FACTORS AFFECTING PHAGOCYTOSIS OF BACTERIA

BY AMOEBAE

by

Savanat Tharavanij. M.B.

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Department of Microbiology,

The University of Adelaide.

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ABSTRACT.

Survival of salmonellae in the cytoplasm and in the vacuole of Amoeba proteus was measured after the bacteria were introduced into the cells by means of a micro-injection technique, or after they had been phagocytosed naturally.   It was shown that amoebae were able to kill these bacteria under either condition, but the rate of killing was faster after natural phagocytosis.   Mammalian opsonins did not enhance bactericidal effectiveness in either situation, but the additional presence of complement significantly reduced their survival rate after micro-injection.

Influence of the nucleus on killing of bacteria in the cytoplasm of Amoeba proteus was investigated.   It was shown that the nuclear half exerted significantly greater bactericidal effect than the cytoplasmic half when the cells were challenged at 24 hours after cutting, but not when they were challenged at 2 hours after cutting: this was attributed to the effect of starvation and the changes associated with cellular regeneration.

The location of bacteria or bacterial spores injected into the cytoplasm of Amoeba proteus was determined electronmicroscopically after various periods of time and the evidence for de novo formation of vacuolar membranes around the injected bacteria or spores is presented.

Attempts to induce more efficient bactericidal ability in Amoeba proteus, either by repeated injections of bac-

teria into the cytoplasm or by prolonged exposure of amoebae to bacterial substrates, were not successful.

Selection of bacterial food by soil amoebae was studied. At the level of food seeking, it was demonstrated that some edible bacteria were able to attract amoebae chemotactically, whereas inedible organisms failed to be so. After treatment with mammalian antibody, however, the inedible bacteria became chemotactic. In general, chemotactic principles were associated only with living bacterial cells since chemotaxis was lost after the bacteria were killed. Many species of soil amoebae were shown to respond to the same chemotactic stimuli.

At the level of ingestion, soil amoebae were able to feed preferentially on edible bacteria which were present together with inedible organisms in a mixed colony. The mechanisms whereby this was achieved are discussed.

The killing rate of edible and inedible bacteria was comparable, provided that the number of bacteria ingested per amoeba was not too great. On the contrary, killing of inedible bacteria proceeded at a slower rate if the organisms were ingested in large numbers.

The factors affecting chemotaxis, adherence, phagocytosis and intracellular killing of Gram negative bacteria by amoebae are discussed by comparison with existing knowledge of these same phenomena as found in mammalian phagocytes.

This thesis contains no material previously submitted for a degree in any university, either by the candidate or by any other person, except where due reference is made in the text of the thesis.

(Signed)

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INTRODUCTION.

Although a considerable amount of information is available about the function of phagocytic cells of multi-cellular animals, particularly in relation to host defence, our knowledge is still very incomplete regarding the way in which phagocytic cells recognise particles, and the fate of the ingested material. As in so many other fields of biological science, it is possible that a comparative study of phagocytic cell physiology and function, at different levels of phylogeny, would contribute to our understanding of this problem. The free living amoebae and the macrophages of the reticulo-endothelial system of mammals are particularly suitable for such a study. Phagocytosis is the principal means by which amoebae obtain nutrients. These protozoa feed by ingesting microorganisms, such as ciliates, or decomposing organic materials from an environment heavily laden with bacteria. It therefore seems inevitable that some bacteria will be taken into the cell during the act of phagocytosis or pinocytosis. In the case of Amoeba proteus it is not known what happens to bacteria that enter the cell in this manner, but it is reasonable to suppose that mechanisms exist whereby these organisms are either killed or eventually discarded. In the literature surveyed, particular attention has been paid to the mechanisms leading to phagocytosis and intracellular degrad-

ation of particles in relation to what is known to occur in higher vertebrates.

The process of phagocytosis in higher vertebrates occurs in three separate stages: the attraction of cells to an area of microbial invasion (chemotaxis), the act of phagocytosis itself and the degradation of the ingested materials within vacuoles or their eventual egestion, in whole or part, from the phagocyte.

Chemotactic movement of protozoa.

Information concerning chemotaxis in protozoa is very fragmentary. Stahl (1884) discovered that an extract of dead leaves had the property of attracting plasmodia, the amoeboid organisms belonging to the class Myxomycetes, whereas solutions of salt, sugar and numerous other materials repelled them. Metchnikoff (1893) reported that stagnant infusion of dry leaves exerted less chemotactic effect on a plasmodium of Physarum than a filtrate of the same infusion. This was attributable to the presence of bacteria in the former. Repulsion by bacteria was only relative since the plasmodium moved more readily towards the stagnant infusion than the fresh infusion of dead leaves which contained no bacteria (Metchnikoff, 1893). When a plasmodium of Didymium farinaceum was placed on a slide immersed in various concentrations of hydrochlorate solution of quinine, it was found that it reacted negatively by moving away

from the solution at a concentration of 0.05% or higher;
but at a concentration of 0.005% or less the plasmodium
advanced and even inserted processes into the solution
(Metchnikoff, 1893).   Jennings (1931) described the res-
ponse of paramecia to a wide range of unfavourable intens-
ities of certain stimuli, as an 'avoiding reaction'.   On
approaching a threshold stimulus, the cilia reversed their
beat and the paramecium withdrew.   If the ciliates were
placed under a cover-slip containing a bubble of oxygen and
a bubble of carbon dioxide, they accumulated near the latter.

Schaeffer (1916a) described behaviour of _Amoeba
proteus_ towards various substances, both soluble and insol-
uble; he maintained that they were able to sense particles
such as carmine, carbon, glass, silicic acid, etc., or sol-
uble substances, e.g. tyrosine, held in a capillary tube at
a distance of 60-100 μ.   They responded positively by send-
ing a main or lateral pseudopod to touch the object; this
might or might not be followed by food cup formation.   Ed-
wards (1923) tested the effect of chemicals on locomotion
in _Amoeba proteus_ by applying various materials locally to
the cell membrane by means of a capillary pipette.   He
found that with a weak alkaline solution streaming of
pseudopods stopped and that at the point of contact a local
protuberance developed which was subsequently transformed

into an advancing pseudopod.   Weak acid, on the other hand,
induced a small protuberance but this did not develop into
a normal pseudopod, i.e. the protuberance gradually dis-
appeared and normal streaming resumed.   In 1925 Edwards
reported that food cups could be induced in Amoeba proteus
by various salt solutions and consequently the uptake of
fluid was accomplished in the absence of food particles.
This worker demonstrated that certain salts were better
inducers than others, the best being a mixture of equal
amounts of N/300-N/500 NaCl, LiCl and $CaCl_2$.   Pseudopod
and food cup formations were recently reported to have been
induced by extract of Tetrahymena pyriformis and Hydra vir-
idans and by heparin (Jeon and Bell, 1962;   Bell and Jeon,
1962).   When these substances were held in capillary tubes,
the stimulus was effective up to 50 μ from the test amoeba.
Response to heparin varied according to the concentration
used.   At a concentration of 3 x 10^{-5}M the amoeba was stim-
ulated and a pseudopod was formed, but at a concentration
of 10^{-4}M it was repelled.   Pseudopod induction in these
instances was unlikely to be due to a convection current at
the tip of the pipette since the control capillary tube,
filled with the medium suspending the amoeba, did not stim-
ulate pseudopod formation even at a distance of 1-2 μ. from
the cell.   Bell (1963) suggested that the inducers acted
by depolarising the surface membrane.   This has been sup-

ported by Bingley, Bell and Jeon (1962) who showed that hydra extract reduced the membrane potential of _Amoeba_ _proteus_ by approximately 70%. Jeon (1963) claimed that Ca ions were not required for the process of pseudopod induction since this occurred in the presence of 10^{-3}M versene. The mechanism of membrane depolarisation is not known. Bell (1963) has suggested that the inducer either alters the permeability of the membrane or changes the properties of poly-ions on the cell surface, or both.

