhering to macrophages and initiating the process of entry. Fig. 42 shows that fixed parasites can be taken up by either their flagellar or posterior ends, while Figs. 43 and 44 show two immotile promastigotes each being engulfed somatic end first. This finding was the reverse of what would normally be seen if motile promastigotes were used which are generally taken up by their flagella. A single parasite is seen in Fig. 45 being engulfed simultaneously by two adjacent macrophages, one attempting to engulf the flagellum and the other the body of the parasite. Such instances are common with dense macrophage monolayers and are a further indication that all parts of the surface of fixed parasites are equally adhesive to macrophages. As a consequence of this randomization of parasite attachment. There is no overall uniformity in the sequence of events, in contrast to the uptake of

unfixed parasites, engulfment can begin at any part of the parasite and proceed to completion.

When exposure of macrophages to fixed parasites was allowed to proceed for longer periods another interesting aspect emerged: in striking contrast to their normal counterparts, fixed parasites became progressively internalized without any interruption. After 1 and 2 hours partially engulfed parasites became less and less evident until eventually at 4 hours most parasites were internalized completely (Figs. 46, 47).

3. Engulfment of Parasites by an Alternative Mechanism

The principal feature of parasite engulfment was the formation of tubular structures in the form of lamellar sheaths. In most stages the plane created by the leading edge of the lamellar sheath would be at right angle to the longitudinal axis of the parasite. This meant that as a rule engulfment would proceed by elongation of these tubular structures. In two cases, however, some evidence was obtained to suggest that engulfment could proceed by another mechanism. Here, the lamellar process assumed the shape of a sheet which surrounded the parasite from three sides (see Figs. 48, 49). The two fringes of the lamellar sheath eventually overlapped each other as seen in Fig. 49 although no evidence of direct fusion of the two membrane flaps could be found. This mode

of lamellar activity gave the impression that engulfment can also proceed by a mechanism similar to wrapping.

4. Effect of Temperature on Parasite Entry

A comparison of macrophage monolayers challenged with parasites at temperatures of 37° and 24°C showed no significant difference in the pattern of parasite engulfment as seen by scanning electron microscopy. At a temperature of 14°C the lamellar activity of the macrophages was apparently suppressed. Of the greatly reduced number of adherent parasites, none were seen to have been internalized. Fig. 50 shows parasites superficially attached to a macrophage at 14°C. One of the parasites is apparently attached by its body.

5. Effect of Cytochalasin D on Parasite Entry

Examination of macrophages with scanning electron microscopy revealed a fundamental difference between the surface morphology of normal and cytochalasin D treated cells. A half hour treatment of macrophages with 5 µg/ml cytochalasin D and further incubation for 20 minutes with a parasite suspension containing the same concentration of the drug resulted in a smooth surface (Fig. 51), which had lost the microvilli and filopodia so characteristic of normal untreated macrophages (Fig. 52). The scrutiny of rare cases of adherent parasites showed that macrophages did not produce any lamel-

lar processes in response to the attached parasites.

6. Topography of Parasite Attachment to Macrophages

A persistent feature of parasite attachment to macrophages was the predilection of parasites for marginal zones of macrophages. This is well illustrated in Figs. 52-55 where a multiplicity of parasites are associated with one or a few macrophages. The majority of parasites seem to confine themselves to the peripheral areas of fully spread cells, which often results in the juxtaposition of numerous parasites in rather limited areas of the macrophage surface.

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## DISCUSSION

### I. Characteristics of Parasite Attachment to Macrophages

#### 1. Kinetic Aspects of Parasite Attachment

a) Relationship of parasite attachment to time of exposure and volume of assay medium

The *in vitro* exposure of macrophages to parasites at increasing intervals of time showed that parasite binding by macrophages increased as a result of longer periods of exposure. However, this increase was largely confined to the initial part of a one hour period of exposure, since the sharpest increase in macrophage parasitization took place in the first five minutes. In the ensuing periods the increase gradually declined until there was very little increment in macrophage parasitization in the last half hour of exposure.

The relatively rapid uptake of parasites by macrophages can be attributed to the motility of the promastigotes in normal culture conditions which increases the probability of their collision with macrophages. The progressive decline in the rate of macrophage parasitization in later periods is probably due to the decrease in the concentration of free promastigotes as more and more parasites become irreversibly associated with macrophages.

Exposure of macrophages to the same load of parasites suspended in different volumes of assay medium did not reflect a clear

cut relationship between parasite binding and the volume of the suspension. The inverse but weak relationship between volume of suspension and macrophage parasitization observed at 10 minutes of exposure did not hold for the longer periods of exposure. This deviation is contrary to what would have been expected if the approach and collision of parasites to macrophages were governed by the force of gravitation alone.

The relative independence of the time of exposure and volume of assay suspension of parasite binding enables one to choose a fairly short period of exposure that makes numerous trials feasible and allows a small volume of assay medium to be used which obviates the use of very heavy parasite suspensions and permits the accurate handling of promastigote suspensions. A short period of exposure also allows a better detection of changes in the rate of parasite binding where any reduction in such a rate due to experimental conditions is less likely to be compensated for by the sheer length of exposure.

b) Effect of temperature

The incubation of promastigotes with macrophages at three different temperatures showed that parasite binding was strongly dependent on temperature. The preincubation of macrophages at either 37°C or 10°C before exposure to parasites at 37°C produced similar results which shows that parasite binding is continuously dependent on temperature and the effect of low temperature is reversible.

The attachment phase of phagocytosis, in contrast to engulfment is believed to be in general independent of temperature (Jones, 1975; Silverstein et al., 1977). The number of specific examples that support such a generalization is nevertheless limited. One such example is the attachment of Mycoplasma pulmonis to macrophages (Jones et al., 1972). On the other hand there are more numerous examples of temperature dependent attachment of particles to phagocytes. The Fc receptor-mediated attachment of erythrocytes to macrophages which was previously considered to be temperature independent (Munthe-Kaas, 1976; Griffin et al., 1975), was recently shown to be temperature dependent (Passwell et al., 1978). Other examples of temperature dependent attachment are the attachment of complement-coated erythrocytes (Lay and Nussenzweig, 1968), glutaraldehyde-fixed erythrocytes (Rabinovitch, 1967), bacteria, such as Escherischia coli (Brumfitt et al., 1965) and Corynebacterium parvum (Ogmundstottir and Weir, 1976), zymosan particles (Michl and Silverstein, 1976), and concanavalin-A mediated attachment of yeast to macrophages (Bar-Shavit and Goldman, 1976). A recent study by Baker and Liston (1978) has reported that the attachment of Trypanosoma dionisii is unaffected by lowered temperature.

An increase in the rigidity of the macrophage would be one simple explanation to account for the observed decrease in

parasite binding at low temperatures. Macrophages that lack global plasticity would be less able to expand areas of mutual contact with parasites. This would make adhesions susceptible to disruption by mechanical forces such as parasite motility and the washing of the macrophage monolayer at the end of the assay.

It is quite logical to suppose that sufficient areas of mutual contact are necessary for stable adhesion as has been shown in several morphological observations of cellular adhesions (Garrod and Born, 1971; Van Blitterswijk et al., Lloyd et al., 1976) as well as in the attachment of Leishmania promastigotes to macrophages (Merino et al., 1977). However, if such an effect were the only factor in the inhibition of macrophage adhesiveness then such a property would be expected to provide us with a critical temperature below which the binding capacity of the cell would vanish abruptly. Such a threshold is known to exist for the engulfment stage of phagocytosis at 18°C (Silverstein et al., 1977). No such threshold could be found in the present study since parasite binding was found to have a linear relationship with temperature. A significant decrease could already be observed at intermediate temperatures which would not be expected to cause appreciable rigidity of the macrophage. Since a decrease in the deformability of the cell is a physical phenomenon it would be expected to operate in all instances when low temperature

is applied. A few examples of adhesion at low temperature such as wheat germ agglutinin - mediated cell agglutination (Stanley and Carver, 1978) concanavalin-A mediated phagocytosis of yeast cells (Goldman and Bursuker, 1976) and attachment of fibroblasts to glass surface (Grinnell, 1974) argue against such a simplistic interpretation for the effect of low temperature. In this context it is of interest to mention that incubation of macrophages at 10°C was found to cause the appearance of extensive surface folds when observed by scanning electron microscopy. Such an effect does not agree well with the view that low temperature might render cells more rigid.

Another possible interpretation for the effect of low temperature could be given in terms of the physical state of the lipid bilayer of the cell membrane. Decreased fluidity of the membrane was implicated in inhibition of cellular adhesion (Martinozzi and Moscona, 1975; Lloyd et al., 1976) and phagocytosis (Bar-Shavit and Goldman, 1976) at low temperatures, who suggested that reduced fluidity of the lipid bilayer would discourage cell adhesion by restricting the mobility of cell surface receptors and their rearrangement to a distribution conducive to adhesion. A clustered distribution of antigenic sites on the cell surface at high temperatures was correlated with normal cell to cell adhesion and a uniformly diffuse distribution at low temperatures to reduced adhesion (Nicolson, 1972).

According to the fluid mosaic model of the plasma membrane the lipids of most functional membranes exist in a fluid state at physiological temperatures. At low temperatures e.g. at 4°C the fluidity of the lipid bilayer is greatly reduced due to a shift from a fluid to a more solid state (Singer, 1974). On the basis of physical and physiological data it has been suggested that there are two independent phase transitions with characteristic temperatures in membranes of homeothermic animals (Wisniewski et al., 1974); a lower one below which all membrane lipids are in the solid state, and a higher one above which all the membrane lipids are in the fluid state. Between these two temperatures, separate solid and fluid domains form by a process of lateral phase separations (Shimshick and McConnell, 1973).

A reduction in the fluidity of the membrane could impose two types of restrictions on membrane dependent phenomena. A putative cell surface receptor responsible for cellular adhesion may suffer a restriction in translational mobility either by being excluded in discrete patches of fluidity as a result of lateral phase separation or be immobilized in its native distribution due to a general solidification of the lipid bilayer (Frye and Edidin, 1970; Petit and Edidin, 1974). A second type of restriction would result in the inhibition of conformational changes in the membrane receptor due to increased viscosity of the membrane lipids (Singer, 1974).

Direct evidence for the control of cellular adhesion by membrane fluidity phenomena has not been conclusive yet. A report by Ueda et al., (1976) suggests a correlation between changes in cellular adhesiveness and the characteristic temperatures of membrane fluidity, although no such correlation could be found in the presence of magnesium ions. Further support for this view comes from the work of Rittenhouse and Fox (1974), who found that experimentally induced changes in the critical temperature of adhesiveness of red blood cells. On the other hand, Curtis et al. (1975) and Hoover et al. (1977) could not find a direct correlation between changes in membrane fluidity caused by fatty acids of varying chain length and the adhesiveness of cells treated in such a way. Curtis et al., (1975) have raised a theoretical problem in the interpretation of cellular adhesiveness in terms of increased or decreased fluidity. If major redistribution of membrane receptors in the lipid bilayer is operating in cellular adhesion then increased fluidity and hence increased receptor mobility might discourage cell to cell adhesion by increasing the tendency of the receptors to disperse back into their native distribution just as decreased fluidity and receptor mobility would discourage cell adhesion by preventing their assembly into a more favourable distribution.

Although cell deformability and membrane fluidity are fundamental physical properties of cells which may alter cellular

behaviour at different temperatures, the existence of other cellular functions which are also sensitive to temperature does not allow the interpretation of the effect of temperature by physical phenomena alone.

The temperature dependence of parasite adhesion may also imply the involvement of active cellular metabolism although the specific nature of such a process is difficult to identify by the effect of temperature alone. At the intracellular level the normal function of the cytoskeletal structures, microfilaments (Stossel and Hartwig, 1976) and microtubules (Roth, 1967) are known to be inhibited by low temperature. At the cell surface level the effect of low temperature has been interpreted by many investigators as evidence for a bridging mechanism that involves a chemical interaction between receptors on opposite sides, possibly regulated by an enzyme. A corollary of chemically induced cellular adhesion would be a requirement for the synthesis of a bridging molecule. Based on the temperature sensitivity of cellular adhesion Moscona (1961) has suggested the requirement for a synthetic step for the production of a macromolecular bridging agent. This explanation has been rejected by Steinberg (1962) and Edwards and Campbell (1971) on the grounds that physiological temperatures were required only during the adhesion process. A similar observation mentioned in this study plus the lack of effect of cycloheximide, an inhibitor of protein

synthesis, on parasite binding discourage the adoption of a view that implicates the synthesis of a cementing agent. The insensitivity of cellular adhesion to inhibition of protein synthesis has led to a similar conclusion (Kemp et al., 1967; Weiss and Chang, 1973). However, these findings do not rule out a chemical interaction between pre-existing surface receptors. Temperature sensitivity of the interaction may imply the consumption of energy or its control by an enzymatic reaction. Such a conclusion has been drawn from the logarithmic rate of cellular aggregation plotted against absolute temperature (Arrhenius plot) by Jones and Morrison (1969). The linearity of parasite binding as a function of temperature makes the involvement of a chemical reaction a strong possibility.

It becomes clear after consideration of the various aspects of temperature sensitive cellular behaviour that no single mechanism can be favoured by the effect of temperature. Such information however clearly points to the likelihood that parasite binding is a complex phenomenon that involves several parameters of cellular physiology.

c) Effect of chemical fixation

The inhibition of parasite binding by fixation of the macrophage and not of parasites clearly indicates that the temperature sensitivity of the parasite macrophage interaction

is a property of the macrophage and not the parasite.

The main constituent of cells with which the aldehyde fixatives glutaraldehyde and formaldehyde are thought to react are proteins (Bowes and Cater, 1966). The reactivity of these aldehydes with proteins is attributed to the carbonyl groups in these compounds which react readily with free amino groups (Feeney et al., 1975) and sulfhydryl groups (Habeeb and Hiramoto, 1968) of protein molecules. Both fixatives are capable of inducing intermolecular cross linking (Avrameas and Ternynck, 1969; Feeney et al., 1975). In the case of glutaraldehyde this is due to the presence of two reactive carbonyl groups in each molecule, whereas in formaldehyde this takes place by the formation of methylene bridges (French and Edsall, 1945).

It is generally believed that these fixatives preserve a great deal of the antigenicity of proteins and cell surfaces. In the case of protozoan parasites Trypanosoma and Leishmania preservation of surface antigenicity has been ascertained by Nantulya and Doyle (1977) and Dwyer (1977), by antigen-antibody reactivity and lectin agglutinability respectively. Several other studies have shown similar results after fixation of mammalian cells with glutaraldehyde or formaldehyde (Sabatini et al., 1963; Anderson and Dresser, 1971; Bubbers and Henney, 1975). An important factor in the preservation of surface antigenicity is the concentration of the fixative to be used.

Concentrations above 0.1% of either fixative are generally detrimental to surface antigenicity (Bubbers and Henney, 1975; Tokoyasu and Singer, 1976).

Fixation with glutaraldehyde or formaldehyde is in general inhibitory to cell-to-cell adhesion. A few examples are $C_3$ mediated cytoadherence (Dierich and Reisfeld, 1975), concanavalin A mediated agglutination of cells (Inbar et al., 1973; Martinozzi and Moscona, 1975), antigen antibody mediated agglutination of erythrocytes (Victoria et al., 1974) and binding of aggregates of 3T3 cells to fibroblast layers (Cassiman and Bernfield, 1976).

The inhibition of all agglutinability by fixation of the cell surface has been interpreted as evidence for a requirement for lateral mobility of surface binding sites (Inbar et al., 1973; Rosenblith et al., 1973). These and other ultrastructural studies have shown that fixation prevents rearrangement of surface binding sites (De Petris and Raff, 1974). This has led to the conclusion that clustering of surface binding sites is a prerequisite for cell-to-cell binding (Nicolson, 1972; Dierich and Reisfeld, 1975; Inbar et al., 1973). This theory has later been challenged by some investigators Temmink et al., 1975; Ukena et al., 1976), who found no direct correlation between agglutinability and surface distribution of lectin binding sites in agglutinating cells.

Since fixation of parasites does not alter their ability to bind to macrophages, the mobility or redistribution of parasite surface binding sites does not seem to be necessary for successful binding. It also follows that receptors for parasite antigens on the macrophage surface do not have to redistribute to form a patch or a cap unless the density of receptors on the macrophage surface is less than the density of reciprocal receptors on the parasite surface, in which case macrophage receptor redistribution becomes necessary.

Rutishauser and Sachs (1974, 1975) have reported a similar asymmetric effect of glutaraldehyde fixation on cell-to-cell binding by concanavalin A. They found that fixation of lectin coated cells did not prevent their binding to uncoated cells whereas fixation of the uncoated cells abolished their ability to bind the lectin coated cells. With respect to fixation, parasites resemble lectin coated cells and macrophages uncoated cells. Based on these findings they concluded that neither capping nor patching of surface receptors was necessary for concanavalin A mediated cell-to-cell binding but that short range lateral mobility of concanavalin A receptors would be necessary for their realignment on one cell into a position favourable for the formation of multiple bridges with receptors on the other cell. In view of the similarity of results obtained from fixation of parasites and macrophages to those obtained by Rutishauser and Sachs, their proposed model, pos-

tulating the short range realignment of surface receptors seems to be the most relevant explanation for the effect of fixation on parasite-to-macrophage binding.

Inhibition of cell-to-cell adhesion by aldehyde fixation has also been interpreted in terms of decreased deformability of fixed cells that are unable to develop mutually aligned areas of contact (Van Blitterswijk et al., 1976). While a decrease in deformability of fixed cells cannot be ruled out entirely, such a contingency is thought to be unlikely in view of the low concentration of glutaraldehyde used. A dose-dependent effect of glutaraldehyde on cell deformability has been shown by Kataoka et al., (1975). A more plausible alternative explanation for the effect of glutaraldehyde could be based on the inhibition of an enzyme that takes part in parasite binding. The activity of such a hypothetical membrane bound enzyme may be susceptible to aldehydes if its reactive group is an amino or sulfhydryl group, with which glutaraldehyde reacts readily. The activity of such an enzyme could also be suppressed by a more subtle mechanism such as restriction of its conformational mobility in the plane of the membrane which may be necessary for the expression of its activity (Singer, 1974). Such an effect would most probably be exerted through the cross-linking activity of aldehyde fixatives.

2. The Role of Macrophage Physiology in Parasite Binding

Results obtained from the challenge of macrophages with parasites in the presence of several metabolic inhibitors showed that parasite binding was significantly inhibited by sodium fluoride, Iodoacetamide and sodium azide whereas potassium cyanide and 2,4 dinitrophenol were without any significant effect.

Phagocytosis is usually accompanied by the expenditure of metabolic energy. In peritoneal macrophages this energy is provided by the glycolytic pathway. Oxidative phosphorylation, another source of metabolic energy does not affect phagocytosis significantly (Allison and Davies, 1975). Consequently, phagocytosis is sensitive to inhibitors of glycolysis such as NaF, Iaa and 2 Deoxy-Glucose, while it is insensitive to inhibitors of oxidative metabolism such as KCN, DNP and antimycin (Sbarra and Karnovsky, '959; Sbarra and Shirley, 1963; Oren et al., 1963; Michell et al., 1969).

Results obtained in this study broadly agree with previous findings about phagocytosis in that parasite binding is affected by inhibitors of glycolysis and unaffected by inhibitors of oxidative phosphorylation. They do not agree with the generally accepted view that only the engulfment stage of phagocytosis requires metabolic energy whereas the attachment stage is independent of such a requirement (Simson and Spicer, 1973;

Silverstein et al., 1977). It should be pointed out, however, that some of the studies on the metabolic requirements of phagocytosis, do not differentiate between attachment and uptake and may, therefore, represent a reduction in particle attachment as well.

Two of the metabolic poisons producing an effect on parasite binding are known to inhibit glycolysis. Sodium fluoride inhibits the activity of enolase an enzyme of the glycolytic pathway by limiting the availability of magnesium ions that are required for its activity (Yudkin and Offord, 1973). Iodoacetamide which blocks free sulfhydryl goups (Anfinsen and Haber, 1961) inhibits the activity of 3 phosphoglycerate dehydrogenase (Silverstein et al., 1977), another key enzyme in the glycolytic pathway. The mode of action of sodium azide is somewhat different although less clear. It is known to inhibit at least two peroxidatic enzymes which are not related to glycolysis (Dri et al., 1979). Since these metabolic inhibitors readily penetrate cells their effect suggests participation of events that take place inside the cell to events that take place on the cell surface. In the case of Iodoacetamide, this was possible to show by comparison with another sulfhydryl blocking agent p-chloro mercuri benzene sulphonic acid (PCMBS) which poorly penetrates cells and has little effect on phagocytosis (Tsan et al., 1976).

Some attempts have recently been made to correlate the effects of these metabolic inhibitors with other aspects of macrophage metabolism. These include hexose transport and sugar phosphorylation (Gee et al., 1974) and ATP content and cell deformability (Mazur and Williamson, 1977). Based on the effect of metabolic inhibitors on macrophages, parasite binding does not seem to be affected by either hexose transport or sugar phosphorylation. DNP and KCN, two chemicals which decreased hexose transport and phosphorylation substantially, did not affect parasite binding significantly. On the other hand, the ATP content of macrophages seems to play a role in parasite binding. Thus NaF and Iaa which caused significant decreases in ATP content of macrophages (Mazur and Williamson, 1977) also reduced parasite binding substantially, whereas KCN which had no effect on ATP content was also without effect on parasite binding. This relation could presumably be explained by the fact that glycolysis is the main source of metabolic energy in peritoneal macrophages (Allison and Davies. 1975. Oren et al., 1963). A recent study, however, has shown that in certain cases it is not possible to demonstrate a one to one relationship between ATP content and phagocytosis (Michl et al., 1976) This had led to the opinion that it is too simplistic to assume that the effect of these metabolic inhibitors is confined to a single metabolic process in the cell (Silverstein et al., 1977. Rabinovitch and DeStefano, 1974) Magnesium, whose availability is curtailed by fluoride is most

probably involved in many other enzymatic reactions which are not related to anaerobic glycolysis. The same applies to Iodoacetamide which blocks sulfhydryl groups that are known to be the active group of numerous other enzymes. The two types of cytoskeletal elements, microfilaments (Perry and Cotterill, 1962) and microtubules (Burchill et al., 1978) are also known to be sensitive to sul ydryl blocking agents. However, it is safe to assume that some biochemical step is involved in normal parasite attachment to host macrophages.

The microfilament inhibiting agents cytochalasins B and D showed a drastic effect on parasite uptake. Since the observed effect represented a decrease in the total number of parasites associated with macrophages, these drugs must therefore, affect the attachment of parasites to macrophages. Previous studies on interaction of various protozoan parasites such as Leishmania (Alexander, 1975), Toxoplasma (Ryning and Remington, 1978) and Trypanosoma (Alexander, 1975; Nogueira and Cohn, 1976; Baker and Liston, 1978), have stressed the effect of these drugs on parasite entry but report no effect on parasite attachment. Although this discrepancy in results is not entirely clear several factors may contribute to the apparent lack of effect of cytochalasin on parasite attachment reported in those studies. The unusual motility of Leishmania promastigotes may impose greater restrictions on the macrophage's

binding capacity making it more susceptible to the action of these drugs. The longer period of exposure to parasites employed in some studies may contribute to increased adherence by mechanical means such as may result from insertion of parasites between the cell and the substratum. In some other studies no total estimates of parasite to macrophage association were mentioned. Since parasites must have attached before being internalized, a significant effect on parasite attachment by cytochalasin could be overlooked in the absence of such total estimates.

The effect of cytochalasin B on phagocytosis has been studied extensively, and its inhibitory effect is now quite familiar (Allison et al., 1971; Davis et al., 1971; Davies et al., 1973; Axline and Reaven, 1974). The same applies to its congeneric analogue cytochalasin D (Mimura and Asano, 1976; Atkinson and Parker, 1977) which shows a similar dose dependent effect. The general belief is that cytochalasin B affects the engulfment stage of phagocytosis without any effect on the attachment stage (Davis et al., 1971; Malawista et al., 1973; Davies et al., 1973) although some of the more recent studies show a partial effect of cytochalasin B on particle attachment (Helentjaris et al., 1976; Munthe-Kaas, 1971) The inhibitory effect of cytochalasins B and D on cell adhesion is also well known (Sanger and Holtzer, 1972; Helentjaris et al., 1976; Weber et al., 1977; Miranda et al., 1974a).

The mechanism of action of these drugs has been studied in detail by several groups. In a similar manner these two drugs affect cellular motility and other cellular activities by their interaction with microfilaments (Wessels et al., 1971; Miranda et al., 1974). Both drugs also share a common binding site on the cytoplasmic face of the plasma membrane (Lin and Snyder, 1977; Tannenbaum et al., 1977). However, the manifestations of their effect on microfilaments is somewhat dissimilar. Cytochalasin D causes the hypercontraction of microfilaments of the cell cortex and prevents their relaxation (Miranda et al., 1974b) whereas cytochalasin B inhibits the temperature dependent gelation of actin in vitro by preventing the cross-linking of actin by actin binding protein (Hartwig and Stossel, 1976). Cytochalasins also interfere with a few other aspects of cell physiology. A prominent effect on cellular metabolism is the inhibition of hexose transport by cytochalasin B (Gee et al., 1974; Kletzien and Perdue, 1973). The inhibition of phagocytosis by this drug however, is unlikely to be a result of its interference with sugar transport since concentrations 10 times less than those needed to inhibit phagocytosis are sufficient to inhibit sugar transport (Axline and Reaven, 1974, and present observations). Furthermore, cytochalasin D has been shown to be a poor inhibitor of sugar transport (Miranda et al., 1974a; Tannenbaum et al., 1974). Other metabolic processes in the macrophage

like sugar phosphorylation and ATP production do not seem to be affected significantly by cytochalasin B at least (Gee et al., 1974; Mazur and Williamson, 1978). Two other metabolic processes related to phagocytosis, hexose monophosphate pathway (Bauduin, 1975) and superoxide anion production (Nakagawara et al., 1976) are also unaffected by cytochalasin B and D respectively. The lipid fluidity of membranes has also been shown to be unaffected by cytochalasin B (Oppenheimer et al., 1977) but cell deformability is increased by the same drug (Mazur and Williamson, 1978). The last effect is likely to be the result of microfilament disruption by cytochalasin B. It appears, therefore, that inhibition of microfilament function is the most probable cause of the observed effect of cytochalasins on parasite binding.

The exact mechanism by which microfilaments take part in cellular adhesion is far from clear. The prominence of microfilament networks beneath the surface of cells in areas of contact with the adherent particles has been taken as an indication for their participation in particle binding (Reaven and Axline, 1973; Berlin and Oliver, 1978). By means of fluorescent antibody staining Lazarides and Burridge (1975) have shown the prominence of $\alpha$ actinin filaments at sites of cell-to-cell adhesion which suggests the direct participation of microfilamentous structures in cell adhesion.

The role of microfilaments in particle binding has been depicted in a model by Vasiliev et al. (1976) which also explains the increased adhesiveness of the cell periphery. According to this mechanism the increased adherence of particles to peripheral parts of cells is explained by anchorage of surface receptors to cortical microfilaments which takes place optimally in the peripheral parts of the cell, and leads to stable particle binding. In other areas of the cell surface where no such linkage is possible particles can be detached even by minor mechanical stress.

This mode of particle adherence requires the transmembrane linkage of microfilaments to binding sites on the cell surface. Indirect experimental evidence has long suggested such a linkage in view of the inhibitory effect of cytochalasin on cell surface phenomena which,in addition to cell adhesion and phagocytosis, include ligand induced redistribution of surface receptors 'capping' (de Petris, 1974), the rearrangement of cell surface label (Brown and Revel, 1976) and concanavalin A inhibition of cellular adhesion (Letourneau, 1979). Recently, direct ultrastructural evidence for the transmembrane linkage of microfilaments with surface receptors has been provided to support such a notion (Ash and Singer, 1976; Geiger and Singer, 1979; Singer, 1979).

The inhibition of parasite binding by cytochalasin, therefore,

can be explained by its effect on the anchorage of surface recognition receptors to cortical microfilaments. The normal functioning of such a linkage can be prevented in two ways. If direct transmembrane linkage is preexisting, cytochalasin may modulate the function of cell surface receptors by its effect on the polymerization cycle of microfilaments. This in turn would prevent the rearrangement of cell surface receptors to a conformation favourable for stable parasite-to-macrophage association. On the other hand, if no such prior linkage exists, these drugs may prevent the anchorage of the surface receptors to microfilaments, either by blocking their binding sites on the cytoplasmic face of the membrane or by restricting the realignment of the contractile proteins to a position that supports receptor anchorage.

At a wide range of concentrations the anti-microtubule agent colchicine showed a biphasic effect on parasite binding. At low concentrations ($10^{-3}$ + $10^{-5}$M) it produced a moderate inhibition which amounted to 30% whereas at the concentration of $10^{-2}$M it produced a much greater effect of 70% inhibition. Since at the lower concentrations of $10^{-5}$ or $10^{-6}$M colchicine binds only to microtubules (Borisy and Taylor, 1967) and is sufficient to cause their disappearance (Pesanti and Axline, 1976) it is concluded therefore, that microtubules as such would only moderately influence parasite binding. The

mode of action of colchicine at high concentrations is at present difficult to speculate on.

Opinions on the effect of colchicine on phagocytosis have varied considerably. One group of investigators have found no significant effect by colchicine on phagocytosis and have concluded that phagocytosis is a microtubule independent phenomenon (Malawista et al., 1967; Bhisey and Freed, 1971; Pesanti and Axline, 1975). Another group have reported a significant effect by colchicine which varied between 30-50% inhibition at low concentrations (Stossel et al., 1972; Ukena and Berlin, 1972 ; Mimura and Asano, 1976).

Results obtained in this study favour a significant effect for colchicine in phagocyte function. In a similar fashion to some of the findings in the above mentioned studies this effect manifested itself in a decreased number of parasites associated with macrophages which suggests the susceptibility of parasite binding to colchicine. Some previous evidence also suggests such an inhibitory action on particle attachment to macrophages (Atkinson and Parker, 1977) and on cell adhesion in general (Penny et al., 1966; Kolodny, 1972; Shields and Pollock, 1974).

The role of microtubules in attachment and phagocytosis as a whole has been obscure. A few recent studies provide some

insight into the role of microtubules in phagocytosis. It is now known that phagocytosis is accompanied by a rapid assembly and disassembly of microtubules associated with the centriole (Burchill et al., 1978). This finding has been extended by Berlin and Oliver (1978) who have shown that congregation of microfilaments beneath areas of particle adherence to macrophage coincides with the disappearance of microtubules from the same region. They have suggested that the role of microtubules in determining surface topography and probably phagocytosis emerges as one that controls the distribution and function of microfilaments. In the absence of either microtubule assembly or its clearance from the peripheral parts of the cell the orderly progression of microfilament aggregation and dispersal is either lost or prolonged. It would be tempting then to postulate that the role of microtubules in parasite attachment is reflected similarly in an indirect manner in which the normal function of microfilaments is affected by microtubule assembly and disassembly. In this respect the findings of Mimura and Asano (1976) on the effect of colchicine on phagocytosis lend some support to such a hypothesis. They have found that at concentrations which affect only microtubules, colchicine acts synergistically with suboptimal concentrations of cytochalasin D to inhibit phagocytosis. The elucidation of the mechanism

of this synergism is likely to shed more light on the role of microtubules in phagocytosis.

### 3. Surface Characteristics of the Interaction of Parasites with Macrophages

The four surface reactive agents, periodate, neuraminidase, trypsin and concanavalin A shared the common characteristic of producing an asymmetric effect on parasite binding. In other words, their effect depended on whether they were applied to parasites or macrophages.

The pre-treatment of macrophages with sodium metaperiodate resulted in a moderate but significant inhibition of parasite binding. The concentration of reagent used in this study is generally believed not to affect the viability (Remold, 1973; Ogmundsdottir et al., 1978) or the physiological responsiveness (McClain et al., 1975) of macrophages and lymphocytes. The inhibitory effect manifested itself in the percentage of parasite binding cells rather than a uniform decrease in parasite binding. This was borne out by the fact that the average number of parasites associated with each macrophage among infected ones was the same in treated and untreated monolayers.

Pretreatment of parasites with the same concentration of the drug, on the other hand, increased their adherence to the macrophages. This increase was confined to infected macro-

phages alone since it did not result in any increase in the percentage of macrophages that bound parasites indicating that treatment of parasites with periodate did not increase the ability of macrophages to recognize parasites. Pretreatment of parasites and macrophages confirmed this point once more. A similar decrease in the percentage of parasite binding macrophages took place but the average number of parasites per infected macrophage actually went up. The effect of periodate oxidation of the macrophage, therefore, appears to be an all or none effect since it only affected the percentage of infected macrophages whereas oxidation of parasites appears to produce a quantitative effect since it only increased the number of attached parasites but did not increase the percentage of infected macrophages. The dual nature of the effect of oxidation by periodate suggests the involvement of two steps in parasite binding in which oxidation of the macrophage surface affects the first one and oxidation of the parasite surface the second.

Similar results on the effect of periodate on phagocyte function had been obtained before with a few exceptions. Bona et al. (1968); Ofek et al. (1977) and Ogmundsdottir et al. (1978) all found a decrease in the binding capacity of cells after treatment with periodate. All these studies show the effect of periodate on the phagocyte and convey similar find-

ings. On the other hand Czop et al. (1978) have found that periodate oxidation of erthrocytes increases their uptake by monocytes, while pretreatment of either Mycoplasma or tracheal epithelium with periodate decreases the bacteria to cell association substantially (Powell et al., 1976).

The action of periodate on cell surface chemistry has been studied in some detail and is thought to affect selectively the sialic acid residues of cell surface glycoproteins (Liao et al., 1973; Zatz et al., 1972). The selectiveness of the effect of periodate to sialic acid is suggested by the finding that pretreatment of cells with neuraminidase, an enzyme that removes terminal sialic acid from cell surface polysaccharide moieties, abolished the responsiveness of cells to subsequent stimulation with periodate (Novogrodsky and Katchalsky, 1972). Oxidation with periodate results in the elimination of the two terminal hydroxyl groups from sialic acid with conversion of the remaining terminal hydroxyl group into aldehyde. On this basis Ogmundsdottir et al. (1978) have argued that particle binding by macrophage depends on the presence of terminal hydroxyl groups since their elimination from the macrophage surface by periodate results in diminished particle binding. However, the enhancement of parasite binding by oxidation of the parasite surface by periodate suggests that the role of sialic acid may be more than a presentation of free hydroxyl groups.

The potentiating effect of periodate on parasite adhesion can be explained in two ways. Firstly, the generation of a terminal aldehyde group on the parasite surface may increase the adhesiveness of parasites to macrophages due to the reactivity of the carbonyl group of aldehydes with free amino groups of proteins. However, this is not very likely since the newly formed carbonyl groups can easily be blocked by amino acids or serum proteins found in the extracellular medium. There is the possibility though that such a reaction, especially with serum proteins, might itself increase the adhesiveness of parasites. Secondly, the removal of the two terminal hydroxyl groups might render the modified sialic acid more restrictive in a hypothetical chemical reaction responsible for parasite adhesion in which sialic acid is involved.

Treatment of macrophages and parasites with neuraminidase, an enzyme that removes sialic acid residues caused the exact reverse of the effect produced by treatment with periodate. Thus, pretreatment of macrophages with the enzyme increased their ability to bind parasites whereas pretreatment of parasites with the same enzyme reduced their binding by macrophages. The potentiating effect of neuraminidase on particle binding by macrophages is well known (Knop *et al.*, 1978; Ogmundsdottir *et al.*, 1978). However, its effect on phagocytizable particles has been different from what was observed

with parasites in this study. Treatment of erythrocytes with neuraminidase is known to enhance their uptake by phagocytes (Lee, 1968; Czop et al., 1978).

The effect of exogenous neuraminidase on intact cells is attributed to removal of terminal sialic acid residues from cell surface glycoproteins (Lloyd, 1975). A half hour treatment of cells with the same concentration of enzyme used in this study was reported to cause the removal of 60% of accessible surface sialic acid (Tsan and McIntyre, 1976). The presence of sialic acid on the surface of Leishmania promastigotes (Dwyer, 1977) and peritoneal macrophages (Despont et al., 1975) is now confirmed.

Since sialic acid is thought to be the main determinant of cell surface charge negativity (Cook et al., 1961; Skutelsky and Danon, 1969), the increase in adhesiveness brought about by neuraminidase has often been interpreted in terms of changes in the electrostatic properties of cell surfaces, (Ogmundsdottir et al., 1978 ; Galili et al., 1978). The electrostatic interpretations of the effect of neuraminidase on cellular adhesion have been challenged by many investigators: Vicker and Edwards (1972) have argued that changes in surface electric charge cannot explain the increased difference in adhesiveness between transformed and normal fibroblasts since neuraminidase is expected to decrease the difference in surface

charge density between the two types of cells and hence decrease the difference in their adhesiveness rather than increase it. Knop et al. (1978) have also rejected an electrostatic interpretation in view of their finding that the potentiation in bacterial attachment to the neuraminidase treated macrophages reaches its maximum 2 hours after completion of neuraminidase treatment which suggests the involvement of a metabolic event in this phenomenon. A simple electrostatic interpretation for the effect of neuraminidase on parasite binding is not sufficient to explain the different effects of neuraminidase on parasites and macrophages. If surface charge negativity were the only decisive factor in parasite-to-macrophage adhesion then a similar effect of neuraminidase on macrophages and parasites should have been obtained.

Alternatively, the enhancement in adhesiveness by removal of sialic acid is explained by the exposure of other carbohydrate antigenic sites such as galactose groups (Lloyd and Cook, 1974) which are situated at subterminal positions in polysaccharide side chains (Spiro, 1973). Treatment of cells with neuraminidase makes them agglutinable with Soybean agglutinin a lectin which binds to B-N-acetylgalactosaminyl groups (Nicolson, 1973). Two recent studies provide some indirect evidence which suggests that galactosyl groups

may be determinants of leishmanial antigenicity (Ghose, 1976) and infectivity (Dawidowicz, 1975).

A mere protective role for sialic acid either through its negative charge or by masking other protentially adhesive carbohydrate binding sites is not sufficient to explain the results obtained in this study. In both cases, neuraminidase would be expected to show similar effects on parasites and macrophages. The results obtained with periodate treatment further emphasize this point. Treatment of parasites with periodate would not be expected to increase parasite binding since it neither removes sialic acid nor affects its negative charge, whereas treatment of parasites with neuraminidase which presumably achieves both, would be expected to increase their binding rather than decrease it.

It should be pointed out that in most of the studies on the effect of neuraminidase or periodate on phagocytosis, attention was given to the effect of either reagent on one partner in the phagocytic reaction. This usually involved the phagocyte (Ogmundsdottir et al., 1978; Knop et al., 1978; Ofek et al., 1977) or less frequently the phagocytized material (Lee, 1968; Czop et al., 1978). Therefore, no complementarity of effect could be found among the above mentioned studies that deal with the effect of neuraminidase or periodate on phagocytosis. However, an example of complementary effect

for neuraminidase could be found in a study by Pricer and Ashwell (1971) on the uptake of circulatory glycoproteins by liver cells. They found that treatment of the glycoproteins with neuraminidase promoted its uptake by the liver whereas treatment of the liver tissue with the same enzyme prevented the uptake of desialylated glycoprotein. They have interpreted their findings as a structural requirement for sialic acid in the binding of glycoprotein to liver cells, and suggested the participation of a glycosyltransferase in this interaction.

The complementarity of the effects of neuraminidase and periodate strongly suggests the involvement of the transglycosylation of surface sialic acid between parasite and macrophage surface. According to an enzymatic model for cellular adhesion originally proposed by Roseman (1975) an enzyme on one cell would interact with a substrate on another cell to form an activated intermediate or a high-energy complex. The energy of this complex would hold the cells together (Bosmann, 1977). Adoption of this mechanism for parasite binding would assume the presence of glycosyltransferases on the macrophage surface at least. The presence of sialyltransferase on surfaces of several cell types (Despont et al., 1975; Painter and White, 1976) in addition to neuraminidase (Pricer and Ashwell, 1971; Mitsuo et al., 1974; Bosmann, 1974) make a similar supposition about macrophages

possible. If such enzymes exist, the removal of sialic acid from the macrophage surface may enhance parasite adhesion by stimulating sialyltransferase activity which results in increased association with parasite surface sialic acid.

Other explanations for the effect of neuraminidase on parasite adhesion can be considered although none of them seem to be satisfactory. It has been proposed that neuraminidase affects the topography of membrane glycoproteins. Removal of highly negatively charged sialic acid residues from these glycoproteins would decrease their mutual repulsion and cause them to aggregate in clusters (Lloyd, 1975). The same criticism levelled against an electrostatic interpretation applies to this sort of interpretation. If the increased adhesiveness observed after neuraminidase treatment had been a result of the clustering of membrane glycoproteins periodate would have been expected to show the same effect, since the generation of aldehyde groups would also be able to cause clustering of surface receptors due to its reactivity with amino groups of proteins (Novogrodsky and Katchalski, 1972).

Another interpretation for the effect of neuraminidase was proposed by Weiss (1965) based on the finding that neuraminidase increased the deformability of cells. However, it is not yet clear how cell deformability would affect phagocytosis since cytochalasin B which inhibits phagocytosis and cell

adhesion was also found to increase macrophage deformability (Mazur and Williamson, 1978).

Pretreatment of macrophages with trypsin greatly reduced their ability to bind parasites while pretreatment of parasites with the same concentration of trypsin did not produce any appreciable effect on their attachment to macrophages.

The trypsinization of macrophages has been previously shown to abolish their capacity to bind a variety of phagocytizable particles. These include latex particles (Tsan and McIntyre, 1975), bacteria (Allen and Cook, 1970), unopsonized erythrocytes (Czop et al., 1978) and complement coated erythrocytes (Griffin et al., 1975). An exception is antibody mediated attachments of erythrocytes which is not sensitive to trypsin (Lay and Nussenzweig, 1969). Recently, it has been shown that pretreatment of macrophages with trypsin abolishes their ability to bind Trypanosoma cruzi (Nogueira and Cohn 1976) and Trypanosoma dionisii (Baker and Liston, 1978) trypomastigotes and epimastigotes.

The effects of trypsin on macrophage metabolism were studied in some detail by Ulrich (1976) and Tsan and McIntyre (1976) who found a 50% decrease in glucose oxidation and ATP production but no significant effect on cellular viability. However, the susceptibility of cellular adhesion to trypsin has gene-

rally been interpreted as evidence for the involvement of surface proteins or glycoproteins in such a phenomenon. (Moscona, 1961; McClay et al., 1977). By its proteolytic action, it is expected to cause the degradation and release of the exposed portion of most integral proteins of the cell surface (Singer, 1974). As a result of this action macrophages require a lag period to repair surface components (Werb, 1975). Such a repair is sensitive to inhibitors of protein synthesis (McClay et al., 1977).

The inhibition of parasite binding by treatment of macrophages with trypsin strongly suggests that surface integral proteins are responsible for parasite binding. The reason for the lack of effect of trypsin on parasites is not clear. The dense carbohydrate matrix on the promastigote surface reported by Dwyer et al. (1974) might have a protective role against degradation by trypsin. A phenomenon of similar nature has been reported by Yamada et al. (1979). An alternative explanation for this phenomenon can be supplied by the effect of trypsin on the surface topography of cellular membrane. Nicolson (1972) and Rosenblith et al. (1973) have shown that proteolysis increases the mobility of surface receptors and enhances their agglutinability of cells by concanavalin A. The greater effect of trypsin on the macrophage surface would, therefore, imply a stricter requirement for a certain topography which

is disrupted by proteolysis. It would also imply that no such strict topographic requirement is necessary for the parasite surface. Studies with glutaraldehyde fixation have already raised the possibility for such a topographic requirement for parasite binding.

There is yet another aspect of macrophage physiology which has been shown to be susceptible to trypsin. Pollack and Rifkin (1975) found that trypsin can rapidly be interiorized by cultured cells and cause disruption of microfilaments. On this basis Revel et al. (1974) have explained the inhibition of cell-to-substratum adhesion by trypsin. This effect is quite likely to have taken place by trypsinization of macrophage and since the participation of microfilaments in parasite binding has been established by the effect of cytochalasin such a mode of action for the inhibition of parasite binding by trypsin is also likely. The requirement for a surface protein in parasite binding can be demonstrated if the period of recovery from trypsin injury is estimated. Microfilaments need less time to recover from the effect of trypsin than the synthetic repair of surface proteins would.

Pretreatment of macrophages with a low dose of concanavalin A reduced parasite binding to residual levels. Since this inhibition took place in the absence of the lectin during the assay it must, therefore, be the result of its direct inter-

action with macrophage surface components. Pretreatment of parasites with the same concentration of the lectin did not produce a comparable effect. It is not clear whether the inhibitory effect of lectin treatment of parasites is due to the interaction of the lectin with the parasite surface or to the agglutination of the parasites that is caused by the lectin. It may be that large clumps of parasite are more difficult to be bound by macrophages for purely mechanical limitations. For this reason the inhibition of parasite binding by concanavalin A treatment of parasites is not considered to be very informative.

The various effects of concanavalin A are mediated by its interaction with the glycoproteins of the cell surface. More specifically this lectin binds to the mannose and glucose residues of the polysaccharide chain of glycoproteins (Nicolson, 1974; Goldstein and Hayes, 1978). Studies on the effect of concanavalin A on phagocytosis have not as a whole given uniform results. The phagocytosis of latex particles by polymorphonuclear leukocytes (Berlin, 1972) and macrophages (Friend et al., 1975) was found to be significantly inhibited by concanavalin A. A few other studies, on the other hand, fail to show any effect of the lectin on the phagocytosis of antibody coated erythrocytes (Edelson and Cohn, 1974) and latex particles (Edelson and Cohn, 1974;

Goldman, 1974b). The binding of streptococci to epithelial cells is also unaffected by concanavalin A (Ofek et al., 1977) although the binding of Escherischia coli to the same cells could be prevented by the lectin. Furthermore, Allen et al. (1971) have made the interesting observation that while treatment of macrophages with concanavalin A did not prevent the attachment of unopsonized bacteria, the attachment of opsonized bacteria was greatly reduced. In contrast to this, concanavalin A inhibition of the phagocytosis of latex particles was found to take place in the absence of added serum only, whereas in its presence uptake was not inhibited by the lectin (Goldman, 1974b).