Mast (1932) has shown that light attracts or repels amoebae depending on the intensity of light and the species tested. He reported that _Amoeba proteus_ reacted positively to very weak illumination and negatively to moderate or intense illumination, a finding strikingly different from that observed with _Amoeba dofleini_ which responded positively to light even of moderate intensity. By means of a camera lucida technique, Mast showed that the plasmagel of amoebae exposed to light became thicker in relation to the plasmasol, and he concluded that light had a gelating effect on the plasmasol.

Chemotactic movement of mammalian phagocytes.

Migration of mammalian leucocytes towards bacteria and other chemotactic substances has been amply documented (McCutcheon, 1946; Harris, 1954; Boyden, 1962). The actual mechanisms which act to promote the migration of

cells, however, are not known. McCutcheon (1955) post-
ulated that a chemotactic component from the serum was
adsorbed on to the surface of bacteria or particles, thus
creating a concentration gradient of the chemotactic prin-
ciple which induced leucocytes to migrate. McCutcheon
discounted the possibility of a concentration gradient of
substances diffusing from bacteria on the grounds that max-
imal chemotactic responses were obtained when the prepara-
tion was freshly made. Recently Boyden (1962) has shown
that chemotaxis of human polymorphonuclear leucocytes was
elicited by a heat-stable intermediary product formed as a
result of activation of a heat-labile component of fresh
serum by an antigen-antibody complex.

Clark and co-workers (1936) showed that polymorpho-
nuclear leucocytes were attracted by dead cells, droplets
of cream or egg yolk, yet they did not engulf these sub-
stances. Conversely many particles, such as carbon and
silica, which do not necessarily cause chemotaxis, may be
taken up readily as a result of chance encounters (Fenn,
1923). It seems likely, therefore, that phagocytosis of
inducing particles is not an inevitable consequence of leuco-
cyte chemotactic response. This is also true in the case
of amoebae. Schaeffer (1916b) observed that attraction of
Amoeba proteus or Amoeba dubia to particles of carmine did

not necessarily lead to their ingestion. If a particle was phagocytosed on the first encounter, decision to ingest the same particle on a subsequent encounter seemed to depend on the physiological condition of the amoeba, i.e. the degree of hunger. It was found that an amoeba was attracted strongly by a tyrosine grain which caused a food cup to be formed, but this did not always lead to the ingestion of the particle.

Phagocytosis in amoebae.

Rhumbler (1910) recognised four different types of food intake in amoebae, namely:

1. Import. The food comes into contact with the surface membrane of the amoeba and sinks into the body without any noteworthy movement of the phagocytes.

2. Invagination. The particle is in contact with the surface membrane which enfolds it and then invaginates into the interior of the cell.

3. Circumfluent. The surface of the amoeba flows over and encloses the particle in full contact with it, resulting in a corresponding change in the shape of the amoeba.

4. Circumvallation. The particle is not in immediate contact with the amoeba but pseudopods are put out to enclose it in a vacuole.

Food ingestion, similar to import, has been observed in Entamoeba histolytica by Hopkins and Warner (1946). This

was seen to occur in a region just forward of the posterior
end of the cell. Comandon and De Fonbrune (1953) have
photographic records of Bacillus megaterium passing through
the cell membrane of Amoeba phagocytoides into the cyto-
plasm during the 'import' type ingestion. Two or three
minutes after entry, vacuoles were formed surrounding the
ingested organisms.

Invagination is common among amoebae with thick
pellicles. Penard (1905) observed that in Amoeba terricola
the prey came into contact with the thick pellicular surface
which was subsequently invaginated; the prey thus ingested
was enclosed within a portion of the original pellicle. In
many species of amoebae, showing invagination, a region
known as a 'temporary pharynx' was formed at the site of
ingestion, and it was shown that this region would take up
stain more intensely than other regions of the cell (Ivanic,
1933, 1936; Wenrich, 1941). The active contractile pro-
cess along the region of 'cytostome' of Amoeba verricosa has
been reported by Comandon and De Fonbrune (1936).

Circumfluens and circumvallation are the most fre-
quent forms of feeding in free living amoebae, e.g. Amoeba
proteus, the food of which consists mainly of ciliates;
feeding is normally accomplished by means of the formation
of a 'food cup', the term coined by Schaeffer to include both
processes (Schaeffer, 1916b). He commented that these two

processes did not have any essential difference other than
a reflection of the condition of the amoeba, i.e. the degree
of hunger. He observed that food cups varied greatly in
method of formation, structure and size, depending upon the
nature of the object to be ingested and the general degree
of hunger of the amoeba. Normally a food cup is formed by
two opposing pseudopods enclosing the prey and fusing at
their tips. Occasionally the two encircling pseudopods do
not develop but ingestion takes place by means of a thin
sheet of membrane spreading over the prey to form a food cup.
It is inevitable that fluid is incorporated together with
food particles into the vacuoles. However, ingestion of
fluid in the absence of food cups can be achieved by the
process of pinocytosis which was first described by Mast and
Doyle (1934) who observed that in a solution of 2% albumin
or a hypertonic solution of NaCl, numerous tubes filled with
fluid were formed and these later disintegrated releasing
the fluid contents into the cytoplasm. The sequence of
events was similar to pinocytosis in rat peritoneal macro-
phages described by Lewis in 1931. Pinocytosis in amoebae
has been extensively reviewed recently by Chapman-Andresen
(1962).

Factors affecting phagocytosis.

It is realised that many factors influence the func-
tional capacity of both vertebrate phagocytic cells and pro-
tozoa. For the sake of convenience these factors can be

loosely divided into two main groups, i.e. those which change the general physiological state of phagocytes and those which are believed to change the physico-chemical characteristics of the particles to be phagocytosed. In many cases the ingesting cells and the ingested particles are both modified by the factors under study which promote phagocytosis. The following sections will consider in detail agents which mainly owe their phagocytosis promoting effects to changes on the surface of particles to be ingested and also agents which alter cell physiology. This review, however, will mainly concern amoebae.

Factors which promote phagocytosis by acting mainly on the surface of ingested particles.

A. Mammalian phagocytes.

It is generally accepted that pre-coating of bacteria by serum opsonin is usually, if not always, a necessary requirement for efficient phagocytosis (Rowley, 1962). This concept is also applicable to non-living particles such as carbon and starch (Nelson and Lebrun, 1958), and bentonite (Potter and Stollerman, 1961). The requirement of complement for the full opsonic effect of normal serum has been shown by Ecker and Lopez-Castro (1947). These authors showed that phagocytosis of $Micrococcus\ candidus$ by human polymorphonuclear leucocytes was markedly decreased if C'_1, C'_2 and C'_4 components of complement were removed from human

serum used as a source of opsonins, whereas removal of C_3 component was without effect. A similar result was reported by Rytel and Stollerman (1963) who studied the effect of complement on phagocytosis of bentonite particles coated with denatured gamma globulin by human polymorphonuclear leucocytes. Northrop and De Kruif (1922) showed that opsonins produced physico-chemical changes on the surface of bacteria as measured by alteration in electrophoretic mobility. Various changes of surface properties of mycobacteria after exposure to normal and immune serum were reported by Mudd and co-workers (1929), the most striking changes perhaps being alterations in cataphoretic mobility and partition of cells at an oil-water interface. This was explained by a decrease in zeta-potential and an alteration in wetting properties of the cell surfaces; the latter change resulted in the mycobacteria becoming hydrophilic. Mudd et al. (1934) postulated that all the changes were due to deposition of serum on the bacterial surface, so that the physico-chemical characteristics of the bacterial cell wall resembled those of denatured globulin. A correlation has been shown between zeta-potential and the degree of phagocytosis of bacteria in NaCl solution; in general the lower the zeta potential, the greater the phagocytosis (Mudd et al., 1934). However, when the serum used to treat mycobacteria was either heat-inactivated normal serum or ageing immune serum, the surface properties of the bacteria were

altered as described by Mudd and co-workers (1929), but
phagocytosis was not enhanced (Lucke et al., 1929).   It was
pointed out by Mudd and co-workers (1929) that phagocytosis
could not be formulated in terms of the properties of the
sensitising substances alone, but depended both on the prop-
erties of the sensitising substance and on the intrinsic
properties of the bacteria undergoing sensitisation. They
drew attention to the fact that a virulent strain of Myco-
bacterium tuberculosis could be ingested readily by rabbit
polymorphonuclear leucocytes in the absence of antiserum
even though these organisms possessed a relatively high zeta-
potential, whereas Mycobacterium avium, which had a relative-
ly low zeta-potential, was phagocytosed poorly (Mudd et al.,
1934).   It has been shown by Lucke and co-workers (1929)
that physico-chemical changes at the surfaces of mycobacter-
ia treated with normal human and normal rabbit sera were
comparable, but when these two groups of bacteria were ex-
posed to rabbit leucocytes there was a marked predilection
for cells opsonised with the homologous serum.   Recent stud-
ies of Rowley (1964, personal communication) showed that the
amount of antibody required in promoting phagocytosis and
killing of E. coli by mouse peritoneal macrophages in vivo
was very small, i.e. 10-15 molecules of 19S antibody or 5000
molecules of 7S antibody per one bacterium.   It is conceiv-
able that these small numbers of antibody molecules cannot
cover the entire surface of bacteria and hence any change

of surface charge as a result of this opsonisation should
be exceedingly small.   It follows that opsonin is unlikely
to owe its phagocytosis promoting effect solely on the
change on surface charge of bacteria.

   B. Amoebae.

      There is fragmentary information concerning fact-
ors which act on the particles and render them susceptible
to phagocytosis by amoebae. Pavolova (1938) reported that
ingestion of red blood cells by 3 strains of Entamoeba
histolytica was enhanced by normal horse serum. The degree
of enhancement was higher if the serum was inactivated at
56° for half an hour.   Drozański and Drozańska (1961)
claimed that a water extract of Aerobacter aerogenes, pre-
pared by repeated freezing and thawing, and an extract of
yeast cells when added to amoeba culture, stimulated in-
gestion of heat-killed Gram-negative organisms by soil am-
oebae.   He also showed that the feeding index of yeast
cells killed at 100°, which were normally not ingested, was
raised when the extract of aerobacter was added.   He sug-
gested that the way in which the extract of aerobacter pro-
moted phagocytosis in soil amoebae could be compared to the
effect of serum opsonins acting on bacteria to promote phag-
ocytosis by mammalian leucocytes.   The suggestion that cer-
tain factors, occurring in the amoebae environment or even
on the surface of amoebae themselves, could promote phagocy-

tosis is supported by the observation of Ray (1951). She showed that a strain of limax amoebae was able to agglutinate flagellated bacteria on their surfaces, whilst non-flagellated organisms were unaffected. The bacteria were first seen adhering at random to the surface of the amoeba, but were subsequently re-orientated so that a bacterial clump was formed at the tail region of the cell where phago-cytosis was most active. The adhering force was not great since the agglutinated bacterial mass was easily detached from the amoeba on application of gentle pressure.

Factors which modify cell physiology thereby leading to an enhancement of phagocytosis.

This review will be confined to physical factors affecting phagocytosis in Amoeba proteus.

1. Physiological state. Mast and Hahnert (1935) drew attention to the fact that Amoeba proteus does not feed readily on ciliates unless attached to a suitable surface. Starvation promoted feeding provided that the deprivation of food did not last longer than 5-8 days. Qualitative cytochemical determinations of RNA, DNA and sulphydryl compounds in starved Amoeba proteus were reported by Heller and Kopac (1955). These authors showed that during five to thirteen days of starvation there was a progressive reduction in cytoplasmic RNA, a relative increase in nuclear DNA and nucleolar RNA, and an increase in both cytoplasmic and

nuclear sulphydryl compounds. No changes occurred in the plasmalemma of <u>Amoeba proteus</u> during starvation (Heller, 1959) and the net protein levels remained stable (Cohen, 1959), but cytoplasmic glycogen became undetectable after starvation for three days (Brachet, 1955).

2. <u>Effect of temperature on phagocytosis.</u>

It has been shown that in <u>Amoeba proteus</u> the frequency of ingestion of chilomonads is closely correlated with the temperature (Mast and Fennel, 1938). These authors reported that the rate of ingestion was maximal at 26° but decreased to zero at 40°. A similar finding was obtained by Leische (1938) who studied the rate of ingestion of paramecia. These findings were almost in full accord with the effect of temperature on locomotion as shown by Mast and Prosser (1932). In <u>Amoeba proteus</u> temperature has also been shown to influence the second stage of pinocytosis, i.e. interiorisation of the loaded membrane, but the first stage - adsorption of inducers to the membrane - is independent of temperature (Schumaker, 1958; De Tara and Rustad, 1959; Chapman-Andresen, 1962). Moreover, Chapman-Andresen (1962) showed that at a lower temperature there was a delay in channel formation and a decrease in the total number of channels formed, whereas at higher temperatures there was no delay in formation, but the total number of channels decreased. Maximal channel formation occurred at 23°. It

has also been shown that viscosity of the cytoplasm of
Amoeba proteus varied according to the temperature. Thorn-
ton (1932) equated viscosity of the plasmagel of Amoeba
proteus with the time of centrifugation needed to displace
cytoplasmic granules down to the lower half of an amoeba
when the speed of centrifugation was constant (4084 g).
He found that viscosity decreased as the temperature in-
creased and that maximal viscosity was obtained at 4°. A
similar result was reported by Mast and Prosser (1932) who
employed a different measurement technique which was based
on the frequencies of rupture of the plasmagel sheet.

In mammalian phagocytes the degree of phagocytosis
follows closely the Laws of Thermodynamics (Fenn, 1922).
This author re-examined the results obtained by Madsen and
Watabiki (1919) who studied phagocytosis of staphylococci
and E. coli by leucocytes at various temperatures. It was
found that there was a surprisingly uniform temperature co-
efficient over the entire range of 5° to 35°, and it was
concluded that the rate of phagocytosis was doubled by a
rise in temperature of 10°.

3. Effect of pH on phagocytosis.

Mast and Fennel (1938) recorded the frequency of
ingestion of chilomonads by Amoeba proteus in a solution of
0.002 N and 0.005 N NaCl within the pH range 4.4 to 8.3.
It was shown that the frequency of ingestion was minimal at

pH 4.4 but rose to a peak at pH 5; this was followed by a further rise at pH 6 and 6.5, and gradually became minimal again at pH 7.7 and 8.3.

Pinocytosis in Amoeba proteus induced by a 0.125 M NaCl solution was also influenced by hydrogen ion concentration, the greatest response being obtained at pH 6.1 (Chapman-Andresen, 1962). This author also showed that pinocytotic response was modified by the pH of the medium in which the amoebae were maintained before the test. Thus the response was maximal if the amoebae were cultured at pH 6.0 to 6.5; a significantly lower response was seen if the protozoa were cultured at pH 5.0 - 5.5 or 7.0 - 7.5.