At first glance these results seem to lack consistency if taken as a whole. In some cases, the effect of concanavalin A depends on the nature of the particle being ingested or the presence or absence of serum or opsonin. Clearly, these results do not give the impression that concanavalin A is a nonspecific inhibitor of phagocytosis with a broad action on phagocyte function. Conversely, they suggest that concanavalin A probably acts on the cell surface level and that its effect is rather specific and subtle. That such a low dose as 10 ng/ml could be so effective in preventing parasite attachment is another indication of the specificity of the action of this lectin on parasite uptake.

The interference with the binding of parasites to host cells caused by concanavalin A could imply two possibilities. Firstly, both concanavalin A and parasite antigenic sites could share the same binding site on the macrophage surface. This in turn could take place in two ways. Both ligands may share the same area of the hypothetical molecule on the macrophage surface or residue of that molecule in which case the lectin and parasite antigen would show true cross reactivity. Alternatively the lectin and parasite antigens share distinct parts on residues on the macrophage surface receptor and the two would then show partial cross reactivity. Secondly, concanavalin A may not share the same binding sites with parasite surface antigens at all.

If concanavalin A shares the same binding site with parasites on the macrophage surface, inhibition of parasite binding may take place either by simple blockage in case of true cross reactivity or by steric hindrance in case of partial cross reactivity. In either case it is not likely that concanavalin A could prevent parasite binding in such ways, since it should itself cause parasite attachment through concanavalin A receptors on the parasite surface. It is known that Leishmania promastigotes bind concanavalin A avidly (Dwyer, 1977; Dawidowicz et al., 1975). Furthermore, there are several examples of concanavalin A induced attach-

ment and ingestion of different particles (Allen et al., 1971; Goldman and Cooper, 1975; Goldman and Bursuker, 1976), which support this contention. However, it is possible in the case of partial cross reactivity that concanavalin A may restrict the mobility and rearrangement of cell surface ligand by cross linking these ligands. This may prevent parasite binding if mobility of macrophage surface receptors is required for successful binding. Recent work carried out by Rees et al. (1977) on the mechanism of attachment of cells to inert substratum suggest that lateral rearrangement of surface receptors is necessary for cell adhesion.

There is yet another possibility that may arise from the interaction of concanavalin A with macrophage surface receptors. Some recent evidence shows that inhibition of intercellular adhesion by concanavalin A requires certain conditions such as temperature, metabolic energy and microfilament function, which suggest that actual inhibition of adhesion is caused by events that are secondary to lectin binding (Letourneau, 1979). One visible consequence of concanavalin A binding to the cell surface is the disappearance of bound concanavalin A with its receptors from peripheral parts of the cell surface (Goldman, 1976; Brown and Revel, 1976; Vasiliev et al., 1977). Thirty minutes after binding of concanavalin A to the macrophage surface 50% of the lectin is interiorized with concommitant

loss of membrane into the cell interior (Goldman, 1974a; Goldman and Raz, 1975). This takes place mainly at the peripheral areas of the macrophage surface. Such disappearance of lectin receptors could result in diminished parasite adherence in the case of cross reactivity. The distinct preference shown by parasites for peripheral areas of the macrophages add support to such an interpretation.

If concanavalin A does not share binding sites with parasite antigens then inhibition could take place by interiorization of parasite binding sites by concanavalin A induced membrane internalization. This is in fact an extension of the mechanism of inhibition through receptor internalization discussed above. Alternatively concanavalin A may affect cytoskeletal function through a transmembrane linkage of surface receptors with microfilaments and modulate macrophage surface activity on a global scale reducing surface adhesiveness or cytoskeletal grip (Rees et al, 1977; Ash and Singer, 1976), both of which may be necessary for successful parasite binding.

4. Extracellular Chemical Requirements for Parasite Adherence

The study of the role of various components of the medium in the attachment of parasites produced three main findings. Firstly, independence of parasite attachment as such from

the ingredients of Eagle's medium, but its dependence on most components of Earle's balanced salt solution; secondly the requirement for Eagle's medium in the long term maintenance of macrophages for the expression of full parasite binding capacity; thirdly, the requirement for serum in the assay medium. The two principal requirements for normal parasite attachment are, therefore, serum and components of Earle's balanced salt solution. No difference could be detected in parasite attachment if Eagle's medium were replaced by Earle's balanced salt solution in the assay medium. However, if macrophages were maintained in Earle's solution instead of Eagle's medium a moderate reduction in parasite attachment could be detected. This effect could not be reversed by re-introducing Eagle's medium in the assay medium. Since amino acids are the main additional ingredients found in Eagle's medium, it was thought that their presence might be required for the synthesis of a protein factor by macrophages that takes part in parasite binding. This possibility was tested by preincubating macrophages for 24 hours with cycloheximide, an inhibitor of protein synthesis. This chemical which blocks protein synthesis at the ribosomal stage (Siegel & Sisler, 1965) has been found to inhibit synthesis of a component of complement in macrophages by 80% (Einstein et al., 1976). No inhibitory effect of cycloheximide could be detected. On the contrary,

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some increase in parasite binding could be observed in cycloheximide treated macrophages. If confirmed, this increase could suggest the production by macrophages of a factor that protects them against parasite attachment.

The requirement for serum in parasite attachment seems to be largely confined to the period when the parasite to macrophage interaction takes place and is not related to its familiar role in the general metabolism of cultured cells. Its mere withdrawal from the assay medium was sufficient to reveal its effect in parasite attachment, and its reintroduction into the assay medium to restore parasite attachment to normal levels. Its presence in the extracellular medium is, therefore, sufficient to show its effect on parasite attachment and no opsonization of either parasites or macrophages seems to be required. However, parasite binding is not completely abolished by removal of serum from the extracellular medium. Even after four washes with serum-free medium about a 40% level of parasite attachment could be observed in serum-free medium. The concentration of serum in the assay medium after four washes with serum-free medium would be expected to be no more than 0.01% which would not be expected to sustain such a substantial level of parasite attachment. It is possible that some of the active fraction in serum could remain adsorbed to the macrophage surface and be responsible to the

observed level of parasite attachment. The presence of a plasma protein on the surface of macrophages cultured in the presence of serum has been shown recently to be involved in cell spreading (Colvin et al., 1979).

The study of the effect of divalent cations magnesium and calcium showed that parasite attachment was strongly favoured by the presence of magnesium but very little by calcium. In fact, 90% of the activity shown by the two cations could be attributed to magnesium alone. Calcium, therefore, seems to play a very minor role in parasite attachment. It is also of interest to point out that the sum of the effects of the two cations tested separately was equal to the effect of the two cations tested together in the same assay medium. This shows that calcium cannot replace magnesium and suggests that there is a specific requirement for magnesium. If either of the cations could replace the other, the sum of their separate effects would have been expected to be greater than their coordinate effect.

In most cases, the phagocytosis of particles requires the presence of divalent cations in the extracellular medium. The attachment of Corynebacterium parvum to macrophages (Ogmundsdottir and Weir, 1976), the uptake of opsonized and unopsonized oil particles to polymorphonuclear leukocytes (Stossel, 1973) and the attachment of erythrocytes opsonized with anti-

body and complement (Lay and Nussenzweig, 1968) all depend on the divalent cations calcium and magnesium. An exception is the attachment of glutaraldehyde fixed erythrocytes to macrophages (Rabinovitch, 1967). Their engulfment though, is dependent on divalent cations. In two of the examples mentioned above the attachment of C. parvum and opsonized erythrocytes was primarily dependent on magnesium. On the other hand, the ingestion of opsonized particles was found to be equally stimulated by calcium and magnesium (Stossel, 1973). Magnesium was also found to be more effective in the induction of macrophage spreading (Rabinovitch and Destefano, 1973) and ionophore mediated activation of alveolar macrophages (Hand et al., 1977).

The dependence of cellular adhesion on magnesium and the inability of calcium to replace it has been interpreted as evidence for the metabolic nature of divalent cation involvement instead of an electrostatic bridging mechanism (Rabinovitch and De Stefano, 1973). In the latter mechanism, calcium would be expected to be as effective as magnesium. It is not possible at present to identify the subcellular site at which magnesium is required neither the specific metabolic event that it potentiates to induce parasite attachment. Previous studies on the role of magnesium in cellular metabolism have shown that most of the magnesium would be bound to membranes

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and other cellular constituents in the form of nucleotide complexes such as ATP that act as substrates for a variety of phosphorylation reactions (Kamine and Rubin, 1976). By virtue of its association with ATP magnesium may be involved in the metabolic events that accompany phagocytosis such as glycolysis (Yudkin, 1973) or the temperature dependent gelation of actin (Stossel, 1976). In view of the importance of microfilaments in parasite binding the role of magnesium in the temperature dependent gelation of actin becomes more relevant. However, the dependence of several other enzymatic processes on magnesium makes it difficult to confer an insullar role for magnesium in parasite attachment.

Another ingredient of Earle's balanced salt solution, namely glucose was also found to affect parasite binding to a substantial degree. It too, like serum was needed exclusively during the interaction of parasites with macrophages since preincubation of macrophages in glucose free media did not further affect parasite binding.

The role of extracellular glucose in phagocytosis has not been very clear. The uptake of inert particles opsonized with complement proceed normally in the absence of exogenous glucose (Sbarra and Karnovsky, 1959; Stossel, et al., 1970). The role of glucose is bacterial phagocytosis is less clear. A report by Cohn and Morse (1960) shows a strong dependence of

bacterial uptake by leukocytes on glucose whereas Van Oss (1971) has found only a slight decrease in bacterial uptake in the total absence of glucose.

When either glucose or serum were tested in the absence of the other, each one produced a very small effect on parasite binding. When present together in the assay medium their effect was much greater than the sum of their individual effects. In fact, each ingredient was almost completely dependent on the other for the expression of its effect. A similar finding was reported in the phagocytosis of opsonized bacteria by leukocytes (Cohn and Morse, 1960). More recently, two other cellular phenomena were found to be dependent on the simultaneous presence of glucose and serum. The ingestion but not the attachment of opsonized erythrocytes by macrophages (Michl et al., 1976) and T cell mediated cytolysis (MacLennan and Golstein, 1978).

Since glucose is an ubiquitous source of metabolic energy it becomes necessary to decide whether its requirement in parasite attachment is confined to its traditional role as a source of energy or it is involved in some other way. One of the metabolic pathways in which glucose is utilized during phagocytosis is the hexose monophosphate pathway. Within minutes of challenge with particles, macrophages experience a metabolic burst which is characterised by a several-fold

increase in the oxidation of carbon number one of glucose,a specific indicator of the hexose monophosphate pathway (Rossi et al., 1972; Karnovsky et al., 1975). It is therefore, possible that the strict requirement for glucose in parasite binding is a reflection of the macrophage's need for oxidative metabolism.

To test this possibility, the effect of 2 deoxy glucose, a non-metabolizable analogue of glucose was compared with that of glucose. This compound inhibits glycolysis (Wick et al., 1957), but does not affect the oxidation of Carbon one by the hexose monophosphate pathway (MacLennan and Golstein, 1978). About a 50% reduction in parasite binding was recorded in the presence of equimolar amounts of glucose and 2 deoxy glucose. This suggests that glucose oxidation via the hexose monophosphate pathway is not the reason behind the requirement for glucose. To circumvent the inhibition of glycolytic energy production by 2 deoxy glucose, parasite binding was tested in the presence of pyruvate which is a substrate for the production of lactate, the end product of glycolysis. No improvement in parasite binding could be achieved in the presence of equimolar amounts of pyruvate and 2 deoxy glucose suggesting that anaerobic metabolism is not in itself sufficient to support normal parasite binding. Removal of 2 deoxy glucose from the macrophage cultures after

a one hour period of incubation and its replacement with glucose partially restored the parasite binding capacity of the macrophages suggesting a specific requirement for glucose. It is still possible that some requirement for ATP production by anaerobic respiration is necessary for normal parasite binding since pyruvate is not expected to restore fully the ATP content of macrophages (Michl et al., 1976b) and glycolysis is the chief metabolic pathway for the production of metabolic energy (Karnovsky et al., 1975). To further test this possibility macrophages were challenged with parasites in the presence of several other monosaccharides in glucose free media. Of the several other sugars tested it was found that mannose could support parasite binding almost to an equal degree as glucose. Since mannose cannot restore ATP production by the glycolytic pathway in macrophages (Michl et al., (1976b), its effect clearly suggests that the hexose requirement for parasite binding is for reasons other than energy metabolism. Therefore, taken as a whole, these results suggest that ATP level is not the crucial factor that explains differences in parasite binding in the presence of various carbohydrate metabolites.

One possible mechanism originally proposed by Michl and coworkers (1976b) for the role of glucose in phagocytosis merits special consideration in view of the similarity of their results to the present findings. In their study on

the mechanism of Fc receptor and $C_3$ mediated phagocytosis they found that the ingestion of opsonized erythrocytes was dependent on glucose and prevented by 2 deoxy-glucose. Similarly, the inhibition by glucose could be reversed by mannose. Since the inhibition by 2 deoxy glucose could not be explained by the decrease in the ATP supply in macrophages, a new mechanism, transglycosylation was proposed as an explanation for the role of glucose in phagocytosis. Earlier observations have shown that 2 deoxy glucose inhibits protein glycosylation in a variety of organisms such as yeast (Biely and Bauer, 1971), viruses, (Kaluza et al., 1973) and mammalian cells (Steiner et al., 1973). The inhibition of glycosylation in all these cases could only be reversed by mannose apart from glucose itself. The role of glucose then would be visualized as a ligand which binds to cell surface proteins through glycosyltransferases and mediates the attachment of parasites by a similar enzymatic reaction. The binding of concanavalin A to cell surfaces although not mediated by transglycosylation, requires the presence of mannose residues on the cell surface (Podolsky and Weiser, 1975). Successful parasite binding in the presence of mannose and its inhibition by concanavalin A strongly suggests that mannose and glucose act as structural receptors for parasite binding. A similar finding was reported by Ofek and Beachey (1978) in the binding of Escherichia coli to epithelial cells. Several glycoproteins

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found in the circulation are also bound to the surface of macrophages through carbohydrate receptors such as mannose, glucose and fucose (Stahl et al., 1978; Prieels et al., 1978). However, there are several outstanding issues to be resolved in this type of interpretation. First of all, it is not yet exactly understood how glucose would participate in such a mechanism. The prompt effect of glucose withdrawal from the medium does not fit very well in a typical transglycosylation reaction since the incorporation of carbohydrates into proteins takes several hours to reach significant levels (Steiner et al., 1973). Furthermore the effect of 2 deoxy glucose would be expected to be more lasting if it is incorporated by a transglycosylation reaction. Another point which needs clarification is the substitution of glucose by mannose as the carbohydrate requirement. Does this mean that mannose provides another form of transglycosylation that mediates parasite binding or that it is indicative of a two step reaction in which glucose is first epimerized into mannose before taking part in parasite binding? In spite of these inconsistencies, transglycosylation remains the most viable interpretation in the absence of any alternatives.

The identity of the active fraction in serum which acts synergistically with glucose is not entirely clear. It is not likely to be antibody since foetal calf serum is practically

devoid of immunoglobulins (Kekwick, 1959). A few characteristics of the active fraction however, were possible to define. Its molecular weight is most probably greater than 1,000, the maximum molecular weight for substances that are diffusible through ordinary Visking tubing (McPhie, 1971). Its sensitivity to heat suggests its proteinaceous nature. Since only 40% of its activity was lost upon inactivation at 56°C it is not likely to be complement. Exposure to increasing temperatures revealed that the heat sensitivity of the active fraction was discontinuous. Thus at 60°C 70% of its activity was lost but most of the remaining activity was lost only upon raising the temperature to 80°C, which suggests that there may be two instead of one active fraction. Fractionation by ammonium sulfate also suggests such a possibility. Of the various serum fractions precipated at different salt saturations two fractions showed peak activity but none of the peaks was equal to the activity of whole serum. Since 1 % whole serum was as effective as 10% serum and the various fractions were reconstituted at concentrations equal to those found in 10% whole serum, the failure of any fraction to show an activity equal to whole serum suggests the involvement of two fractions in promoting parasite adherence. However, there is the possibility that these two apparently distinct fractions represent a monomer-dimer situation.

The characteristics of the active fraction of serum that could so far be defined showed some similarity to those of

$\alpha_2$ macroglobulin a macromolecular protein of serum that promotes the phagocytosis of colloidal particles by liver slices (Molnar et al., 1977; Blumenstock et al., 1978). This protein belongs to a group of adhesive glycoproteins called fibronectins of which foetal calf serum is a rich source (Yamada and Olden, 1978). These proteins,that are variously called LETS (large external transformation sensitive proteins), cold insoluble globulin or fibronectin,all represent a single or closely related molecules (Grinnell and Hays, 1978; Yamada and Olden, 1978) which mediate cellular adhesion in a variety of cells and tissues. Several other cellular functions also depend on these proteins such as cell surface morphology and microfilament organization (Ali et al., 1977) and attachment of cells to collagen fibres (Klebe, 1974).

If fibronectins prove to be the active factor in serum they could provide a link between the involvement of serum and microfilament function in parasite binding. It is known that treatment of cells with cytochalasin causes the release of surface fibronectin from cells (Ali et al., 1977; Kurkinen et al., 1978) and their treatment with proteases or anti-fibronectin antibody results in loss of microfilament organization (Pollack and Rifkin, 1975). Furthermore, a transmembrane link between cell surface fibronectin and microfilaments called the fibronexus has been shown by ultrastructural

studies (Singer, 1979). The fibronexus could be a good candidate for the transmembrane linkage of surface receptor to microfilaments implied in the interpretation of the effects of cytochalasin and concanavalin A.

An alternative mechanism for the action of serum could be based on the involvement of a serum enzyme. One such example is plasma transglutaminase-activated factor XIII or von Willebrand factor which covalently crosslinks fibronectin to fibrinogen (Mosher, 1975). Another example is galactosytransferase an enzyme from foetal calf serum which causes the adhesion and flattening of cells to plastic substratum (Shur and Roth, 1975). These two propositions can be combined by postulating the involvement of two serum factors in parasite attachment one a macromolecular ligand such as fibronectin, and the other an enzyme, such as transglutaminase which crosslinks the ligand that attaches to the parasite and/or macrophage surface.

II Mechanisms of Parasite Entry into Macrophages

Examination by scanning electron microscopy of macrophages exposed to parasites for various intervals of time made possible a more detailed study of the mechano-morphological aspects of parasite-macrophage interaction.

The prominent feature of this interaction was the flagellar mode of parasite attachment. Previous studies on promastigote

invasion of host cells using mainly phase contrast microscopy have given conflicting results about the orientation of parasites during initial adherence. Pulvertaft and Hoyle (1960) and Akiyama and Haight (1971) have observed that promastigotes attach to host cells by their somatic ends first, whereas Miller and Twohy (1967) and Merino et al. (1977) have reported flagellar type of parasite attachment. Results obtained by scanning electron microscopy support the findings of the latter group. The main reason for the flagellar mode of parasite adherence is thought to be the parasite's motility. Since leishmanial promastigotes swim with their flagella foremost, the flagellar tip is the most likely part of the parasite to make the initial contact with a relatively sessile macrophage.

To distinguish between a mechanical reason and a genuine surface structural one for flagellar attachment, immobilized promastigotes were tested for comparison with motile promastigotes. Parasites inactivated either by chemical fixation or heat shock were found to attach to macrophages with almost any part of their surface. Both somatic and flagellar adherence could be observed with almost equal frequency. This difference in behaviour clearly favours a mechanical interpretation for flagellar attachment. A mixed pattern of attachment will emerge from the interaction of immobile promatigotes since the flagellar and somatic parts will have an almost equal probability of touching the macrophage first as

they settle under gravity. It might be argued that the random pattern of uptake seen after parasite fixation is due to changes in the antigenic structure of the promastigote surface. However, the exposure of promastigotes to 42$^{o}$C for a brief period would hardly be expected to cause any gross changes in surface chemistry of parasites. Furthermore, the greater sensitivity of the parasite surface to neuraminidase than to trypsin suggests that the adhesiveness of the parasite surface is related to carbohydrate rather than protein structures. The former are relatively insensitive to heat and aldehyde fixation. The fact that fixed parasites could attach to macrophages with an avidity equal to living parasites further emphasizes the insensitivity of attachment promoting structures of the parasite to aldehyde fixation. A mechanical explanation for flagellar adherence, therefore, seems to be the most satisfactory explanation.

The attachment of flagellar tips could take place on any part of the macrophage surface with no preference to any projections. There was, however, a distinct preference for the marginal zones of fully spread macrophages. This observation is in line with the findings of Vasiliev et al. (1976) and Dipasquale and Bell (1974) who have shown that the upper surface of cultured epithelial cells is less adhesive than the marginal zones. Vasiliev et al. (1976) have postulated that the greater adhesiveness of cell margins is due to the ability

of surface recognition sites in such areas to anchor to cortical filaments and thus form stable adhesions. The drastic inhibition of parasite attachment following treatment of macrophages with cytochalasin supports such an explanation for the predeliction of promastigotes to the cell margins.

The remarkable development of extensive membraneous processes around each adherent flagellum was the principal feature of the macrophage's response to parasite adherence. The elaborate development of membraneous processes that took the form of sheaths suggests the active nature of the macrophages' response in engulfing the parasite. In almost every case it was clear that parasite entry was being initiated by these extensions. No sign of flagellar penetration could be seen except in very rare situations. Such a mode of entry is not thought to contribute significantly to the process of parasite entry to host cells. The retraction of the lamellar processes with concomitant sinking of the flagella into the macrophage's body can give rise to the false impression that parasites have penetrated by their flagella if it is not realized that lamellar retraction is a secondary phenomenon that follows the initial extension of the lamellar processes. Since cytochalasin D inhibited all stages of parasite uptake it is possible that both lamellar extension and retraction involve microfilament function. The appearance of microspikes at the edge of the lamellar sheaths that engulf the parasite

provide us with some insight into the mechanism of membrane flow around the parasite. These structures which are thought to take part in several membrane dependent activities including cell attachment and spreading (Carr and Carr, 1970; Witkowski and Brighton, 1972) and particle transport (Albrecht-Beuhler and Goldman, 1976) have been shown by means of transmission electron microscopy to contain a core of microfilament bundles (Buckley, 1975; Burgess and Schroeder, 1977). This further emphasizes the involvement of the cytoskeleton in the mounting of the lamellar response to parasite adherence. Most studies of the morphology of the phagocytic act have failed to demonstrate such structures at leading edges of the lamellar envelope (Aikawa et al., 1977; Munthe-Kaas et al., 1976; Tizard and Holmes, 1974; Orenstein and Shelton, 1977). In one of these studies though (Aikawa et al., 1977) similar structures were seen extending from the macrophage surface and surrounding the base of the engulfed Toxoplasma parasite, without signs of a developing lamellar sheath.

The factor that most probably necessitates the participation of microspikes in membrane flow is the unusual length of the parasite which may be as long as 40 μm in many cases.