Locomotion of Amoeba proteus is also pH dependent (Pitts and Mast, 1933). By recording the movement of amoebae in a balanced salt solution at varying hydrogen ion concentrations, these authors reported that locomotion at pH 4.6 - 5.0 was minimal, it increased as the pH approached 6.2, declined at pH 7.0 and increased again to a second peak at pH 7.5.

4. Effect of ions on phagocytosis.

The frequency of ingestion of chilomonads by Amoeba proteus was shown to be influenced by ions in the medium (Mast and Fennel, 1938). These investigators reported that feeding activity of the rhizopod was greatest in 0.0008 M solution of NaCl and in 0.0005 M solution of KCl, $MgCl_2$ and

CaCl$_2$. They also found that even when the ion concentrations of the test solutions were optimum, each salt caused a different degree of response.

Intracellular killing of bacteria and degradation of ingested material.

A. Mammalian phagocytes. Immediately after phagocytosis ingested particles do not seem to be surrounded by vacuolar space, even though they are enclosed in a membrane which is in intimate contact with their surface (Mudd et al., 1934). These workers suggested that the fully developed vacuole is associated with the process of particle digestion. By means of phase contrast microscopy Marchant (1952) showed that in human polymorphonuclear leucocytes, which had phagocytosed bacteria or collodian particles, vacuoles were only formed around the bacteria. There is good evidence from electron microscopic studies that ingested particles within mammalian cells are surrounded by a membrane even if this is not readily seen by light microscopy (Goodman and Moore, 1956; Parks and Chiquoine, 1956; Essner, 1960; Karrer, 1960; North and Mackaness, 1963). In view of the above findings, it is clear that when bacteria are killed by phagocytes, the events leading to killing occur within the environment of the vacuole. Various methods have been devised for a study of intracellular killing of bacteria in vitro. Wilson et al. (1957) visualised the ingestion of

bacteria by leucocytes under phase contrast. At varying times after ingestion the cell was disrupted by electric current and the viability of the liberated organism was assessed from its ability to multiply in situ. It was shown that the half life of streptococci in human and mouse polymorphonuclear leucocytes was 8 and $6\frac{1}{4}$ minutes respectively. Rowley and Whitby (1959) added bacteria to a monolayer of mouse peritoneal macrophages in the presence of serum antibody. At intervals the number of viable organisms in the extra- and intra-cellular phase was determined by direct plating. By this technique the intra-cellular half-life of E. coli and certain Salmonellae was shown to be approximately 5 - 15 minutes.

The actual mechanism which is operating to achieve intracellular bactericidal effect has not been analysed fully. However, two general possibilities have to be considered, namely, the presence of specific bactericidal substances and the cumulative effect of non-specific factors which change the environment of the vacuoles so that the bacteria fail to survive. At least 3 specific bactericidal principles have been found in mammalian phagocytes: phagocytin (Hirsch, 1956), a bactericidal principle of Fishman and Silverman (1957) and lysozyme (Fleming, 1933; Ridley, 1928).

Phagocytin is an acid soluble protein associated with the granules in polymorphonuclear leucocytes, from which it can be extracted by salt solutions or citric acid. This extract is known to kill a wide range of Gram-positive and Gram-negative bacteria (Hirsch, 1956, 1960). During the uptake of particles by polymorphonuclear leucocytes the granules containing phagocytin rupture near the organisms and liberate their components either into the phagocytic vacuole or in its vicinity. The extent of degranulation varied with the number of organisms ingested (Hirsch and Cohn, 1960).

The antibacterial principle of Fishman and Silverman was prepared from an ultrasonic homogenate of rat polymorphonuclear leucocytes. The bactericidal substance was found to be associated with mitochondria. It was most effective at pH 7 against many microorganisms, particularly Gram-positive cocci. Its activity could be reduced by trypsin and was destroyed by digestion with lipase, suggesting the material to be lipoprotein.

Lysozyme was first identified in polymorphonuclear leucocytes by Fleming (see Fleming, 1933; Ridley, 1928), and in macrophages by Myrvik and Weiser (1955). In spite of its high bactericidal activity for certain Gram-positive cocci, it is relatively inactive for many other microorganisms. In view of the fact that certain bacteria become susceptible to the lytic action of lysozyme at low pH or

in the presence of chelating agents, such as versene
(Repaske, 1956), it is conceivable that this enzyme may
have a more important role in intravacuolar killing than
it was at first believed. Intracellular killing of lyso-
zyme sensitive and resistant strains of Micrococcus lyso-
deikticus by rat peritoneal macrophages and polymorpho-
nuclear leucocytes has been studied by Brumfitt and Glynn
(1961). These workers claimed that the sensitive strain
of M. lysodeikticus was rapidly lysed and killed, but no
appreciable degree of lysis or killing occurred with the
resistant strain. However, after deacetylation by glycine
at pH 11, it was possible to restore the sensitivity to
lysozyme of the resistant strain without loss of viability.
The organisms treated in this way were susceptible to both
intracellular lysis and killing. Pavillard (1963) showed
that lysozyme was probably not a decisive determinant of
intracellular killing, since in spite of a high content of
lysozyme rat lung macrophages were less able to kill Staph-
ylococcus aureus than were peritoneal macrophages which have
low lysozyme activity. Recently Cohn (1964) reported that
lung macrophages from rabbits stimulated with BCG were able
to kill Escherichia coli in vitro as efficiently as oil-
induced peritoneal macrophages or polymorphonuclear leuco-
cytes. It thus appears possible that the bactericidal
ability of lung macrophages in this experiment might be due

to factors associated with metabolic changes after BCG
stimulation and this is not necessarily related to the
content of lysozyme inside the cells.

In view of the fact that certain phagocytes, e.g.
macrophages, are not adequately equipped with bactericidal
substances for a wide spectrum of bacteria, an alternative
possibility that merits consideration is that bacteria may
fail to survive in the vacuolar environment as a result of
a 'cumulative effect' of non-specific substances. Sprick
(1955) determined the vacuolar pH of mouse and guinea pig
peritoneal macrophages from colour changes of ingested heat-
killed Mycobacterium tuberculosis and Mycobacterium smegmatis
which had been previously stained with indicator dyes.   He
observed that a pH as low as 4.5 was common within vacuoles;
however, values below this were rare.   The possibility that
an acidic condition alone is able to exert an antimicrobial
effect has not been favourably received.   Dubos (1954) ad-
mitted that the hypothesis that intracellular acidity is one
of the causes of bactericidal effect in phagocytes is, at
best, a working hypothesis almost devoid of experimental
support.   Looke and Rowley (1962) demonstrated that killing
of Vibrio choleras and Lactobacillus bulgaricus by mouse
peritoneal macrophages was not strikingly different even
though these bacteria possessed different degrees of resist-
ance to an acidic environment.   However, the acid environ-

ment of vacuoles may contribute to the effectiveness of acid hydrolyses which have been shown to be located in the cytoplasmic granules of mammalian phagocytes (Thorbecke, Old, Benacerraf and Clarke, 1961; Cohn and Hirsch, 1960). This correlation has added significance when considered in conjunction with the observation that these cytoplasmic granules lyse into vacuoles containing ingested material (Hirsch and Cohn, 1960; Cohn and Weiner, 1963). Furthermore, electron microscopic examination of phagocytic cells demonstrated the discharge of cytoplasmic granules into vacuoles containing ingested organisms (Lockwood and Allison, 1963; North and Mackaness, 1963).