As originally postulated by Albrecht-Beuhler and Goldman (1976) these microspikes may provide the necessary guidelines for the outflowing lamellae without the apparent need for firm anchorage of the leading edge of the lamellar extension

at all points along the parasite. The function of the microfilament cores of these microspikes with their bases firmly connected to a wider network of filaments at its base could be visualized as a form of scaffolding that supports the progression of the lamellar sheaths. The latter could be achieved by covering the space between microspikes by membraneous flaps by a process similar to webbing.

The time sequence studies of parasite entry showed that the initially rapid engulfment phase soon slowed down, and after only 10 minutes of contact with the parasites the flow of lamellar membrane suffered a sharp decline from which the majority of the macrophages never recovered. The engulfment of a minor proportion of parasites was completed in about one hour but over as long as four hours many parasites still remained extracellular. The reason for this incomplete engulfment is obscure. Stevens and Moulton (1978) have described a similar phenomenon in the phagocytosis of Trypanosoma brucei by mouse macrophages. They have postulated that the size and motility of the parasites prevent complete phagocytosis. The relative ease with which aldehyde-fixed parasites are engulfed suggests that the motility of the parasite is at least partially responsible for this phenomenon, but at the same time it rules out the size of the parasite being of any hindrance to the completion of engulfment. In fact it was possible to find an individual macrophage that had engulfed as many as

six fixed parasites. This shows that uptake is not limited by the availability of macrophage surface membrane required for phagosome formation. This is particularly true of the majority of macrophages which on the average bind 2-3 parasites each. When live preparations were examined under the phase contrast microscope, the extracellular bodies of adherent parasites were seen to oscillate throughout a six-hour period of examination. Such an activity which causes sharp fluctuations in the angle between the parasite body and the macrophage surface may hinder complete engulfment.

The striking morphological features of the macrophages' response to parasite adherence and the greater ease with which fixed parasites were internalized emphasize once more the passive nature of parasite entry into host cells. That parasite entry into host cells could be exclusively mediated by active phagocytosis has already been established in the case of Toxoplasma entry into host cells (Jones et al., 1972). However, the mode of entry of many protozoan parasites is still in some doubt. This is true of Trypanosoma (Alexander, 1975; Kongtong and Inoki, 1975) and Leishmania (Lewis, 1974). Some studies of promastigote entry into host cells conclude that parasites are actively phagocytosed by host cells (Miller and Twohy, 1969; Akiyama and McQuillen, 1972) while other studies are in favour of a more active role for the parasite in its entry to the host cell (Lewis, 1974; Merino,

et al., 1977). It should be pointed out that some of the conclusions in favour of penetration were derived from studies made on cells that were thought to be non-phagocytic. The ability of parasites to enter such cells has therefore, been taken as evidence for active penetration on the part of the parasite. However, the latest consensus about the endocytic character of various cells do not justify such an approach. It is now generally accepted that given the proper stimulus practically any cell is capable of phagocytosis (Silverstein et al., 1977). Cells that are not normally thought of as phagocytes have been observed to engulf inert particles. Two such examples are fibroblasts (Ravinovitch, 1969) and heart cells (Hurle et al., 1978).

It is generally held that phagocytosis can be dissected into its two separate but constituent events: attachment and engulfment. This can apparently be achieved by low temperature (Rabinovitch, 1967; Griffin et al., 1975) treatment of macrophages with cytochalasin (Davis et al., 1971; Munthe-Kaas, 1976), omission of serum (Munthe-Kaas et al., 1976) or fixation of macrophages (Bar-Shavit and Goldman, 1976). Based on the effect of cytochalasins the dissociation of parasite attachment from subsequent entry into host cells has been reported in several parasitic protozoa, including Leishmania (Alexander, 1975), Trypanosoma (Nogueira and Cohn, 1976) and Toxoplasma (Ryning and Remington, 1978).

In contrast to such findings the results obtained in this study failed to establish a clear dissociation between the attachment and engulfment of promastigotes by macrophages. At an ambient temperature of 24°C parasite binding was reduced nearly by half but engulfment did not seem to be affected. At the lower temperature of 10°C engulfment was suppressed but attachment was further reduced to 20% of that observed at 37°C. Likewise, omission of serum from the assay medium reduced parasite attachment by 50-60% without affecting engulfment. On the other hand, treatment of macrophages with cytochalasin D or mild fixation with gultaraldehyde or formaldehyde reduced parasite uptake to residual levels.

It is possible that adhesion between parasites and macrophages is initially mediated by rather weak physical adsorption forces and sufficient area for a stable contact to form requires the active moulding of the macrophage membrane around the parasites. This would increase the area of contact from a cylinder-plane geometry to that of effectively parallel planes. Lowered temperature, macrophage fixation or cytochalasin would thus prevent or interfere with the fluid response of the macrophage surface and, therefore, inhibit the development of a mechanically stable adhesion. Alternatively, these results can be interpreted in the perspective of a physiological mechanism analogous to capping (Berlin and Oliver, 1978). Conditions that restrict changes in surface topography or cyto-

skeletal architecture block the triggering of a local response that would otherwise increase surface adhesiveness or induce pseudopod formation. The latter occurs almost immediately after parasite contact in normal physiological conditions.

## III Overview of Parasite Attachment and Entry Into Macrophages

The study of parasite adherence under various experimental conditions revealed that parasite attachment to macrophages is as complex as the physiology of the macrophage itself. Successful binding depended largely on the physiological response of macrophages whereas parasites seem to participate only in a passive manner; their role confined to the presentation of surface antigens. In general parasite binding was accompanied by two main events, one intracellular and the other on the cell surface. At the intracellular level the macrophages' response required the normal function of microfilaments and possibly microtubules. This was coupled with requirement for a minimum level of energy metabolism by the glycolytic pathway. At the cell surface level binding was initiated by surface carbohydrate structures of parasites and macrophages and required the presence of extracellular glucose, magnesium and a serum factor, having the characteristics of a biochemical interaction and possibly activated by surface enzymes. However, apart from this chemical interaction successful binding seemed to require some degree of macrophage surface

receptor mobility presumably modulated by cytoskeletal structures.

Since the modulation of the cell surface by intracellular events is a physiological mechanism that is involved in a variety of cellular activities (Edelman, 1976), the distinctive feature of the parasite-to-macrophage interaction must belong to the surface recognition reactions between parasites and macrophage. The answer to this problem undoubtedly lies in the surface chemical structure of parasites and macrophages and the extracellular environment that promotes such an interaction.

Based on these factors a schematic model is proposed in an attempt to combine the surface properties of the two organisms with the extracellular requirements for their association.

In this attempt, Roseman's enzymatic model for cellular adhesion (Roseman, 1975; Bosmann, 1977) was used as a guide since experimental findings could only be described adequately by such an approach.

$$\text{Macrophage}_{\text{acceptor}} + \text{monosaccharide (Glucose or Mannose)} + \text{serum protein (ligand)} \xrightarrow[\text{transferase 1}]{\text{glycosyl}} \text{Macrophage}_{\text{ligand}}$$

$$\text{Macrophage}_{\text{ligand}} + \text{parasite}_{\text{donor}} \xrightarrow[\text{transferase 2}]{\text{glycosyl}} \text{Macrophage}_{\text{ligand}}\text{parasite}$$

The acceptor on the macrophage surface is probably a glycosyltransferase such as sialyltransferase or galactosyl transferase.

Donor is sialic acid and/or other surface carbohydrate.

Transferase 1 is serum enzyme

Transferase 2 is macrophage surface enzyme possibly sialyltransferase.

Conditions of the various aspects of parasite attachment to macrophages would lead to the conclusion that surface chemical association is a prerequisite for the stable attachment and engulfment. This could be best explained by a two step attachment process. The first step involves the surface interaction with the help of extracellular substances which trigger the second step an intracellular response culminating in extensive membrane activity and engulfment of the parasite. Although intracellular structures and their normal activity seem to be involved in parasite attachment, the trigger for such activity must arise from the surface interaction since conditions that do not affect intracellular structures still prevent successful parasite attachment. However, since parasite attachment could not be seen under impaired intracellular function these two steps seem to be intimately linked. Alternatively the first step of attachment could have been too weak to withstand the parasites' motility or the rigid experimental procedures employed. It is quite likely, therefore,

that parasite attachment referred to in this study implied both of these constituent steps i.e. initial attachment and stable attachment in the form of engulfment dictated by the shape of the parasite.

The results obtained in this study could, therefore, be explained better in terms of a two step adhesion theory rather than attachment engulfment concept (Rabinovitch, 1967; Jones, 1975). Such an approach is in agreement with the two step adhesion model proposed by Rees and co-workers (1977). According to this model, cell adhesion is controlled by internal structures which confer a grip by the cell on the attachment site and by external structures which confer the cell its stickiness. Engulfment would then correspond to the grip of the cell on the attachment site. Dictated by the shape of the parasite this grip will take the form of engulfment. The engulfment stage of phagocytosis has already been referred to as the second or stable form of attachment (Umbreit and Roseman, 1975) in reference to the work of Rabinovitch (1967). Therefore, engulfment can be taken as an extreme or consummate form of attachment.

Morphological studies have clearly shown the impracticability of attempting to separate attachment from engulfment under a variety of experimental conditions. Such a distinction is, therefore, conceptual rather than operational. It is not

tenable for reasons additional to its lack of applicability in experimental situations.

First, while attachment can be dissociated from engulfment the reverse is not true simply because engulfment cannot take place without prior attachment. The same applies to the first and second steps of a two step attachment theory. Engulfment or stable attachment would, therefore, also include pure attachment or weak attachment as a prerequisite.

Secondly, a model for phagocytosis where engulfment would operate independently of attachment is not consistent with the theory of surface modulation (Edelman, 1976). This theory which is based on a plethora of experimental evidence postulates that surface activities are under the modulating influence of intracellular functions. An attachment/engulfment model of phagocytosis and for that matter, of parasite uptake would not, therefore, be compatible with a theory that links the membrane and cytoplasm in a co-ordinate mode of action.

TABLE 1

Effect of Temperature on the Attachment of Parasites to Macrophages[1]

| Ambient temperature | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage[2] | Parasite attachment index[3] | Per cent of macrophages with one or more parasites[4] | Macrophage parasitization index[5] |
| 37°C | 2.79 ± 0.08 | 1.00 | 91.9 | 1.00 |
| 24°C | 1.51 ± 0.02 | 0.54 | 74.8 | 0.81 |
| 10°C | 0.61 ± 0.06 | 0.22 | 37.6 | 0.41 |

1. Assay conditions: Parasite to macrophage ratio was 5:1 volume of parasite suspension 0.5 ml and period of exposure to parasites 20 minutes.

2. Mean and standard deviation of quadruplicate assays.

3. Ratio of experimental to control values obtained at 37°C.

4. Average of the proportion of infected macrophages in each assay population.

5. Ratio of experimental to control values.

All results differ significantly from each other according to the Wilcoxon Rank Sum Test.

TABLE 2

Effect of Fixation with Glutaraldehyde on the *In Vitro* Uptake of Parasites by Macrophages

| Glutaraldehyde treatment[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage[2] | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control | 3.22 ± 0.16 | 1.00 | 96.4 | 1.00 |
| Macrophages only | 0.26 ± 0.01 | 0.08 | 14.6 | 0.15 |
| Parasites only | 3.16 ± 0.09[3] | 0.98 | 97.9[4] | 1.02 |

1. Parasites and macrophages were pretreated with 0.01% glutaraldehyde prior to the assay.

2. Mean and standard deviation of quadruplicate samples.

3. Eight degrees of overlap of ranks with control.

4. Nine degrees of overlap of ranks with control.

All other results are significantly different from the control values according to Wilcoxon Rank Sum Test.

TABLE 3

Parasite Binding by Macrophages Following Treatment with Different Concentrations of Glutaraldehyde[1]

| Concentration of glutaraldehyde in moles/litre | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.04 ± 0.23[2] | 1.00 | 81.1 | 1.00 |
| $10^{-3}$ | 0.51 ± 0.05 | 0.25 | 27.8 | 0.34 |
| $10^{-4}$ | 0.30 ± 0.07 | 0.15 | 20.0 | 0.24 |
| $10^{-5}$ | 0.52 ± 0.13 | 0.25 | 40.7 | 0.50 |
| $10^{-6}$ | 1.86 ± 0.11[3] | 0.91 | 81.8[4] | 1.01 |

1. Macrophages were pretreated with the various solutions of glutaraldehyde in Eagle's MEM for half an hour, after which they were assayed for parasite binding in standard conditions.

2. Mean and standard deviation of triplicate assays.

3. Two degrees of overlap of ranks with control.

4. Four degrees of overlap of ranks with control.

All other experimental results differ significantly according to the Wilcoxon Rank Sum Test.

TABLE 4

Parasite Binding by Macrophages Following Treatment with Two Concentrations of Acetaldehyde[1]

| Concentration of acetaldehyde in moles/litres | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.17 ± 0.22[2] | 1.00 | 87.4 | 1.00 |
| $10^{-2}$ | 0.62 ± 0.02 | 0.28 | 47.7 | 0.55 |
| $10^{-3}$ | 2.04 ± 0.22[3] | 0.94 | 79.5 | 0.91 |

1. Macrophages were treated with two concentrations of Acetaldehyde in Eagle's MEM for 30 minutes, after which they were assayed for parasite binding in standard conditions.

2. Each value represents the average of triplicate assays.

3. Three degrees of overlap of ranks with control.

All other values differ significantly from that of the control according to Wilcoxon Rank Sum Test.

TABLE 5

Parasite Binding by Macrophages Following Treatment of the Macrophages with Different Concentrations of Formaldehyde[1]

| Concentration of Formaldehyde in moles/litres | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 1.27 ± 0.06[2] | 1.00 | 58.1 | 1.00 |
| $10^{-3}$ | 0.26 ± 0.01 | 0.21 | 14.0 | 0.24 |
| $10^{-4}$ | 0.36 ± 0.05 | 0.28 | 38.4 | 0.66 |
| $10^{-5}$ | 0.76 ± 0.09 | 0.60 | 47.4 | 0.82 |
| $10^{-6}$ | 0.92 ± 0.05 | 0.72 | 48.1 | 0.82 |

1. Macrophages were pretreated with the various solutions of Formaldehyde in Eagle's MEM after which they were assayed for parasite binding in standard conditions.

2. Each value represents the average of duplicate assays. All experimental values differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 6

Effect of 2,4 Dinitrophenol (DNP) and Sodium Azide ($NaN_3$) on Parasite Binding by Macrophages

| Metabolic inhibitor[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.43 ± 0.34 | 1.00 | 92.1 | 1.00 |
| $NaN_3$ ($10^{-2}$M) | 1.15 ± 0.08 | 0.47 | 69.8 | 0.76 |
| Ethanol (1% v/v) | 1.61 ± 0.09 | 0.66 | 76.5 | 0.83 |
| DNP ($10^{-3}$M) + Ethanol (1% v/v) | 1.29 ± 0.15 | 0.80[2] | 71.5 | 0.94[2,3] |

1. Macrophages were pretreated for 1 hour with the inhibitor after which they were assayed for parasite binding in the presence of the same concentration of inhibitor in otherwise standard conditions.

2. Both indices for DNP are based on values obtained for 1% ethanol control.

3. Two degrees of overlap of ranks with control values. All other experimental values differ significantly from control according to Wilcoxon Rank Sum Test.

TABLE 7

Effect of Potassium Cyanide (KCN) and Sodium Fluoride (NaF) on Parasite Binding by Macrophages

| Metabolic inhibitor[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.47 ± 0.33 | 1.00 | 77.61 | 1.00 |
| KCN $10^{-3}$M | 1.95 ± 0.27[2] | 0.79 | 73.68[3] | 0.95 |
| KCN $10^{-4}$M | 2.48 ± 0.19[4] | 1.01 | 78.63[5] | 1.01 |
| NaF $10^{-2}$M | 1.30 ± 0.03 | 0.53 | 63.77 | 0.82 |
| NaF $10^{-4}$M | 1.42 ± 0.07 | 0.58 | 71.71 | 0.92 |

1. Macrophages were preincubated for 1 hour with the inhibitor after which they were assayed for parasite binding in the same concentration of the inhibitor, in otherwise standard condtions.

2. One degree of overlap of ranks with control.

3. Three degrees of " " " " "

4. Four degrees of " " " " "

5. Five degrees of " " " " "

All other experimental results differ significantly from the control.

TABLE 8

Effect of DNP and NaF, and Iodoacetamide on Parasite Binding by Macrophages[1]

| Metabolic inhibitor | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.44 ± 0.17 | 1.00 | 82.2 | 1.00 |
| Ethanol (0.5% v/v) | 2.23 ± 0.18 | 0.91 | 62.7 | 0.76 |
| DNP ($5 \times 10^{-4}$M) and NaF ($5 \times 10^{-4}$M) | 1.29 ± 0.10[2] | 0.53 | 52.3[2] | 0.83 |
| Iodoacetamide ($1.3 \times 10^{-5}$M) | 0.64 ± 0.06 | 0.26 | 35.5 | 0.43 |

1. Macrophages were preincubated for 1 hour in the presence of the inhibitor after which they were challenged with parasites in the presence of the inhibitor.

2. Both indices are based on control values for 0.5% ethanol.

All experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 9

Effect of Cycloheximide on Parasite Binding

| Treatment of macrophages[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.85 ± 0.15 | 1.00 | 87.97 | 1.00 |
| Cycloheximide $10^{-5}$M | 4.26 ± 0.3 | 1.50 | 94.70 | 1.08 |
| Cycloheximide $10^{-6}$M | 3.34 ± 0.55[2] | 1.17 | 91.12 | 1.04 |

1. Macrophages were incubated in culture medium containing the appropriate concentration of cycloheximide for 24 hours before being challenged with parasites.

2. One degree of overlap with control.

All other experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 10

Effect of Various Concentrations of Cytochalasin B (CB) on Parasite Binding by Macrophages

| Treatment of macrophages[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 1.73 ± 0.05 | 1.00 | 68.1 | 1.00 |
| DMSO (1% v/v) | 1.92 ± 0.10[2] | 1.11 | 59.2[2] | 0.87 |
| CB 10 µg/ml | 0.44 ± 0.07 | 0.26 | 24.3 | 0.36 |
| CB 5 µg/ml | 0.44 ± 0.06 | 0.25 | 23.3 | 0.34 |
| CB 1 µg/ml | 0.71 ± 0.28 | 0.42 | 30.1 | 0.44 |
| CB 10 µg/ml wash | 1.68 ± 0.09[3] | 0.97 | 65.4[4] | 0.96 |

1. Macrophages were preincubated for one hour in the presence of the drug after which they were challenged with parasites in the same concentration of the drug. To test the reversibility of the action of the drug, macrophages were incubated for another hour in the absence of the drug and challenged with parasites in normal conditions.

2. One degree of overlap of ranks with control

3. Two degrees of " " " " "

4. Four degrees of " " " " "
All other results differ significantly from control according to Wilcoxon Rank Sum Test.

TABLE 11

Effect of Various Concentrations of Cytochalasin D (CD) on Parasite Binding by Macrophages

| Treatment of macrophages[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 1.93 ± 0.2 | 1.00 | 84.3 | 1.00 |
| CD 10 μg/ml | 0.44 ± 0.12 | 0.23 | 25.0 | 0.30 |
| 1 μg/ml | 0.77 ± 0.07 | 0.40 | 46.8 | 0.56 |
| 0.1 μg/ml | 1.73 ± 0.07[2] | 0.89 | 68.0 | 0.81 |
| CD 10 μg/ml, 1 hour wash | 1.89 ± 0.29[3] | 0.98 | 72.6 | 0.86 |

1. Macrophages were preincubated in various concentrations of the inhibitor for one hour after which they were challenged with parasites in the same concentration of the inhibitor.

2. One degree of overlap of ranks with control.

3. Four degrees of " " " " "

All other experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 12

Effect of Different Concentrations of Colchicine on Parasite Binding by Macrophages

| Treatment of macrophages[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.62 ± 0.22 | 1.00 | 91.3 | 1.00 |
| Colchicine $10^{-2}$M | 0.89 ± 0.19 | 0.34 | 42.5 | 0.46 |
| $10^{-3}$M | 1.82 ± 0.16 | 0.69 | 70.7 | 0.77 |
| $10^{-4}$M | 1.85 ± 0.1 | 0.71 | 78.0 | 0.85 |
| $10^{-5}$M | 1.85 ± 0.1 | 0.71 | 81.9 | 0.90 |

1. Macrophages were preincubated in various concentrations of the inhibitor for 30 minutes after which they were challenged with parasites in the presence of the same concentration of the drug.

   All experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 13

The Attachment of Parasites to Macrophages Following Treatment of Macrophages and Parasites with Sodium Metaperiodate ($NaIO_4$)

| | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| Treatment with Periodate[1] | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.31 ± 0.29[2] | 1.00 | 79.4 | 1.00 |
| Parasites only | 2.91 ± 0.19 | 1.26 | 80.0[3] | 1.01 |
| Parasites and macrophages | 1.91 ± 0.15[4] | 0.83 | 57.0 | 0.72 |
| Macrophages only | 1.55 ± 0.13 | 0.67 | 53.6 | 0.68 |

1. Parasites and macrophages were treated with $2 \times 10^{-3}$ M $NaIO_4$ for 10 minutes at 22°C prior to the assay.
2. Each value represents the average of quadruplicate assays.
3. Ten degrees of overlap of ranks with control.
4. One degree of overlap of ranks with control.

All other experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 14

Parasite Attachment to Macrophages Following Treatment of Parasites and Macrophages with Neuraminidase

| Neuraminidase treatment[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.11 ± 0.14[2] | 1.00 | 71.4 | 1.00 |
| Parasites (0.2 units/ $10^6$ organisms) | 1.53 ± 0.27 | 0.72 | 57.2 | 0.80 |
| Macrophages (0.2 units/ $10^6$ organisms) | 2.92 ± 0.07 | 1.38 | 81.1 | 1.14 |
| Macrophages (0.1 units/ $10^6$ organisms) | 2.89 ± 0.14 | 1.37 | 78.9 | 1.11 |

1. Parasites and macrophages were preincubated with the enzyme for 30 minutes at 37°C.

2. All experimental results differ significantly from the control according to Wilcoxon Rank Sum Test. Mean and standard deviation of triplicate assays.

TABLE 15

Parasite Attachment to Macrophages Following Treatment of Parasites and Macrophages with Trypsin

| | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| Trypsin treatment[1] | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 2.37 ± 0.30[2] | 1.00 | 91.9 | 1.00 |
| Parasites | 2.10 ± 0.18[3] | 0.89 | 78.3 | 0.85 |
| Macrophages | 0.86 ± 0.07 | 0.36 | 39.0 | 0.42 |

1. Parasites and macrophages were preincubated with 500 ug/ml of the enzyme for 30 minutes at 37°C.

2. Each value represents the average of quadruplicate assays.

3. Three degrees of overlap of ranks with control.

All other experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 16

The Effect of Concanavalin A on the Attachment of Parasites to Macrophages

| Concanavalin A treatment[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None (control) | 3.08 ± 0.05[2] | 1.00 | 80.3 | 1.00 |
| Macrophages only | 0.81 ± 0.02 | 0.26 | 38.9 | 0.48 |
| Parasites only | 1.88 ± 0.22 | 0.61 | 44.2 | 0.55 |
| Parasites and macrophages | 0.40 ± 0.14 | 0.13 | 21.1 | 0.26 |

1. Macrophages and parasites were pretreated with 10 ug/ml of the lectin for 30 minutes at 37°C and were then either washed free of the lectin or assayed in the presence of the same concentration of the lectin.