B. Free living protozoa. There is ample evidence from phase contrast and electron microscopic study that the structure of the membrane lining of pinocytotic and food vacuoles of Amoeba proteus or Chaos chaos is very similar to that of the plasmalemma (Brandt, 1958; Mercer, 1959; Roth, 1960; Brandt and Pappas, 1962; Hayward, 1963). Similarity between vacuolar and cell membranes in hartmanellar amoeba has also been reported (Vickerman, 1962). Brandt and Pappas (1962), by means of an electron microscope study, showed that the freshly formed pinocytotic vacuole of Chaos chaos was bound by the intact fringe-like structure characteristic of the plasmalemma. A subsequent change involved an increase in the surface to volume ratio and a concomitant

disappearance of the hair-like structures leaving a resid-
ual vacuolar lining similar to a unit membrane as described
by Robertson (1960).   By analogy it is likely that bacteria
are killed in amoebae within the vacuolar environment.

In 1893 Metchnikoff pointed out that ingestion and
killing of bacteria was ubiquitous among free living proto-
zoa and that destruction of intracellular bacteria by proto-
zoa was not merely the means whereby these cells were pro-
tected from infection, but was also a mechanism for their
obtaining nutritive materials.   He observed that during
the digestive process, the ingested organisms were seen to
be isolated in the cytoplasmic vacuoles.   These organisms
could be easily stained with vesuvine solution, a dye which
did not stain them when living in their natural conditions.
Hofer (1889) found that the more the bacteria were altered
in the interior of rhizopods, the more easily they were
stained with aniline dye.   A change in staining properties
of bacteria after phagocytosis in paramecium and vorticella
has also been reported (Metchnikoff, 1893).   Mast (1947)
observed that certain rod-shaped bacteria underwent a morph-
ological change in the vacuoles of Paramecium aurelia when
the vacuoles decreased in size, about 7 minutes after being
formed;   he believed that this indicated bacterial killing.
However, no quantitative studies of the rate of intracell-
ular killing in protozoa have been reported, nor has any

bactericidal principle been demonstrated. Since protozoa
in general and amoebae in particular have been shown un-
equivocally to kill bacteria and degrade the bacterial
substrates (Metchnikoff, 1893; Mouton, 1902; Oehler,
1916; Singh, 1941), the mechanisms whereby these effects
are achieved require elucidation. Mast (1942) has exam-
ined the range of vacuolar pH in _Amoeba proteus_ which he
found to vary between 5.6 and 7.3. From these data he
estimated that the pH in the cytoplasm was of the order of
7.4. Chamber, Pollack and Hiller (1927) injected several
indicator dyes into the cytoplasm of _Amoeba dubia_ and found
that the pH was 6.9 ± 0.1. This narrow pH range could be
attributed to the buffering capacity of the cytoplasm (Rez-
nikoff and Pollack, 1928; Pollack, 1928). Using a sim-
ilar dye injection technique, Wiercinski (1944) showed that
the pH in the hyaline region of centrifuged _Amoeba proteus_
was 6.8 ± 0.2. He claimed that centrifugation of the cells
eliminated the error due to the uptake of indicators by
cytoplasmic granules.

Mast (1942) observed that when a living ciliate was
ingested together with neutral red granules or neutral red
solution, the first change in colour was apparent in the
body of the prey and not in the surrounding fluid in the
vacuole. He interpreted this to indicate that acid was
released from the prey and not secreted into the vacuole
from the cytoplasm. He also postulated that death of the

ciliate was due to the ultimate oxygen depletion brought about by respiration of the ciliate and diffusion of oxygen from the vacuole into the cytoplasm of the amoeba.

Mast and Bowen (1944) have also claimed that vacuolar pH was not an important factor in the killing of bacteria, since organisms which were believed to be killed rapidly by _Vorticella semilis_ were seen to thrive in an acidic environment _in vitro_.

Although pH per se may not be important in bacterial killing, it is possible that hydrolytic enzymes, e.g. acid phosphatase, only function efficiently under the acid condition established in vacuoles. This possibility correlates with the finding of Novikoff (1960) that in _Amoeba proteus_ acid phosphatase was most concentrated in the vacuolar region. Recently Müller and co-workers (1962) demonstrated a progressive increase in acid phosphatase around newly formed food vacuoles containing acid killed _Tetrahymena pyriformis_.

Factors affecting intracellular killing of bacteria by phagocytes.

1. Serum antibody.

There is unequivocal evidence that serum factors are able to promote the uptake of many species of bacteria by mammalian phagocytes (Wright and Douglas, 1903; Mudd _et al._, 1934; Rowley, 1962). When phagocytosis is promoted in this

way, bacteria which are susceptible to intracellular bactericidal mechanisms are killed (Rowley, 1958; Cohn and Morse, 1959; Jenkin and Benacerraf, 1960). Whether the enhanced rate of killing, observed for opsonised bacteria in contrast to that of unopsonised bacteria, merely reflects more efficient phagocytosis is difficult to decide, since it is possible that serum factors may also act to facilitate cytopepsis (Miya and Marcus, 1961). A correlation between intracellular survival of Salmonella enteritidis in mouse peritoneal macrophages and the amount of specific antibody used to opsonise the bacteria has been reported by Jenkin and Benacerraf (1960). However, the only certain way in which the contribution of opsonin to intracellular killing can be tested is to study two populations of phagocytes which have taken up equal numbers of opsonised or unopsonised bacteria, but technically this presents many difficulties. Jenkin (1963) was able to overcome these difficulties by allowing lysogenic phage P_{22} to adsorb on to the surface of Salmonella typhimurium, when the phage/ bacterium complex was treated with serum containing opsonin for the phage only, phagocytosis was achieved, but the ingested bacteria survived within the phagocytes. However, if opsonin for the bacteria was also present at the time of ingestion, efficient bacteria killing resulted. The way in which opsonins enhance intracellular killing is not known, but physico-chemical changes on the surface of bacteria

coated with antibody have been reported (Mudd et al., 1934; Berry and Spies, 1949). Inoue and co-workers (1959) found that in the presence of serum antibody, complement and Magnesium ions Escherichia coli were converted by lysozyme into spheroplasts which were subsequently lysed. In view of the fact that this phenomenon did not occur when lysozyme was lacking, it was suggested that antibody and complement acted on the bacterial surface in such a way that the substrate for lysozyme became exposed. These authors also reported that antibody and complement alone could cause damage to the bacterial cells as demonstrated either by a change in staining properties or a fall in total viable counts. Nevertheless, excess lysozyme in the amount found in normal serum i.e. 5 μg./ml. accelerated bactericidal action of antibody and complement.

Antibody and complement have also been shown to enhance the rate of digestion of P^{32} labelled Histoplasma capsulatum by normal and immune mouse peritoneal macrophages, as measured by the rate of release of labelled material into the supernatant at a variable period of time (Miya and Marcus, 1961; Wu and Marcus, 1964).

2. Factors which modify the physiology of the phagocytes.

Of particular interest is the finding that, in the presence of unlimited amounts of opsonin, the rate of killing of Salmonella typhimurium by peritoneal macrophages from

mice, stimulated 24 hours earlier with lipopolysaccharide prepared from Escherichia coli, was significantly greater than by macrophages from normal mice even though the rate of phagocytosis was comparable (Auzins and Rowley, 1961). These authors suggested that enhanced bactericidal activity of phagocytes could be attributed to the high metabolic activity of the cells.