2. Each value represents the average of triplicate assays.

   All experimental results differ significantly from the control and from each other according to Wilcoxon Rank Sum Test.

TABLE 17

The Effect of Various Surface Reactive Agents on the Attachment of Parasites to Macrophages

| Reagent used | Pretreatment of Macrophages: Parasite attachment index | Pretreatment of Macrophages: Composite index of parasite binding[1] | Pretreatment of Parasites: Parasite attachment index | Pretreatment of Parasites: Composite index of parasite binding |
|---|---|---|---|---|
| Sodium Meta-periodate | 0.67 | 0.456 | 1.26 | 1.273 |
| Neuramini-dase | 1.37 | 1.573 | 0.72 | 0.576 |
| Trypsin | 0.36 | 0.151 | 0.89 | 0.757 |
| Concana-valin A | 0.26 | 0.125 | 0.61 | 0.336 |

1. Parasite attachment index X macrophage parasitization index

TABLE 18

Role of the Different Components of the Macrophage Culture Medium in Parasite Binding

| | Incubation conditions | | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|---|---|
| | Maintenance medium[1] | Assay medium[2] | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| A | Eagle's MEM 10% FCS | Eagle's MEM 10% FCS | 1.28 ± 0.12 | 1.00 | 61.4 | 1.00 |
| B | Eagle's MEM 10% FCS | Eagle's MEM | 0.80 ± 0.09[3] | 0.63 | 45.9[4] | 0.75 |
| C | Earle's BSS 10% FCS | Eagle's MEM 10% FCS | 0.84 ± 0.05[5] | 0.66 | 50.9[6] | 0.83 |
| D | Earle's BSS 10% FCS | Earle's BSS 10% FCS | 0.95 ± 0.07 | 0.74 | 48.4 | 0.79 |
| E | Earle's BSS 10% FCS | Eagle's MEM | 0.59 ± 0.15 | 0.46 | 33.4 | 0.54 |

1. Macrophages were maintained in the specified media for 24 hrs before the assay

Table IB (cont.)

2. Macrophages were washed three times with the specified media before being assayed in the same media.

3. Five degrees of overlap with C and two with E.

4. One degree of " " C " three with D.

5. One degree of " " D.

6. Three degrees of " " D.

All other results differ significantly from each other according to Wilcoxon Rank Sum Test.

TABLE 19

Effect of Divalent Cations on Parasite Binding by Macrophages

| Divalent cation content of assay medium[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| None EDTA 2mM | 0.45 ± 0.05 | 0.21 | 29.1 | 0.39 |
| Calcium 2mM | 0.64 ± 0.04 | 0.31 | 41.2 | 0.56 |
| Magnesium 2mM | 1.93 ± 0.13 | 0.91 | 81.1 | 1.10 |
| Calcium + Magnesium 2mM each | 2.12 ± 0.03 | 1.00 | 74.0 | 1.00 |

1. Apart from the stated divalent cation content, the assay medium consisted of 0.90% NaCl, 0.01% KCl, 0.22% $NaHCO_3$ and 5.5mM Glucose.

   All results differ significantly from each other according to the Wilcoxon Rank Sum Test.

TABLE 20

Effect of Glucose Starvation on Parasite Binding

| Macrophage preincubation conditions[1] | Parasite[2] attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| Earle's BSS 2 hr (control) | 2.58 ± 0.43 | 1.00 | 79.5 | 1.00 |
| GF Earle's BSS 2 hr | 0.82 ± 0.32 | 0.32 | 45.1 | 0.57 |
| GF Earle's BSS 30 mins. | 0.74 ± 0.19 | 0.29 | 45.8 | 0.58 |
| GF Earle's BSS 15 mins. | 0.75 ± 0.30 | 0.29 | 41.2 | 0.52 |
| GF Earle's BSS 0 min. | 1.02 ± 0.09 | 0.40 | 47.3 | 0.60 |

1. Macrophages were preincubated in Earle's BSS or Glucose free (GF) Earle's BSS for different intervals of time before assay was performed in the same medium.

2. Promastigotes were washed 3 times in GF Earle's BSS and resuspended in Earle's or GF Earle's BSS.

All experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 21

Effect of Uninactivated Foetal Calf Serum on Parasite Binding

| Macrophage incubation conditions[1] | | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|---|
| Preincubation medium | Assay medium | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| 10% FCS (control) | 10% FCS | 2.27 ± 0.26 | 1.00 | 79.4 | 1.00 |
| 1% FCS 6 hour | 1% FCS | 2.19 ± 0.11[2] | 0.96 | 73.1[3] | 0.92 |
| Serum free 6 hour | Serum free | 1.25 ± 0.22 | 0.55 | 56.0 | 0.70 |
| Serum free 2 hour | Serum free | 1.46 ± 0.19 | 0.64 | 59.0 | 0.74 |
| 10% FCS | Serum free | 1.24 ± 0.14 | 0.55 | 52.6 | 0.66 |

1. Macrophages were washed three times with Eagle's MEM with or without serum and incubated in the same medium for the specified period before being challenged with parasites.

2. Three degrees of overlap with control.

3. Two degrees of overlap with control.

All other experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 22

Synergistic Effect of Glucose and Serum on Parasite Binding

| Composition of assay medium | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| Eagle's MEM + 10% FCS (control) | 1.85 ± 0.41 | 1.00 | 61.11 | 1.00 |
| Glucose + serum[1] | 1.65 ± 0.09[2] | 0.89 | 62.7[2] | 1.02 |
| Serum | 0.42 ± 0.12 | 0.23 | 26.0 | 0.43 |
| Glucose | 0.32 ± 0.05 | 0.17 | 24.0 | 0.39 |
| None | 0.20 ± 0.03 | 0.11 | 14.7 | 0.24 |

1. The basic experimental assay medium consisted of Glucose-free Earle's balanced salt solution (GF EBSS). To this glucose and dialysed foetal calf serum were added at the concentrations of 5.5mM and 10% (v/v) respectively. Macrophages were washed three times in GF EBSS and challenged with parasites immediately.

2. Three degrees of overlap with control.

3. All other experimental results differ significantly from the control according to Wilcoxon Rank Sum Test.

TABLE 23

Effect of 2 Deoxy Glucose on Parasite Binding

| Macrophage incubation conditions[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| 5.5mM Gluc. 1 hr (control) | 1.83 ± 0.07 | 1.00 | 79.8 | 1.00 |
| 5.5mM Gluc. + 5.5mM 2DG, 1 hour | 1.02 ± 0.10 | 0.56 | 55.3 | 0.69 |
| 5.5mM 2DG + 5.5mM pyruvate 1 hour | 0.79 ± 0.10 | 0.43 | 48.4 | 0.61 |
| 5.5mM 2DG 1 hour | 0.61 ± 0.10 | 0.36 | 43.5 | 0.55 |
| 5.5mM 2DG 1 hour → 5.5mM Gluc. 1 hour | 1.22 ± 0.10 | 0.67 | 60.8 | 0.76 |

1. Macrophages were incubated in Glucose free Earle's BSS + 10% FCS containing the stated concentrations of Glucose (Gluc.) or 2 Deoxy glucose (2DG) for the specified periods of incubation after which they were challenged with parasites in the media in which they were last incubated.

All experimental results differ significantly from the control according to the Wilcoxon Rank Sum Test.

TABLE 24

Effect of Various Carbohydrates on Parasite Binding

| Carbohydrate content of assay medium[1] | Parasite attachment: Average number of parasites attached per macrophage | Parasite attachment: Parasite attachment index | Macrophage parasitization: Per cent of macrophages with one or more parasites | Macrophage parasitization: Macrophage parasitization index |
|---|---|---|---|---|
| Glucose (control) | 1.02 ± 0.05 | 1.00 | 45.7 | 1.00 |
| Glucose free | 0.38 ± 0.08 | 0.38 | 19.1 | 0.42 |
| Fucose | 0.12 ± 0.04 | 0.12 | 11.5 | 0.25 |
| Galactose | 0.28 ± 0.08 | 0.27 | 17.7 | 0.39 |
| Xylose | 0.40 ± 0.05 | 0.39 | 18.2 | 0.40 |
| Mannose | 0.96 ± 0.21[2] | 0.94 | 36.4[3] | 0.80 |

1. Macrophages were washed with Glucose free Earle's BSS and challenged with parasites in medium consisting of 10% dialyzed FCS in Glucose free Earle's BSS plus one of the various sugars at a concentration of 5.5mM.

2. Four degrees of overlap of ranks with control.

3. One degree of overlap of ranks with control.

All other results differ significantly from the control according to Wilcoxon Rank Sum Test. Results obtained for Fucose also differ significantly from those of Glucose-free according to the same test.

TABLE 25

Effect of Dialysis on the Activity of Foetal Calf Serum in Parasite Binding

| Fraction of serum used in assay[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| Whole serum control | 2.72 ± 0.28 | 1.00 | 8.62 | 1.00 |
| Retentate | 2.62 ± 0.24[2] | 0.97 | 81.2 | 0.94 |
| Diffusate | 1.35 ± 0.18 | 0.50 | 66.6 | 0.77 |
| Retentate + Diffusate | 2.86 ± 0.16[3] | 1.05 | 94.9 | 1.10 |

1. Macrophages were washed three times with serum free Eagle's MEM before being challenged with parasites in the presence of the different fractions of the serum.

2. Three degrees of overlap with control.

3. One degree of overlap with control.

All other experimental results differ significantly from the control according to the Wilcoxon Rank Sum Test.

TABLE 26

Effect of Heat Denaturation on the Activity of Foetal Calf Serum in Parasite Binding

| Temperature of inactivation[1] | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization |
| activated (control) | 2.02 ± 0.05 | 1.00 | 72.5 | 1.00 |
| 56°C | 1.52 ± 0.07 | 0.75 | 68.2 | 0.94 |
| 80°C | 0.82 ± 0.18 | 0.41 | 48.2 | 0.67 |
| 100°C | 0.78 ± 0.06 | 0.39 | 45.8 | 0.63 |

1. Serum samples were heat inactivated for 30 minutes in a constant temperature bath and used instead of normal serum in experimental assays.

   All experimental results differ significantly from the control according to the Wilcoxon Rank Sum Test.

TABLE 27

Activities of Heat Inactivated Foetal Calf Serum and Partially Purified Albumin on Parasite Binding

| | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| Temperature of inactivation[1] | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| Uninactivated (control) | 1.99 ± 0.09 | 1.00 | 67.0 | 1.00 |
| 60°C | 1.05 ± 0.04 | 0.53 | 47.5 | 0.71 |
| 70°C | 0.95 ± 0.06 | 0.48 | 47.5 | 0.71 |
| 80°C | 0.66 ± 0.05 | 0.33 | 34.3 | 0.51 |
| Albumin 0.2% | 0.90 ± 0.08 | 0.45 | 44.3 | 0.66 |

1. Incubation and inactivation conditions were the same as described in Table 26.

All experimental results differ significantly from the control according to the Wilcoxon Rank Sum Test.

TABLE 28

Activities of Ammonium Sulfate Precipitated Fractions of Foetal Calf Serum in Parasite Binding

| | Parasite attachment | | Macrophage parasitization | |
|---|---|---|---|---|
| Fraction of serum used in assay[1] | Average number of parasites attached per macrophage | Parasite attachment index | Per cent of macrophages with one or more parasites | Macrophage parasitization index |
| Whole serum (control) | 2.46 ± 0.09 | 1.00 | 86.0 | 1.00 |
| 70% | 1.80 ± 0.13 | 0.73 | 70.0 | 0.81 |
| 60% | 1.09 ± 0.09 | 0.44 | 54.1 | 0.63 |
| 50% | 1.58 ± 0.13 | 0.64 | 67.0 | 0.78 |
| 40% | 1.88 ± 0.11 | 0.77 | 77.4 | 0.90 |
| 30% | 1.15 ± 0.08 | 0.47 | 58.8 | 0.68 |

1. The fractions precipitated with various saturations of ammonium sulfate were incorporated in the assay medium at the same concentration as foetal calf serum (10%).

All experimental results differ significantly from the control according to the Wilcoxon Rank Sum Test.

TABLE 29

Properties of the Active Component(s) in Foetal Calf Serum that Promote Parasite Binding

| Treatment of serum | Fraction of serum used in assay | Retention of activity in parasite binding[1] |
|---|---|---|
| None | Whole serum | ++++ |
| Dialysis | Retentate | ++++ |
| | Diffusate | - |
| Heat denaturation | 56°C | ++ |
| | 60°C | + |
| | 70°C | + |
| | 80°C | - |
| Ammonium sulfate Precipitation | 30% | - |
| | 40% | ++ |
| | 50% | + |
| | 60% | - |
| | 70% | ++ |

1. Each plus sign represents 25% of the activity of uninactivated whole serum. Values are approximate and range from complete retention (++++) to complete loss (-) of activity.

TABLE 29

Properties of the Active Component(s) in Foetal Calf Serum that Promote Parasite Binding

| Treatment of serum | Fraction of serum used in assay | Retention of activity in parasite binding[1] |
|---|---|---|
| None | Whole serum | ++++ |
| Dialysis | Retentate | ++++ |
| | Diffusate | - |
| Heat denaturation | 56°C | ++ |
| | 60°C | + |
| | 70°C | + |
| | 80°C | - |
| Ammonium sulfate Precipitation | 30% | - |
| | 40% | ++ |
| | 50% | + |
| | 60% | - |
| | 70% | ++ |

1. Each plus sign represents 25% of the activity of uninactivated whole serum. Values are approximate and range from complete retention (++++) to complete loss (-) of activity.

TABLE 29

Properties of the Active Component(s) in Foetal Calf Serum that Promote Parasite Binding

| Treatment of serum | Fraction of serum used in assay | Retention of activity in parasite binding[1] |
|---|---|---|
| None | Whole serum | ++++ |
| Dialysis | Retentate | ++++ |
| | Diffusate | - |
| Heat denaturation | 56°C | ++ |
| | 60°C | + |
| | 70°C | + |
| | 80°C | - |
| Ammonium sulfate Precipitation | 30% | - |
| | 40% | ++ |
| | 50% | + |
| | 60% | - |
| | 70% | ++ |

1. Each plus sign represents 25% of the activity of uninactivated whole serum. Values are approximate and range from complete retention (++++) to complete loss (-) of activity.

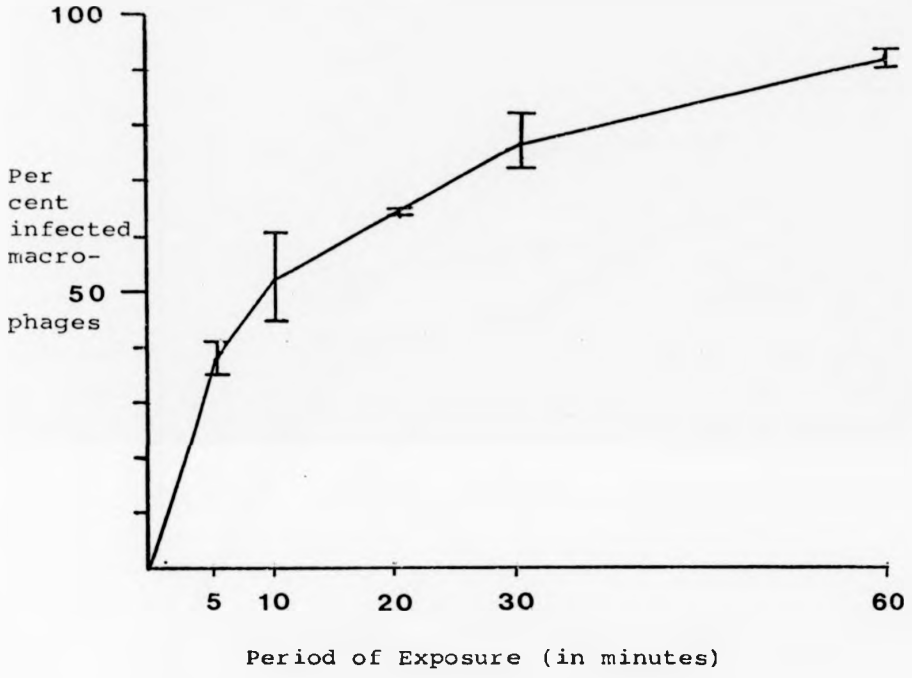



Fig. 1 Relationship of period of exposure to parasites to macrophage parasitization. Percentage of infected cells as a fraction of period of exposure. Macrophages were exposed to parasites at a ratio of five parasites per macrophage. Volume of parasite suspension 1 ml. Each point on the graph represents the average of duplicate assays.

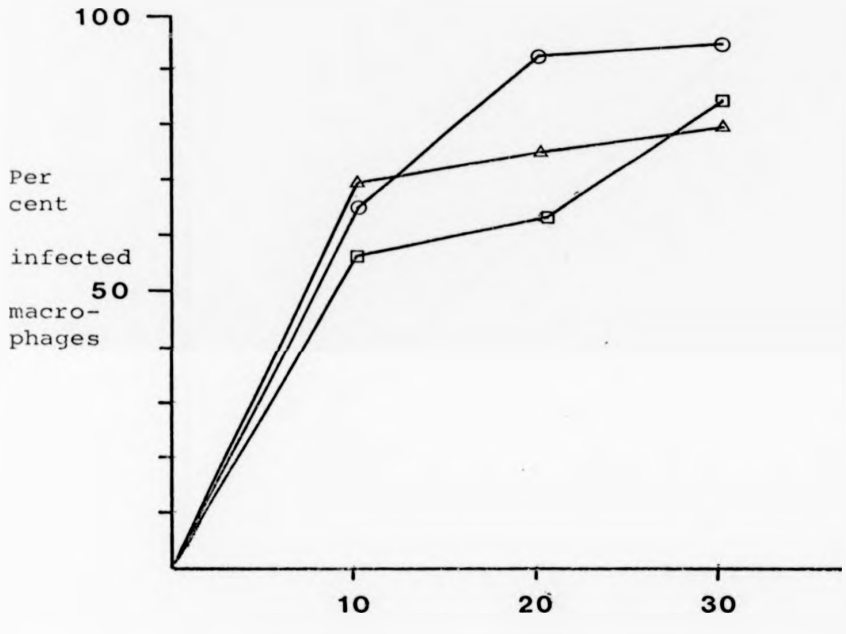



Fig. 2 Relationship of time of exposure to and volume of parasite suspension to macrophage parasitization. Percentage of infected cells as a function of time. Macrophages were exposed to parasites at a ratio of five parasites per macrophage suspended in 0.25 ml (Δ), 0.5 ml (O) and 1 ml (□). Each point on the graph represents the average of duplicate assays.

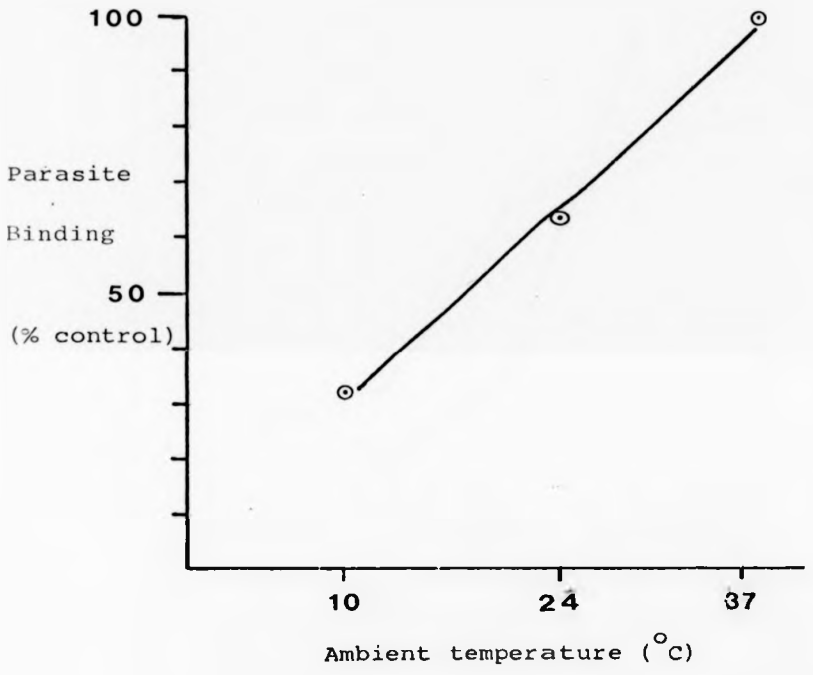



Fig. 3 The effect of temperature on parasite binding by macrophages. Abscissa: Ambient temperature (°C). Ordinate: average number of parasites attached per macrophage (per cent of control, 37°C). See also Table 1.

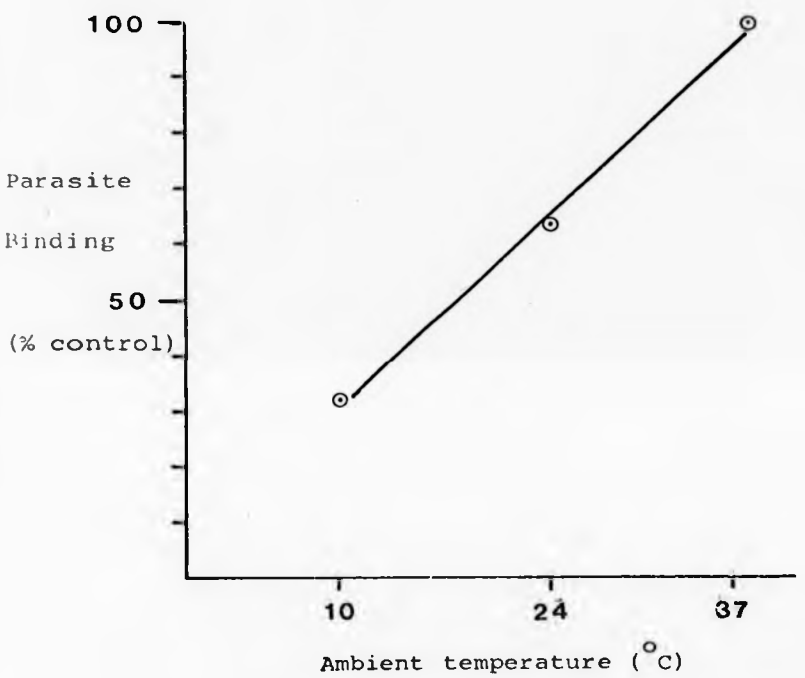



Fig. 3 The effect of temperature on parasite binding by macrophages. Abscissa: Ambient temperature (°C). Ordinate: average number of parasites attached per macrophage (per cent of control, 37°C). See also Table 1.

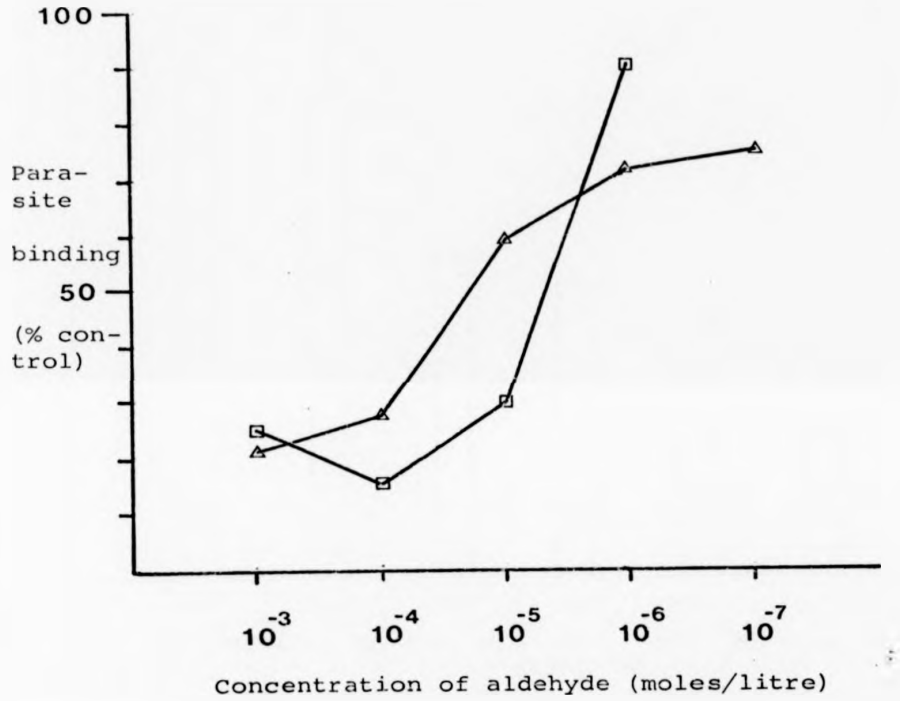



Fig. 4 Comparative dose effects of glutaraldehyde ( □ ) and formaldehyde ( △ ) on parasite binding by macrophages. Abscissa: molar concentration of aldehyde fixative, ordinate average number of parasites attached per macrophage (% of control). See also Tables 3 and 4.

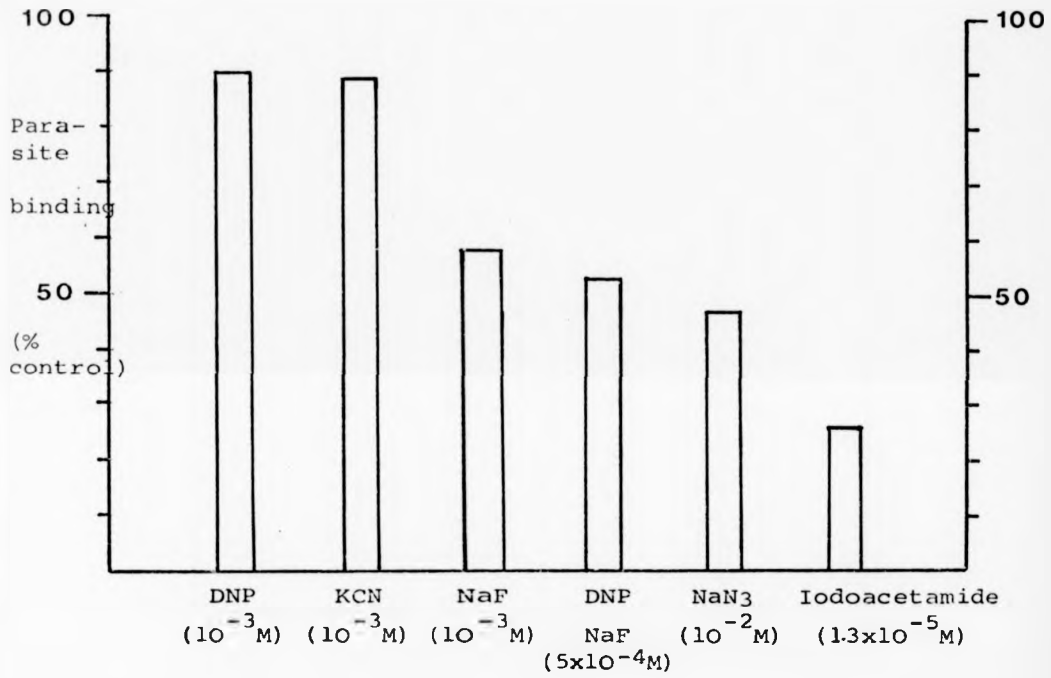



Fig. 5 Effect of various inhibitors of energy metabolism on parasite binding. Average number of parasites attached per macrophage (per cent of control). See also Tables 6, 7 and 8.

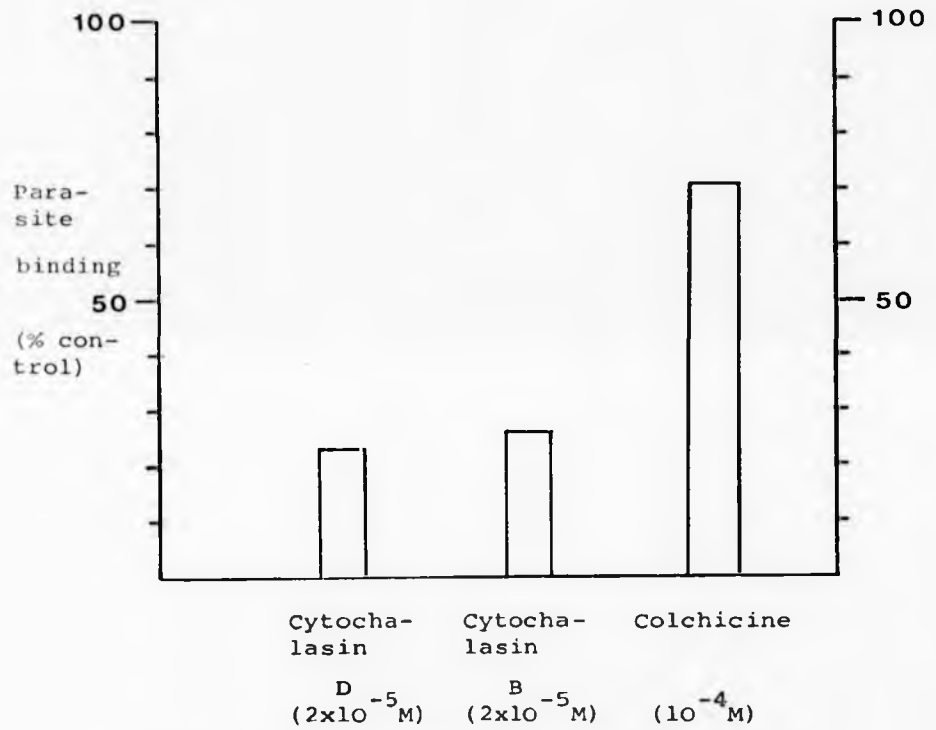



Fig. 6 Effect of inhibitors of the cytoskeletal system on parasite binding. Average number of parasites attached per macrophage (per cent of control). See also Tables 9, 10 and 11.

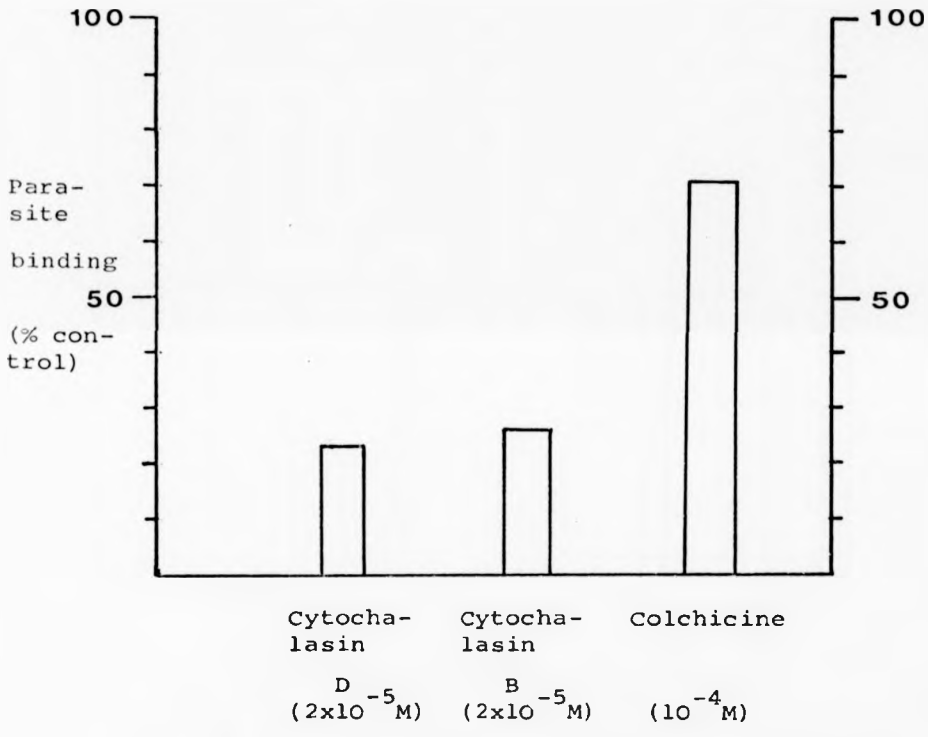



Fig. 6 Effect of inhibitors of the cytoskeletal system on parasite binding. Average number of parasites attached per macrophage (per cent of control). See also Tables 9, 10 and 11.

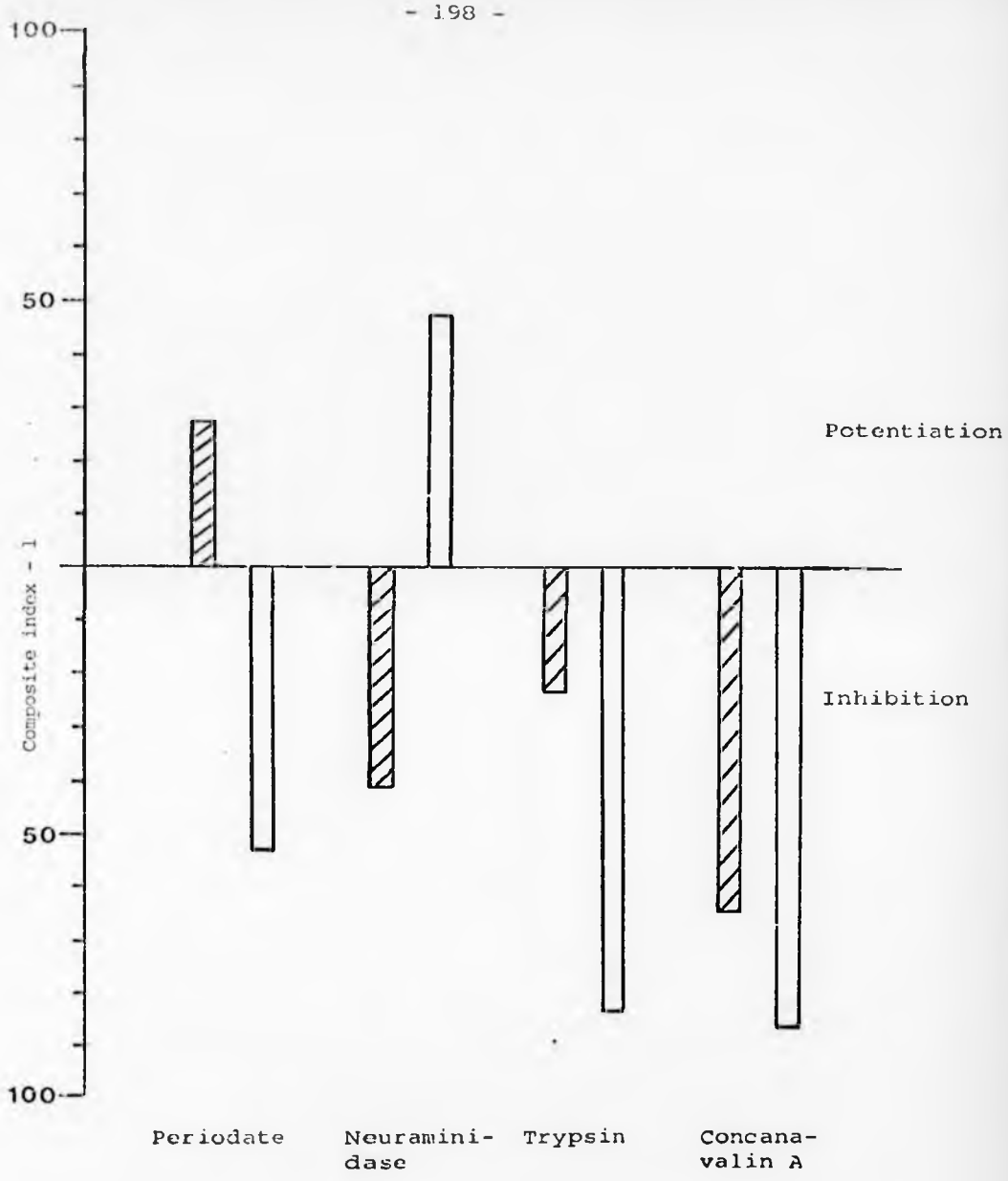



Fig. 7 Changes in parasite uptake after treatment of parasites ▨ and macrophages ▭ with various surface reactive agents. Composite index of parasite binding. (Parasite attachment index x macrophage parasitization index) - 1.

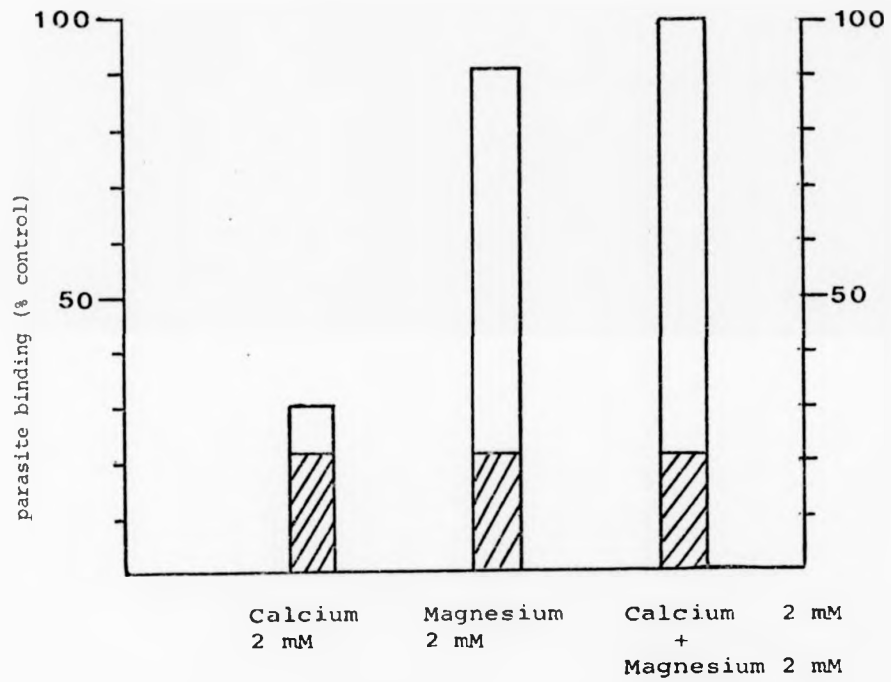



Fig. 8 Effect of divalent cations on parasite binding. Parasite attachment index in the presence of calcium and/or magnesium. Open bars represent the net activities of the divalent cations, hatched bars represent parasite attachment in the absence of divalent cations.

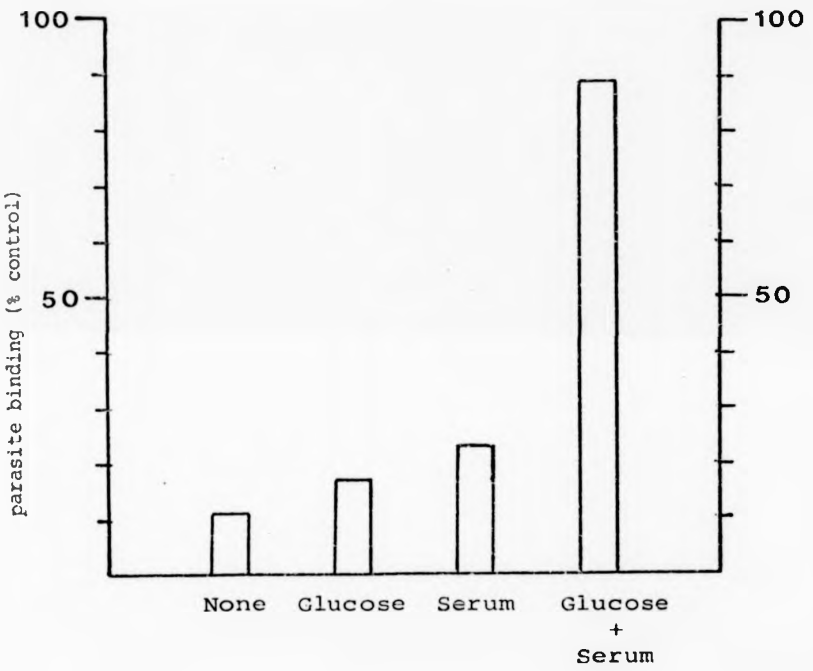



Fig. 9 Synergistic effect of glucose and serum on parasite binding. Average number of parasites attached per macrophage (per cent of control). For other explanations see Table 25.

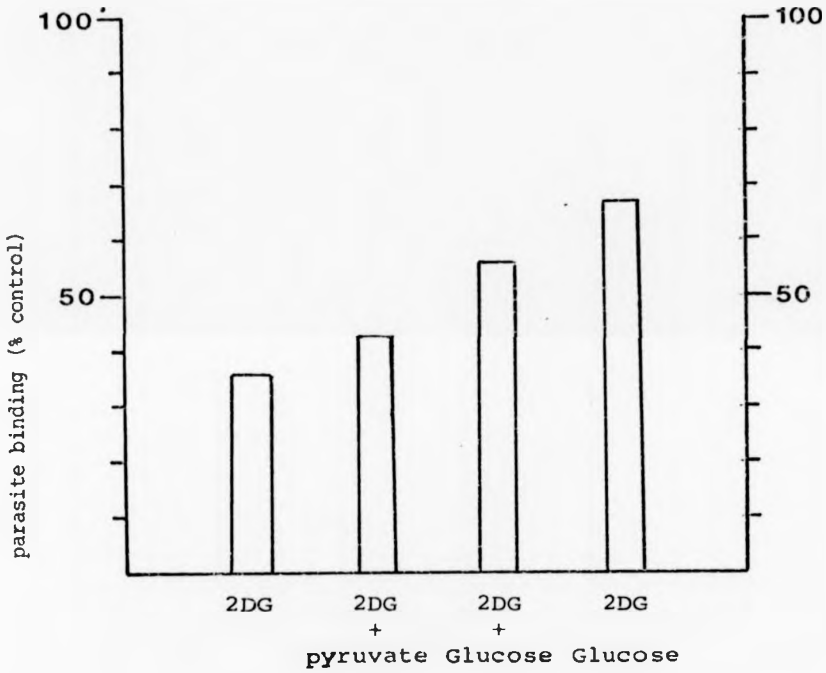



Fig. 10 Effect of 2 Deoxyglucose (2DG) on parasite binding. Average number of parasites attached per macrophage (per cent of control). For other explanations see Table 23.

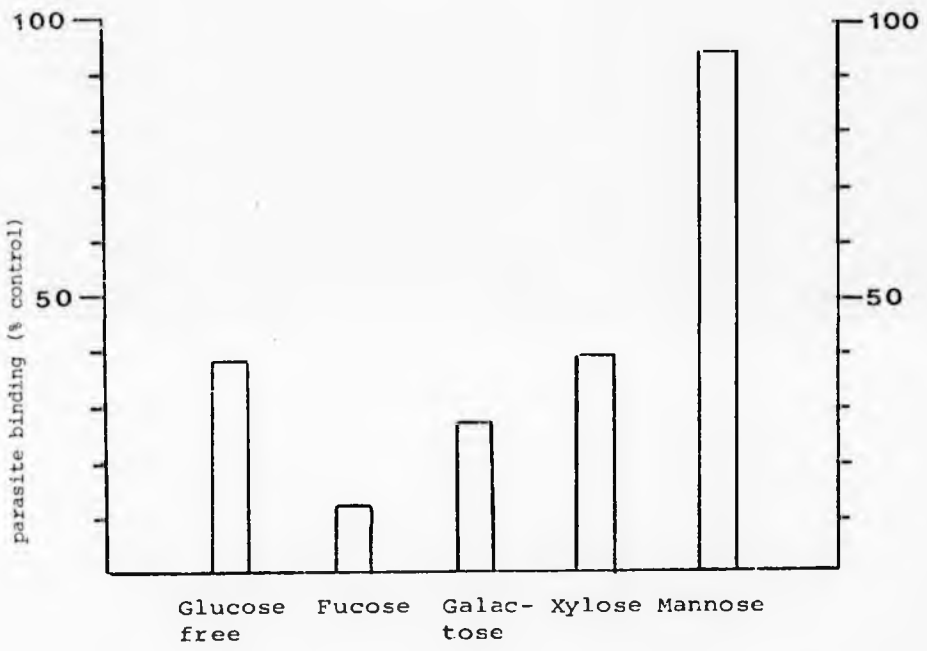



Fig. 11 Effect of various carbohydrates on parasite binding. Average number of parasites attached per macrophage (per cent of control). For other explanations see Table 24.

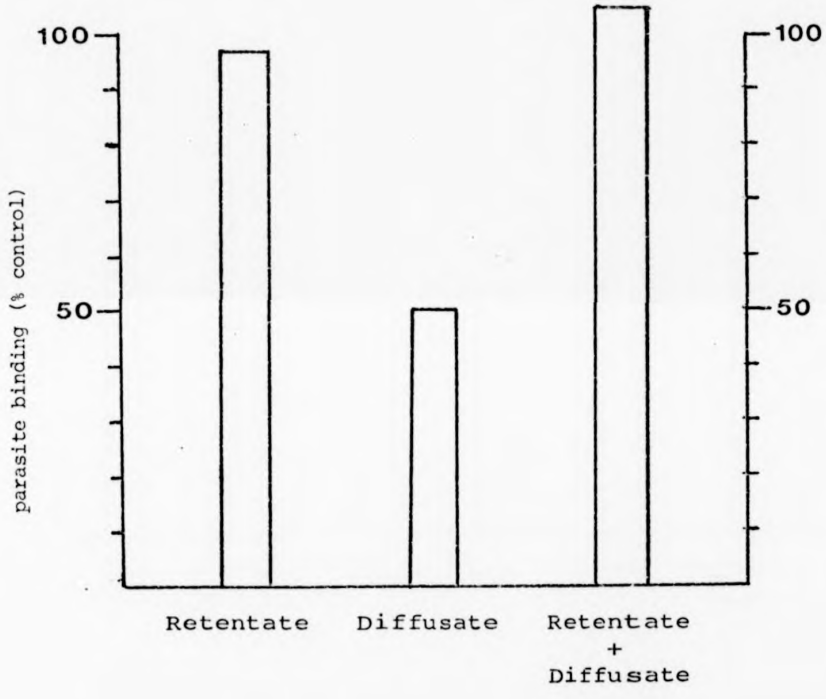



Fig. 12 Effect of dialysis on the activity foetal calf serum in parasite binding. Average number of parasites attached per macrophage (per cent of control). For other explanations see Table 25.