3. Influence of the nucleus on bactericidal mechanisms.

Influence of the nucleus on intracellular killing of the ingested organisms was reported by Clark (1943). He severed Amoeba proteus, which had previously ingested a rotifer, with a small glass rod and found that the prey stayed alive for two days within the enucleate half, although normally it would have died in 1-3 hours. The relation between the nucleus and enzymatic activities in Amoeba proteus has been extensively reviewed by Brachet (1961). In an enucleate half of this protozoon, the level of certain enzymes, such as protease, aldolase, ATPase, remained practically unchanged, whereas that of other enzymes, such as dipeptidase, esterase and phosphatase fell remarkably, especially after the fifth day. The amylase content, on the other hand, increased abruptly and remained constant until the fifth day after which it fell to the same level as that in the nuclear half. Of particular interest is the depletion of the RNA content in the enucleate half as shown by both qualitative and quantitative methods (Brachet, 1955). The

belief that the nucleus plays an important role in RNA
metabolism is shared by Goldstein and Plaut (1955). These
workers showed by means of an autoradiographic technique
that when P^{32} labelled nucleus from an <u>Amoeba proteus</u> was
grafted into a normal unlabelled amoeba or an enucleate half
of a normal amoeba, labelled material became located in the
cytoplasm only. This experiment suggests that the nucleus
actively synthesises RNA and that nuclear RNA is trans-
ferred to the cytoplasm. Since RNA metabolism and protein
synthesis are closely linked (Goldstein, 1958), it is likely
that production of certain enzymes in the enucleate half of
an amoeba would fail, resulting in a reduced ability to
kill bacteria and degrade their substrates. The belief
that the nucleus exerts an influence on cytoplasmic activ-
ity has been supported by electron microscopic studies of
Mercer (1959), who described the presence of small vesicles
(800 angstroms in length) radiating from the nuclear mem-
brane into the adjacent cytoplasm which would be in keeping
with a nuclear secretory activity.

FOOD SELECTION IN AMOEBAE.

The question as to whether protozoa are able to
discriminate in the selection of food particles is one of
considerable interest. Evidence has been presented to show
that certain species can accept some types of food organisms

or particles, but disregard others. This selective feed-
ing has been demonstrated in <u>Stentor coeruleus</u> (Schaeffer,
1910), in bursaria (Lund, 1914) and in a number of par-
amecium species (Metalnikov, 1912; Bozler, 1924; Losina-
Losinsky, 1931; Bragg, 1936). In general, protozoa show
a tendency to ingest materials that are organic in prefer-
ence to those of inorganic origin. Ingestion of particles
by mammalian phagocytes has been shown to be an energy-
requiring process (Stähelin, Suter and Karnovsky, 1956;
Cohn and Morse, 1960); by analogy it seems reasonable to
suppose that phagocytosis occurring in protozoa will also
utilise cellular energy reserves. It is clear that hap-
hazard ingestion of particles, only some of which have
nutritive value, could be a highly wasteful process in
terms of energy expenditure. It follows, therefore, that
the ability to select between a potentially nutritive food
particle and an inert inorganic particle, might endow the
protozoal cell with a survival advantage. The literature
reviewed in the following pages will cover the evidence
that is available in relation to food selection by amoebae
and consider what is known about the way in which such a
selection is achieved.

<u>Choice of food in Amoeba proteus.</u>

Food selection in <u>Amoeba proteus</u> has been extensively
investigated by Schaeffer (1916a, b, 1917). The behaviour

of *Amoeba proteus* towards many substances was studied in
cover-slip preparations in which the test particles could
be placed in any desired position by absorbing fluid from
an appropriate point at the edge of the cover glass. When
a grain of silicic acid was laid in front of a grain of
globulin in the path of a moving amoeba, the cell sent out
pseudopods palpating the silicic acid without ingesting it;
after this the cell moved on until it contacted the grain
of globulin which was promptly ingested. Similar findings
were obtained when a grain of globulin and a grain of carbon
were placed side by side. Occasionally a food cup was
formed over the two particles, but the carbon was pushed
out of the cup whereas the globulin was retained. After
testing a number of substances, Schaeffer (1917) concluded
that *Amoeba proteus* had a predilection for substances which
are of food value. Indigestible materials, such as carbon
and glass fragments, were not phagocytosed unless they were
in a state of motion. Carmine proved an exception to the
rule since it was readily ingested. Schaeffer (1917) sug-
gested that two types of selection existed in *Amoeba proteus*,
viz. organismal and histonic; the former was operating dur-
ing the stage of ingestion and the latter after food had
entered the cell. An example of organismal selection was
a preferential ingestion of globulin rather than carbon, and
that of histonic selection was the expulsion of intracell-
ular carmine 3-14 minutes after it had been taken up.

Schaeffer (1917) also emphasised the importance of the past experience of individual amoebae. He observed that when a grain of globulin and a grain of hematin were lying close together in the path of an amoeba, they were first touched by the advancing pseudopods and then ingested, but in a subsequent encounter with these two substances, the amoeba formed a food cup before touching the particles. In the case of an agitated fragment of glass, ingested by an amoeba for the first time, it was retained in the cell for six minutes. If, however, the same amoeba was induced to phagocytose the same glass fragment, the rejection time was shortened to $3\frac{1}{2}$ minutes. When this glass fragment was presented to the amoeba for a third time, the cell became indifferent and no ingestion occurred.

Mast and Hahnert (1935) demonstrated that Amoeba proteus preferred to feed on colpidia and chilomonas rather than Monas punctum despite similarity in size and shape of these ciliates. When equal numbers of monas and chilomonas were added to amoebae suspended in Hahnert's solution in a hanging drop maintained in a moist chamber, 34 food cups were formed out of 56 contacts with chilomonas, but only 4 were formed out of 66 contacts with monas. These attempts at phagocytosis resulted in the ingestion of 23 chilomonas, but only 1 monas. In the other experiment, when the ratio of chilomonas to monas was 10:7, amoebae ingested almost a hundred times more of the former than of the latter. After

ingestion, chilomonas were seen to remain alive for 5-14
minutes, whereas monas remained alive for more than $3\frac{1}{2}$
hours.

It is clear that Amoeba proteus is able to dis-
criminate between different types of particles, and the
work of Schaeffer suggests that a given amoeba can become
more efficient as a result of previous experiences.   How-
ever, the mechanism of food selection is not known in detail;
it has been stated by Schaeffer (1917) that this cannot be
based solely on solubility, digestibility or chemical prop-
erties of the test particles.   Nevertheless the work of
Bell and co-workers (see a review by Bell, 1963) described
previously in this chapter could provide some understanding
of mechanisms which initiate pseudopod and food cup forma-
tions in relation to food ingestion, but the data presently
available do not adequately explain the entire process of
food recognition.

Choice of food in soil amoebae.

Soil amoebae have been found suitable for studying
selection of food organisms by protozoa, since these amoebae
may be cultured under a variety of conditions, i.e. in syn-
thetic liquid medium with or without bacteria and on agar
in the presence of colonies of an appropriate food organism.

There is good evidence that some species of bacteria
are more suitable than others as food organisms for soil

amoebae (Tsujitani, 1898; Mouton, 1902; Oehler, 1916;
Severtzova, 1928; Singh, 1941; Chang, 1960).   It is
possible that a given species of soil amoebae might be able
to select between suitable and unsuitable bacteria, when
they are present together.   A considerable amount of work
has been done to study this possibility.

Severtzova (1928) prepared streaks of bacteria in
pure culture on solid artificial mediums in a petri dish in
the form of an eight-pointed star.   After incubation to
allow bacterial growth, soil amoebae were placed at the
centre of the radiating lines.   It was found that amoebae
could clear a line of certain types of bacteria faster than
of others and some bacterial lines were quite untouched.
In view of the finding that clearing of the bacterial streaks
was correlated with an increase in numbers of the amoebae
present, it was indicated that the protozoa had the capac-
ity to feed selectively.   In another experiment soil amoebae
were added to a mixture of Escherichia coli commune and
Bacterium ellenbachensis grown on an agar slope;   after in-
cubation at 22° for 6 days the bacteria were washed from
the slope with water and plated for a differential count.
It was found that the proportion of Escherichia coli to
Bacterium ellenbachensis in a control tube was 1:9, whereas
in the tube inoculated with amoebae very few Escherichia coli
were recovered, whilst Bacterium ellenbachensis was present
as an almost pure culture.   This suggested that the amoebae

had fed selectively on _Escherichia coli_ in the original
mixed culture.