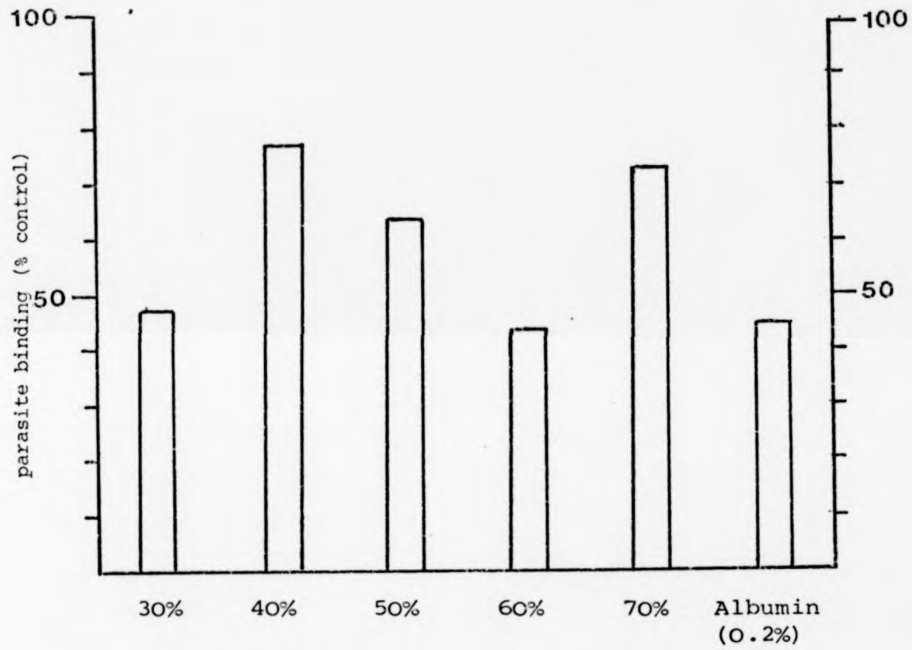



Fig. 13 Activities of various ammonium sulfate precipitated fractions of foetal calf serum in parasite binding. Average number of parasites attached per macrophage (per cent of control). For other explanations see Table 28.

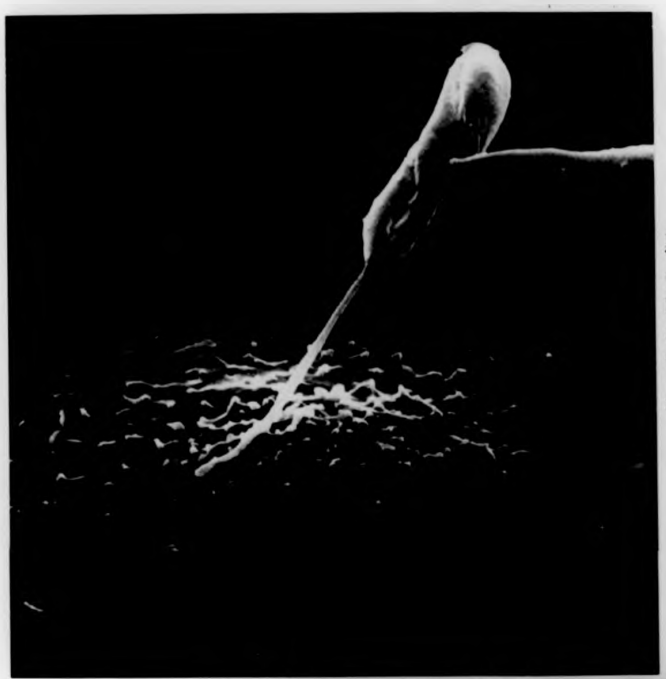


Fig. 14.

The initial contact between a parasite and a macrophage by the flagellar tip.

x 2800

Fig. 15

Another example of flagellar adherence to the macrophage surface.

x 4500

Fig. 16

A detailed view of the initial flagellar contact. Only a small segment of the flagellum (= 2mM) is apposed to the macrophage surface.

x 6400

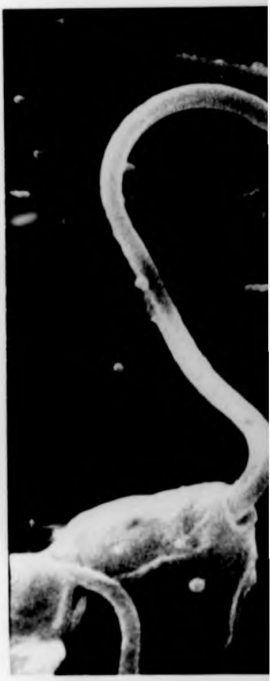


Fig. 17

A similar view of the area of flagellar contact where no response from the macrophage is yet visible.

x 8700

Fig. 18

The beginning of the macrophage surface response to flagellar adherence.

x 8700

Fig. 19

The macrophage's initial response to flagellar contact takes the form of intense membrane ruffling.

x 11600

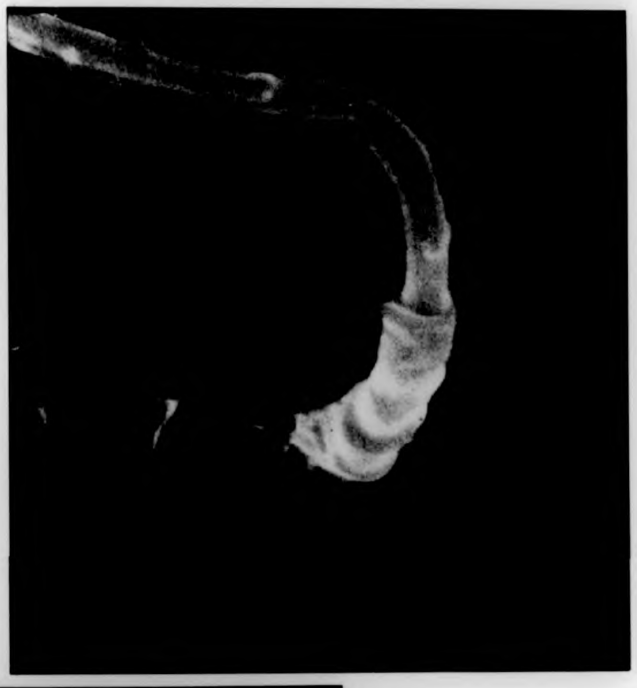


Fig. 20

The macrophage's response develops within 2 minutes into a membraneous sheath that protrudes from the macrophage body.

x 10400

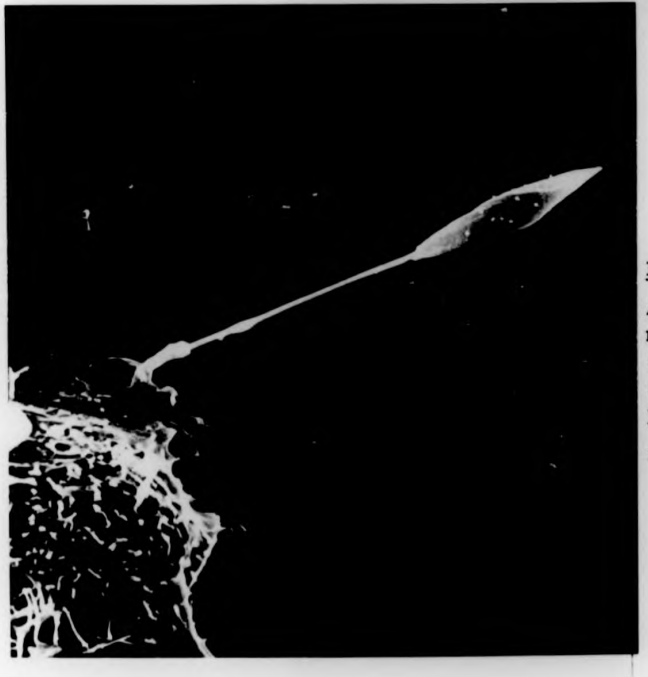


Fig. 21

A promastigote held to the macrophage by the nascent sheath which in the first few minutes covers only a part of the flagellum.

x 1900

Fig. 22

Two adjacent flagella being engulfed by separate membrane sheaths showing some membrane undulation.

x 8700

Fig. 23

Flagellar engulfment by an alternative mechanism: a long segment of the flagellum is embedded in the macrophage surface membrane.

x 3100

Fig. 24

The lamellar sheath has increased in length after 10 minutes of exposure to parasites.

x 1900

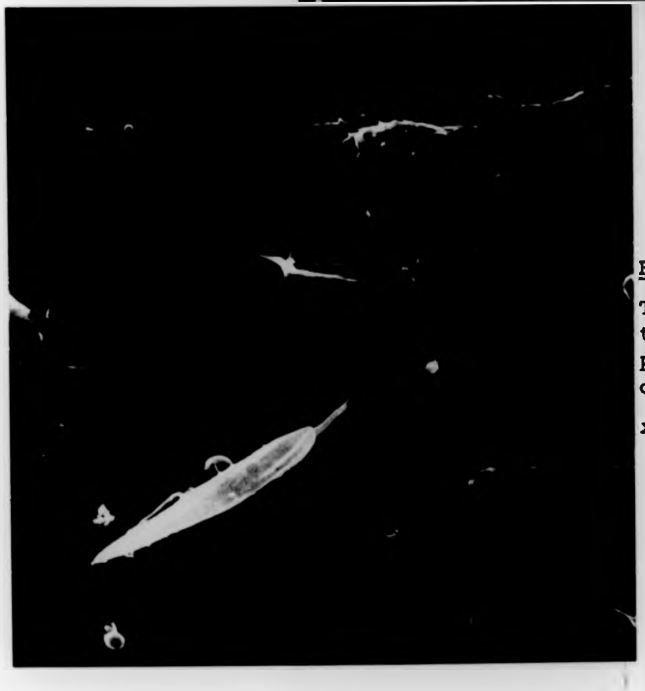


Fig. 25

The peripheral areas of the fully spread macrophage are the main target of parasite anchorage.

x 1700

Fig. 26

The rim of the lamellar sheath is thickened and gives the impression of a rolled shirtsleeve.

x 3500

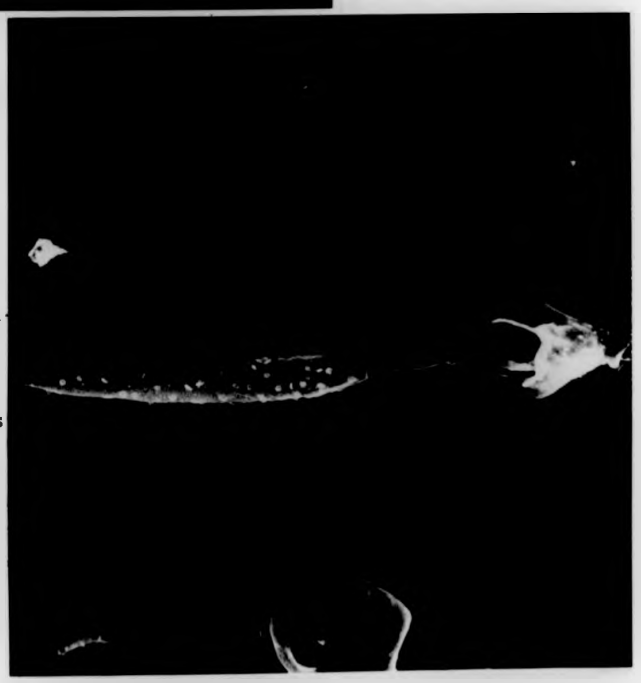


Fig. 27

The advancing lamellar sheath has a widely gaping mouth preceded by several microspikes

x 2500

Fig. 28

The lamellar sheath extends along the flagellum preceded by a filopodial extension.

x 3800

Fig. 29

Two microspikes extend from the leading edge of the lamellar sheath.

x 5800

Fig. 28

The lamellar sheath extends along the flagellum preceded by a filopodial extension.

x 3800

Fig. 29

Two microspikes extend from the leading edge of the lamellar sheath.

x 5800

Fig. 30

A parasite with its flagellum completely encased in a lamellar envelope.

x 2800

Fig. 31

The lamellar sheath covering the flagellum usually extends from the macrophage surface.

x 4200

Fig. 32

After engulfment of the flagellum the lamellar sheath often retracts into the macrophage body.

x 4200

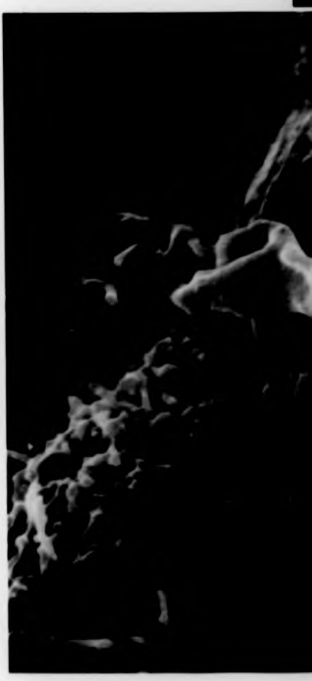


Fig. 33

The parasite body is sometimes engulfed without retraction of the lamellar sheath.

x 3800

Fig. 34

After retraction of the lamellar sheath the parasite body next becomes partially engulfed after 10 minutes of exposure.

x 3100

Fig. 35

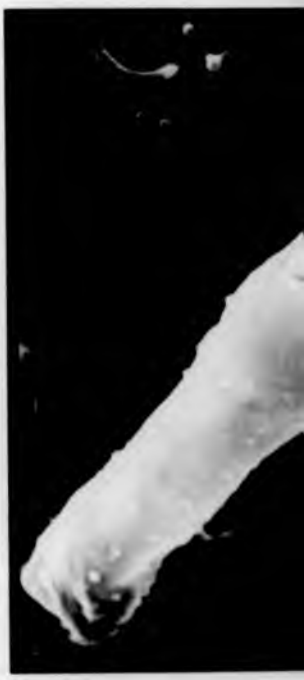
Partial engulfment of a promastigote body by a cup-shaped lamellar process which may become wider due to flexing of the parasite at the flagellar-somatic junction.

x 4500

Fig. 36

Most of the parasite body is engulfed while the lamellar sheath is inflated at the flagellar junction.

x 5800



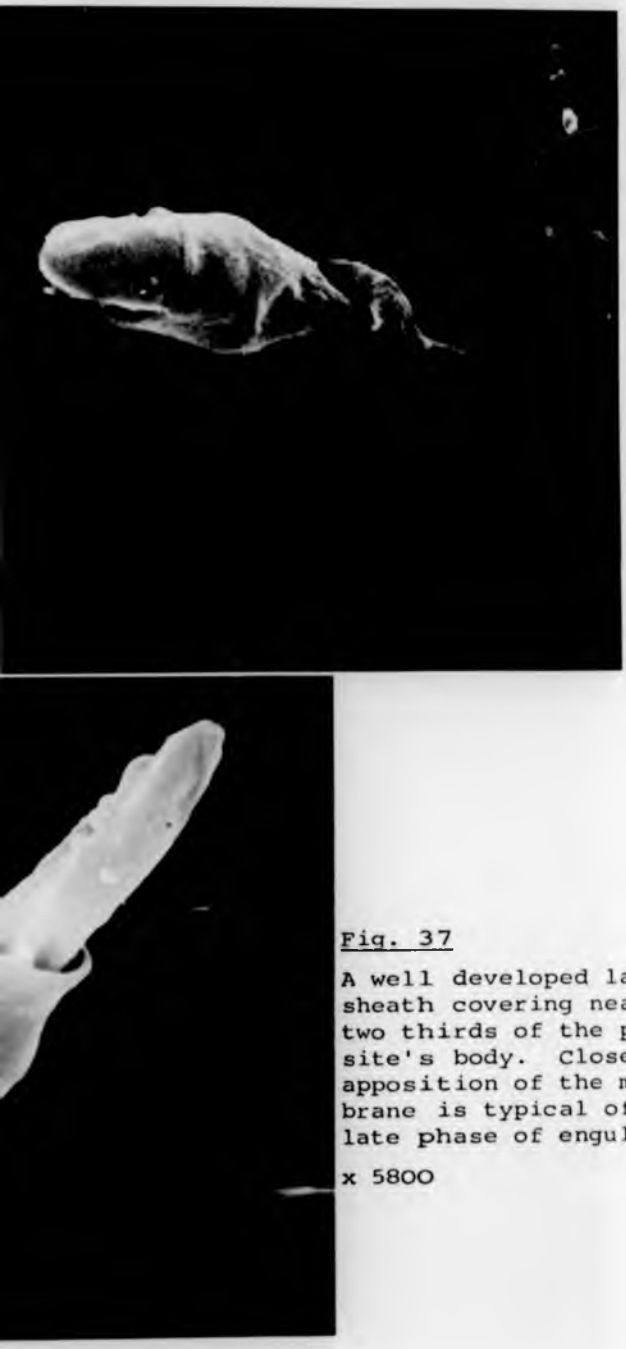


Fig. 37

A well developed lamellar sheath covering nearly two thirds of the parasite's body. Close apposition of the membrane is typical of the late phase of engulfment.

x 5800

Fig. 38

Two microspikes projecting from the leading edge of a lamellar sheath that neatly encases a promastigote's body.

x 5800

Fig. 39

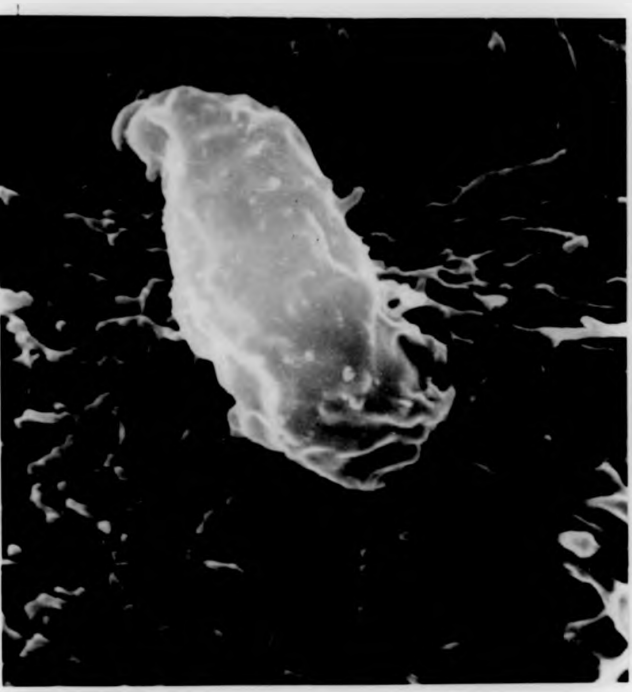
Engulfment of the parasite body by a closely applied lamellar sheath is nearing completion; only a small part is visible.

x 4500

Fig. 40

A completely engulfed parasite after 1 hour of exposure. The flagellum has been drawn into the macrophage.

x 5400



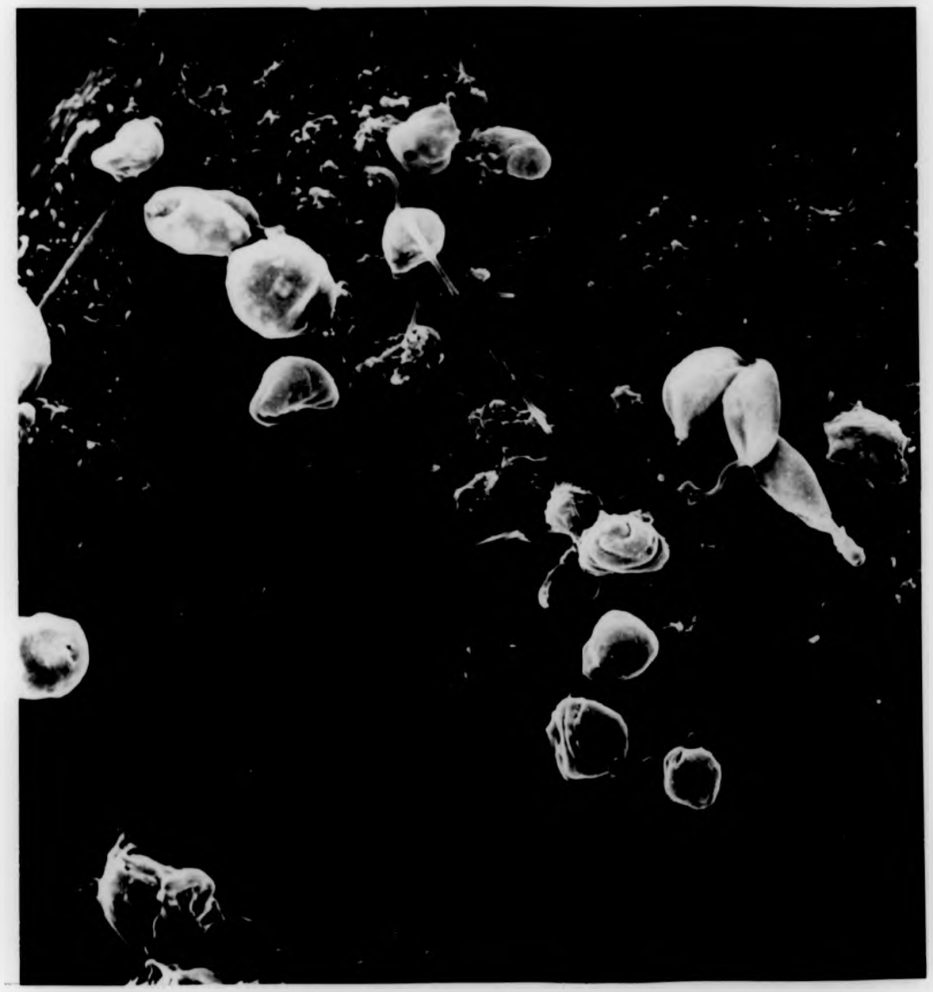


Fig. 41

After 4 hours of their interaction with macrophages most living parasites have still remained partially extracellular.