Following this lead, Singh (1941, 1942, 1945)
showed that some species of bacteria were more acceptable
than others to limax amoebae, and that even within a given
bacterial species, certain strains were more suitable as
food organisms, although no morphological or physiological
differences were apparent between these strains.   When
amoebae were inoculated at one end of the two streaks of
bacteria, lying side by side on an agar plate, the protozoa
first ate the most readily acceptable bacteria and later
migrated to feed on the less acceptable organisms.   If,
however, the amoebae had been previously adapted to grow on
the less acceptable organisms of the two over a long period
of time, it was found that both bacterial streaks were eaten
with equal avidity.   Selection was also apparent when a
mixture of edible bacteria (Bacteria 4045), non-edible bac-
teria (Bacteria 5054) and limax amoebae were inoculated in
damp sterilised soil (Singh, 1941).   This author showed
that while the numbers of edible bacteria were remarkably
reduced, the population of inedible bacteria remained com-
paratively high.

In 1959, Chi, Vogel and Shelokov reported that a
species of acanthamoeba found as a contaminant in a monkey
kidney culture showed preferential selection for nucleated
red blood cells.   When non-nucleated guinea pig red blood

cells were mixed with the amoebae in a hanging drop of
tissue culture medium, the red cells were treated with in-
difference, some red blood cells were occasionally ingested,
but a prompt rejection ensued. However, if nucleated
chicken red blood cells were added, these became attached
to the amoebae and were phagocytosed. After a variable
period of time, the red cells were ejected from the amoeba,
apparently in a denucleated form. Chi and co-workers sug-
gested that acanthamoebae preferred to eat nuclear mater-
ials. This idea gains support from the work of Semenoff
(1938) who showed that during the process of ingestion of
frog red blood cells by Entamoeba histolytica, the nuclear
material was frequently seen to be extracted from the red
blood cells and preferentially ingested.

Possible mechanisms of food selection by soil amoebae.

It is clear that there is a parallelism between
selection and edibility of bacterial food (Singh, 1941).
However, the criteria for bacterial edibility are not rel-
ated to any taxonomic characters, e.g. Gram staining, mot-
ility, slime production, pigmentation, proteolytic power,
ability to nitrify and fix nitrogen (Severtzova, 1928; Van
Rooyen, 1932; Rice, 1935, 1938; Singh, 1941, 1942, 1945).
Good correlation between edibility of bacteria and the
growth rate of amoebae has been demonstrated by Frosch
(1897) who inoculated bacteria-free cysts of soil amoebae
into pure cultures of various bacteria and showed that the

culture of microorganisms did not support the growth of
amoebae equally well.

Cutler and Crump (1928) counted the number of
Hartmanella hyalina in a drop of autoclaved soil extract
to which had been added test bacteria; they reported that
the growth rate of amoebae depended on the number of bacter-
ia present and the species used. When comparable inoculae
of Gram-positive bacilli (Y.B. strain) or Gram-negative
bacilli (S.E. strain) were added to autoclaved soil extract
containing a standard number of soil amoebae, the rate of
amoebic division was greater in the presence of the Y.B.
bacilli, the maximal growth rate being obtained if the ratio
of amoebae to bacteria was 1:1-2 x 10^6. Moreover, the vol-
ume of individual amoebae increased faster when they were
grown with the Y.B. bacilli.

Ray (1951) placed cysts of limax amoebae (Hartmanella
sp.) in a drop of 0.1% yeast extract solution to which she
added various species of bacteria; it was found that the
growth rate of amoebae could be related to the degree of
bacterial motility. In liquid medium it is likely that the
more motile an organism, the greater the chance of contact
with the surface of an amoeba. It has been clearly demon-
strated that when flagellated bacteria touch the surface of
soil amoebae, they are agglutinated and become attached to
the cell (Mouton, 1902; Ray, 1951). By analogy with what

is known about mammalian phagocytes, i.e. that adhesion of
the particles to the cell surface is a pre-requisite for
efficient phagocytosis (Mudd et al., 1934), it is reason-
able to suppose that agglutination of flagellated bacteria
on the amoeba surface would facilitate ingestion.   The
idea that intimacy of contact between amoebae and bacteria
promotes phagocytosis is supported by the finding of Ray
(1951) who showed that two species of bacteria, one of which
was motile and therefore more readily taken up by limax
amoebae in liquid medium, served equally well as food or-
ganisms when amoebae were allowed to contact them on a solid
medium.   This finding suggests that possession of flagella
per se is not a determinant for the pattern of feeding in
soil amoebae under all cultural conditions.

The way in which amoebae cause flagellated bacteria
to agglutinate on contact with their surfaces is not clearly
understood.   Mouton (1902) has suggested that this was
brought about by a secretion from the contractile vacuole.
He claimed that agglutination of Escherichia coli occurred
only in amoebae which had been adapted in these organisms,
but did not occur if amoebae were grown with yeast cells.
On the other hand, Ray (1951) showed that amoebae grown with
Pseudomonas fluorescens on solid mediums agglutinated all
motile organisms tested.   Moreover, there was no evidence
for specific localisation of motile bacteria to the region
of the cell membrane overlying the contractile vacuole.   She

suggested that agglutination could be attributed to a secretion of an adhesive substance on the cell surface or, alternatively, to physicochemical properties of the cell membrane, e.g. electrostatic charge effect.

Four possibilities have been put forward by Cutler and Crump (1928) to explain the different rates of growth observed in soil amoebae with different species of bacteria, or the same species of bacteria after various treatments, e.g. heating.

1. A species may be of a superior food value.

2. A species may be suited to the digestive process of the consumer.

3. Inhibition of growth of amoebae owing to toxic properties of bacteria.

4. For physical reasons bacteria of certain structures cannot be readily ingested.

Out of these possibilities only one, i.e. toxic properties of bacteria, has been the subject of extensive investigation.

Cutler and Crump (1928) grew bacteria in sterilised soil extracts and after varying intervals of time the cultures were filtered and the filtrates tested for their effect on the growth of _Hartmanella hyalina_. It was found that the reproduction rate of amoebae in day-old filtrates was comparable for both readily accepted bacteria (_B. mycoides_)

and less acceptable bacteria (S.E. Bacilli), but was considerably reduced in 2-3 day-old filtrates, depression of growth being most marked in the presence of the filtrate of S. E. bacilli.

Van Rooyen (1932) studied the clearing of virulent and avirulent strains of Staphylococcus by Hartmanella (Acanthamoeba) castellani. He showed that these bacteria resisted clearing, i.e. that they were unacceptable food organisms. However, when these bacteria were killed by heat and washed, or heat-killed only, the amoebae were able to utilise them. If the staphylococci were killed by chemical means, e.g. chloroform or formaldehyde vapour without further washing, they remained unacceptable. Amoebae exposed for 4-24 hours to saline washings from Staphylococcus aureus or other unacceptable bacteria were shown to clear Salmonella typhosa at a much slower rate than control amoebae treated with normal saline only. Van Rooyen concluded that inedibility of certain bacteria was due to diffusible products which damaged or otherwise injured the amoebae. He also attempted to render edible bacteria, e.g. Salmonella typhosa, resistant to ingestion by amoebae by treating them with culture filtrates from inedible organisms, but the bacteria so treated became only slightly more resistant than the untreated controls.

The toxic effect of diffusible and non-diffusible substances from pigmented organisms has been reported by

Singh (1945), who showed that amoebae either died or encysted within 7 days when inoculated on to edible bacteria that had been grown on agar containing diffusible products of Pseudomonas pyocyaneus. Similar results were obtained when amoebae were added to suspensions of crude or purified pyocyanin. This test showed that crude pyocyanin was more toxic than the purified material. Singh also showed that the deleterious effect of Chromobacterium prodigiosum, Chromobacterium violaceum and Bacteria 5654 on soil amoebae was due to action of a water insoluble pigment and not to a freely diffusible exotoxin, since these organisms became partially or completely accepted by soil amoebae when their ability to produce pigment had been lost as a result of frequent subcultures.