x 1900

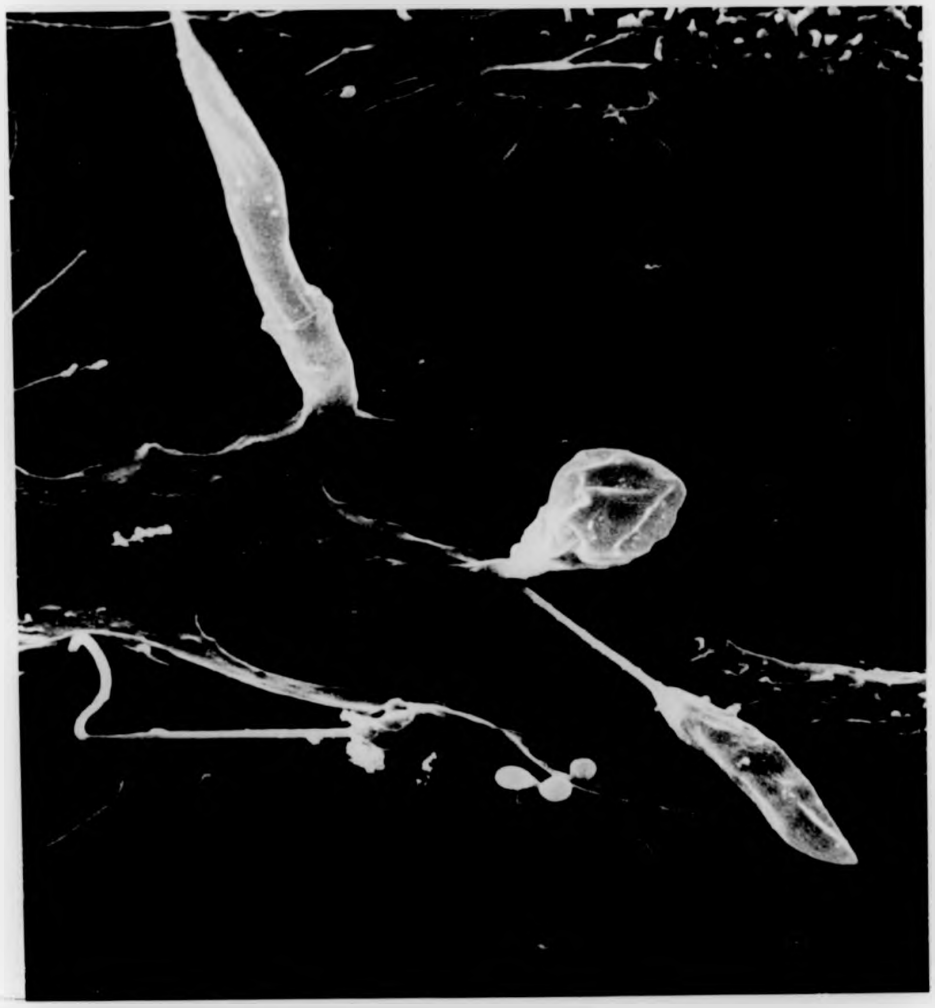


Fig. 42

The peripheral part of a macrophage engulfing 4 fixed parasites. Three have been engulfed flagellum first but the fourth is being engulfed body first.

x 2700

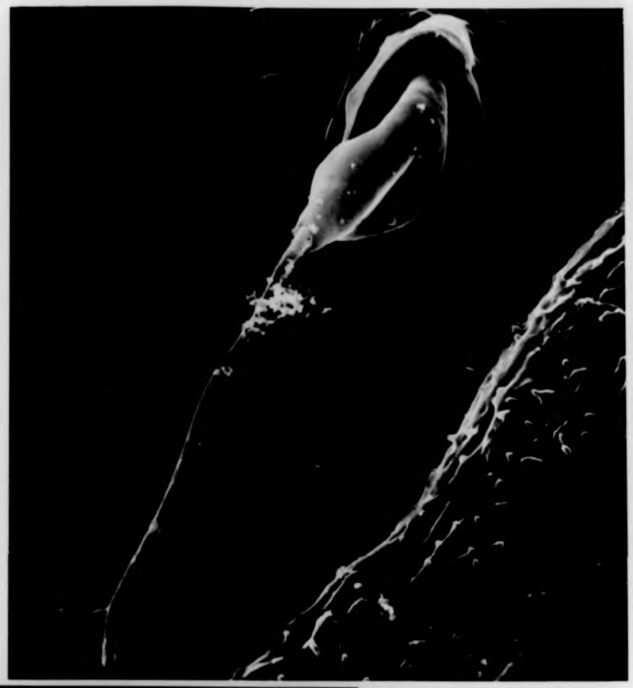


Fig. 43

A heat inactivated promastigote being engulfed somatic end first.

x 4100

Fig. 44

The body of a fixed parasite being engulfed first. Note 3 microspikes extending from the closely adherent lamellar envelope.

x 4500

Fig. 45

A single fixed parasite being engulfed by two macrophages. One engulfing the flagellum and the other the body of the parasite.

x 2800

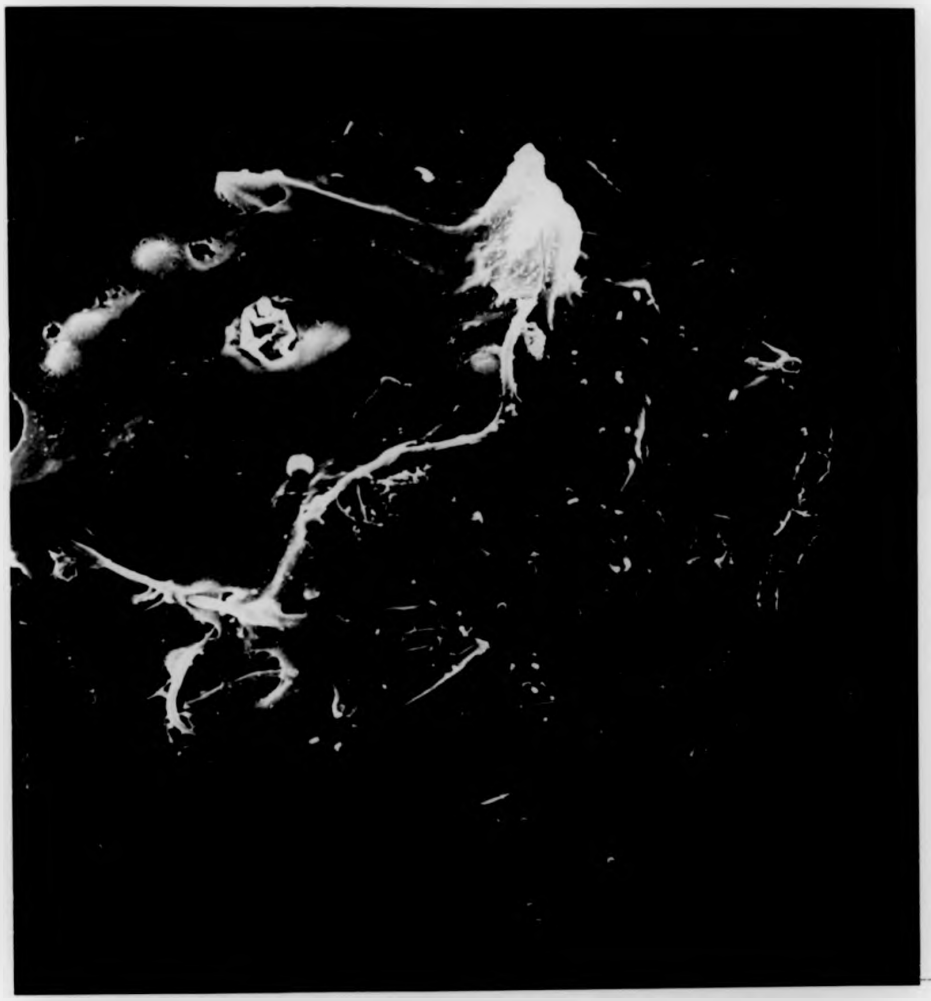


Fig. 46

A single macrophage has successfully engulfed five fixed parasites after 2 hours of exposure.

x 2700

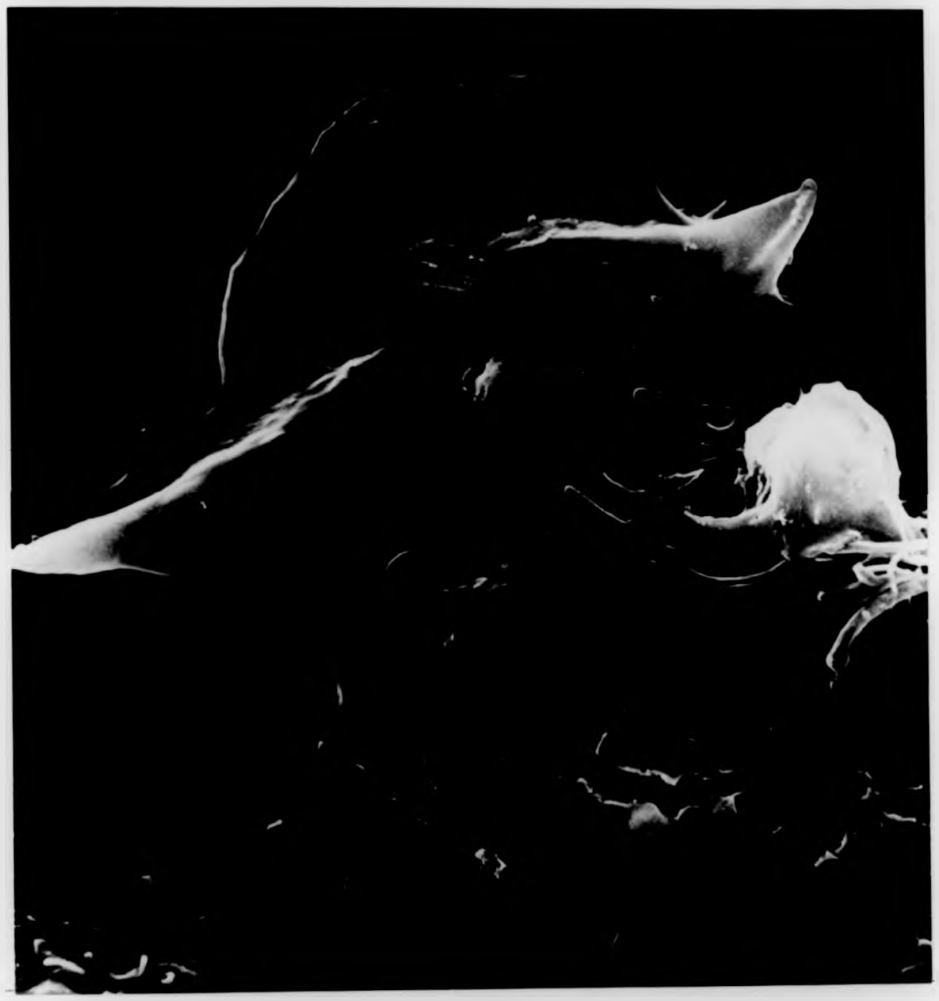


Fig. 47

A single macrophage with three intracellular parasites after 4 hours of exposure.

x 3400

Fig. 48

Engulfment by wrapping; the two flaps of a membraneous sheet encircle the parasite body.

x 3400

Fig. 49

The two flaps of the membrane sheet wrapping the parasite body overlap, but apparently do not fuse.

x 3100

Fig. 50

Two promastigotes attached to the surface of a macrophage at 14°C. Note the absence of any surface response from the macrophage.

x 2800.

Fig. 51

The appearance of macrophages after 40 minutes of treatment with 5 µg/ml cytochalasin D. Note the flattened and smooth surface of the macrophages devoid of microvilli.

x 1300.

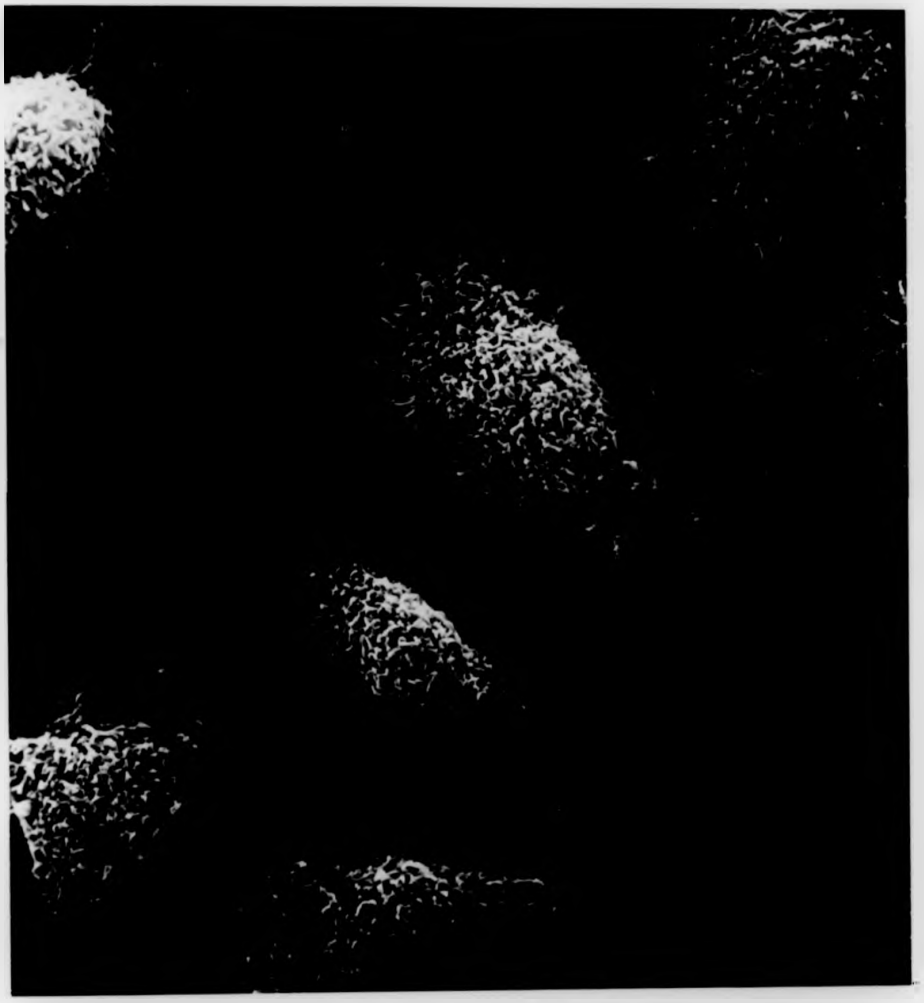


Fig. 52

Untreated macrophages under normal culture conditions. The cells possess numerous microvilli on the upper surface and filopodia can be seen extending from their peripheries.

x 1300

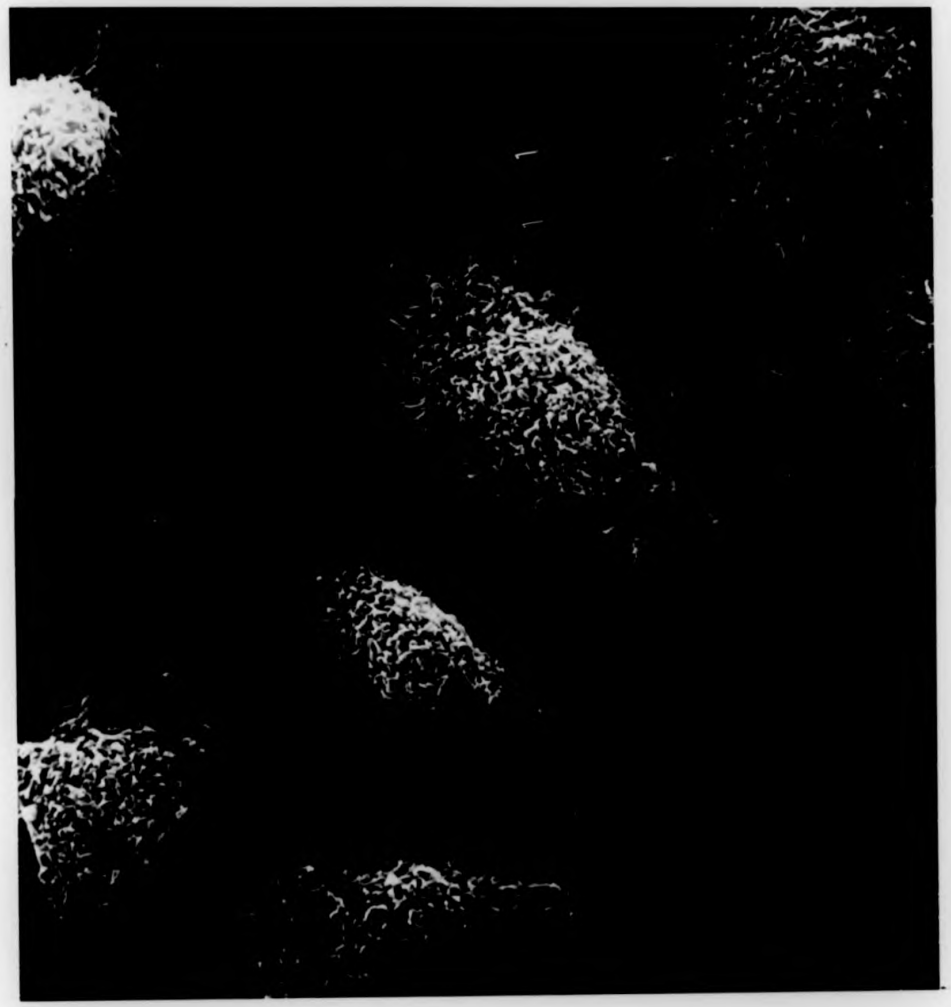


Fig. 52

Untreated macrophages under normal culture conditions. The cells possess numerous microvilli on the upper surface and filopodia can be seen extending from their peripheries.

x 1300

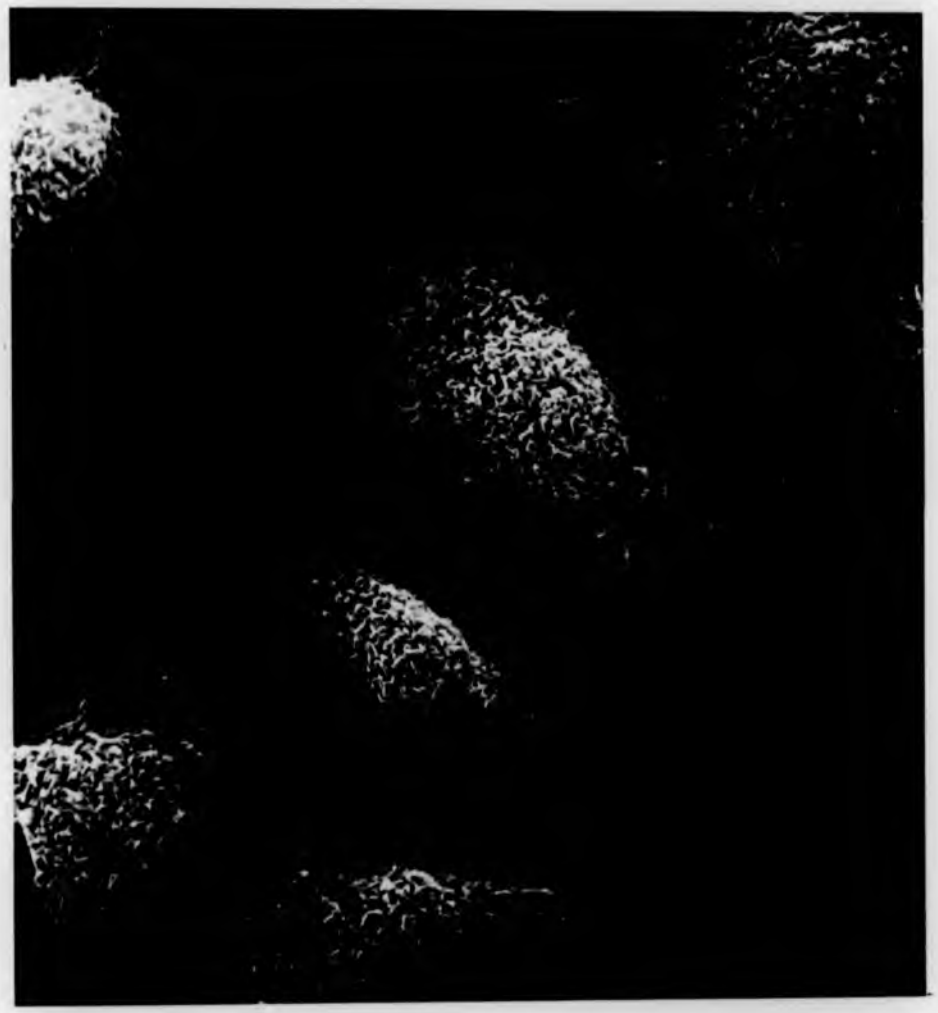


Fig. 52

Untreated macrophages under normal culture conditions. The cells possess numerous microvilli on the upper surface and filopodia can be seen extending from their peripheries.

x 1300

Fig. 53

Four promastigotes have converged on a localized area of the macrophage surface which lies in a peripheral zone.

x 4000

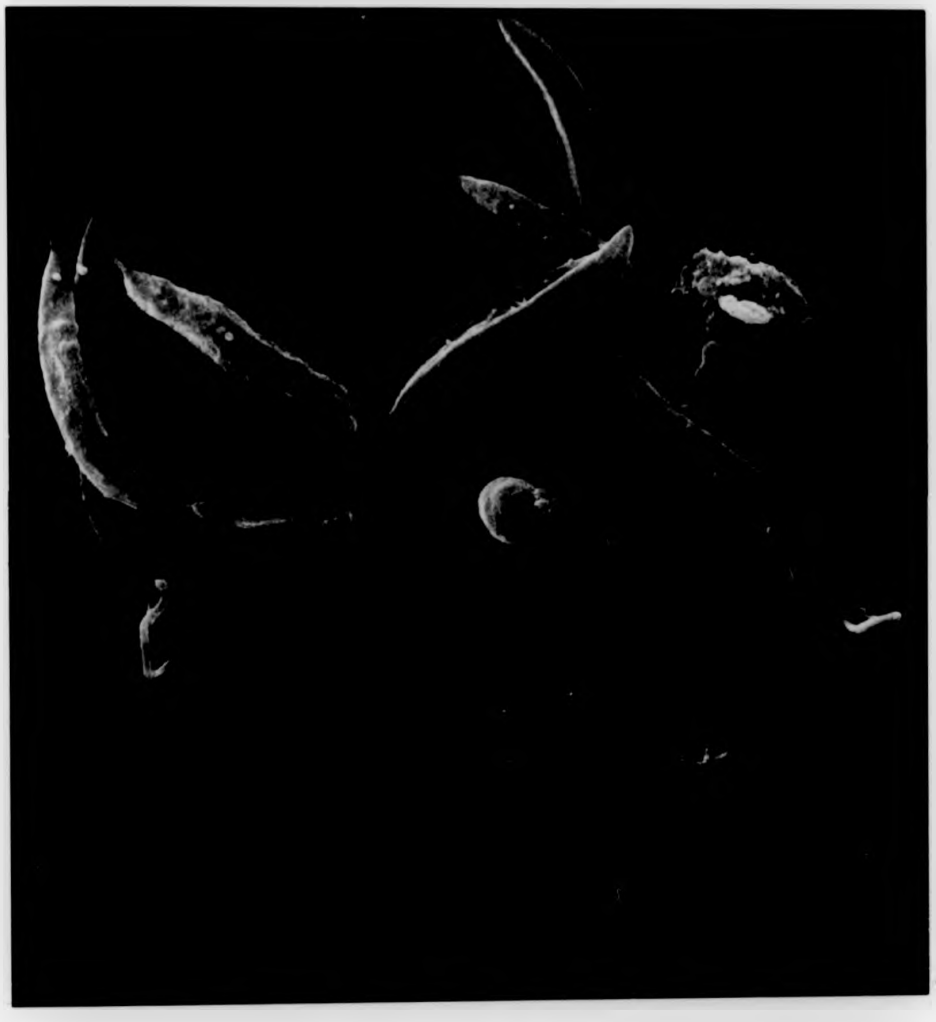


Fig. 54

A single macrophage to which numerous parasites have attached predominantly at the peripheral zones.

x 2700

Fig. 55

A panoramic view of a cluster of macrophages to which parasites have attached mainly at the intercellular boundary zones.

x 1900

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