Dudziak (1962) has shown that the majority of strains of mycobacteria are unable to support the growth of soil amoebae on solid mediums; however, Mycobacterium pelligrino is an exception since it is able to do so in the case of three species of amoebae tested, i.e. Acanthamoeba castellani, Hartmanella rhysodes and an unidentified amoeba B. It is clear that mycobacteria in general do not produce diffusible materials that are toxic for soil amoebae since these protozoa are able to grow in the presence of mycobacteria if a suitable food organism is present, i.e. Aerobacter aerogenes. Investigations of the reasons why mycobacteria are unacceptable as a food substrate have shown

that many of them contain toxic materials that could be
extracted with fat solvents or which are exposed in the
bacterial residue after attempted extraction, e.g. extrac-
tion of Mycobacterium tuberculosis H37Rv results in the
bacterial residue becoming acceptable as a food substrate
for soil amoebae, whereas the ether soluble waxes are toxic
when tested against amoebae.   If Mycobacterium phlei is
extracted in a similar manner, no ether soluble toxic frac-
tion can be demonstrated, but the bacterial residue now ex-
hibits a toxic effect when added to soil amoebae cultures.
The criteria for toxic effect in these experiments were in-
hibition of amoebae division and encystation.

It is possible that failure to ingest food organisms
may also be related to food selection, since it is known
that edible bacteria could be rendered unacceptable by heat
treatment (Oehler, 1916; Drożański and Drożańska, 1961).
Drożański and Drożańska reported that the growth of limax
amoebae was poor in the presence of a wide range of bacteria
killed at 100° suspended in normal saline; however, if an
extract of Aerobacter aerogenes, prepared by repeated freez-
ing and thawing, was added to the culture a faster rate of
growth was measured, but at no stage did the rate approach
that of amoebae grown with living organisms.   In the light
of the finding that this extract increased the phagocytic

index of soil amoebae for heat-killed yeast cells, it is
reasonable to suppose that the uptake of heat-killed bac-
teria was promoted in a similar fashion.

Drożański (1963) has extended his study on the ef-
fects of heating in modifying the acceptability of Aero-
bacter aerogenes as a food organism for soil amoebae; he
showed that although intact bacteria killed by boiling at
100° did not support the growth of amoebae maintained in
normal saline, an aqueous extract of disrupted heated aero-
bacter acted as an adequate source of nutritive material
when added to the amoebae in normal saline.   Drożański
suggested this finding indicated that the heat-killed intact
aerobacter retained its potential nutritive value, but that
the process of heating caused changes at the bacterial sur-
face which resulted in its failure to be ingested by soil
amoebae.

In summarising the above survey, it is clear that
soil amoebae show discrimination in feeding, but the way in
which they do this is only partially known.   It is hoped
that the experiments described in this thesis will allow
better understanding of food-choice mechanisms and indicate
if selection operates at the level of food-seeking, i.e. is
related to the chemotactic response of amoebae, whether
there is preferential ingestion of different bacterial
species, or whether certain types of bacterial cells are
resistant to intracellular killing and cannot, therefore,

be adequately degraded to supply nutritive materials. It is also hoped that this study may throw some light on the broader problem of the evolution of recognition mechanisms at the single cell level.

CHAPTER II.

MATERIALS AND METHODS.

All the glassware used for handling amoebae were
boiled in a mixture of Calgon (Albright and Wilson (Aust.)
Pty. Ltd.) and sodium metasilicate solution for 20 minutes,
followed by rinsing with glass distilled water 12 times.
The procedure was similar to that described by Hanks (1955).

Amoeba proteus.

The Dawson strain of A. proteus used in this study
was kindly provided by Professor J. F. Danielli and Dr.
S. E. Hawkins, King's College, London. The protozoa were
maintained in modified Chalkley's medium to which wheat
grains and a ciliate tetrahymena were added as described by
Hawkins (1962, personal communication). This technique was
essentially similar to that described by Prescott and James
(1955) except for the different culture medium used. Mod-
ified Chalkley's medium was made up as follows:-

NaCl	80 mg.
NaHCO₃	4 mg.
KCl	2 mg.
Na₂HPO₄.12H₂O	1 mg.
Glass distilled water	1 litre

NaCl — 80 mg.
NaHCO$_3$ — 4 mg.
KCl — 2 mg.
Na$_2$HPO$_4$.12H$_2$O — 1 mg.
Glass distilled water — 1 litre

Autoclaved at 121° at the pressure of 15 lbs. per
sq. in. for 10 minutes.

Calcium salt in the original formula described by

Chalkley (1930) was omitted since it has been claimed by Hawkins (1962, personal communication) that this ion increases rigidity of the plasmagel and therefore is potentially able to cause difficulty during micro-injection.

The amoebae were kept in 6 inch diameter petri dishes at 20° to which washed tetrahemenae were added daily. The ciliates were grown separately in axenic condition at 20° in 1 per cent. proteose-peptone solution for 1-2 weeks, they were washed free from proteose-peptone by centrifugation for 3 minutes at 1000-1500 r.p.m. and finally resuspended in Chalkley's medium before addition to the culture of amoebae.

Soil amoebae.

Two species of soil amoebae used extensively in this study were:

1. __Hartmanella astronyxis__ (Ray: Cambridge collection of protozoa and algae No. 1534/1). These amoebae could be grown axenically in a liquid medium or together with bacteria on a solid medium.

Two liquid media employed were -

a) Proteose peptone glucose solution (PPG), (Band, 1959), prepared as follows:-

NaCl	120 mg.
$MgCl_2.6H_2O$	3 mg.
$CaCl_2$	3 mg.

$FeSO_4$	3 mg.
Na_2HPO_4	142 mg.
KH_2PO_4	136 mg.
Difco Proteose-peptone	10 gm.
Glucose	18 gm.
Glass distilled water	1 litre

Final pH = 6.8

Autoclaved at 15 lbs. per sq. in. at 121^0 for 10
minutes.

Amoebae were grown in 20 ml. of this medium in a
4 oz. medicinal bottle placed horizontally at 20^0. The
fluid level in the bottle did not exceed 1 cm. It was
found that luxuriant growth of amoebae was obtained only
when killed bacteria were supplemented. As a rule, Pseudo-
monas fluorescens killed by heat at 56^0 for $1\frac{1}{2}$ hours was
added to give a bacterial concentration of approximately
5×10^7 per ml. (Drożański and Drożańska, 1961).

b) 3 per cent. yeast extract medium, made up as follows:-

Yeast extract (Oxoid)	30 gm.
Glass distilled water	1 litre

Autoclaved at 15 lbs. per sq. in. at 121^0 for 10
minutes.

The growth of amoebae in this medium was poor and
hence it was used only in the early part of the study re-
ported in Chapter VI. The solid medium employed in these
studies was 0.1 per cent. yeast extract agar (Ray, 1951),

prepared as follows:-

Yeast extract (Oxoid)	1 gm.
Agar	15 gm.
Glass distilled water	1 litre

Pseudomonas fluorescens was first streaked on this medium
in a Petri dish in the form of 2 bands crossing each other
at the centre of the plate.   After incubation at 30° over-
night, a loopful of amoebae containing approximately 300
cells were inoculated at the centre and the plate was in-
cubated at 20°.

Subcultures of amoebae were done every month.

2.   Hartmanella rhysodes (Singh;   Cambridge collection
of protozoa and algae No. 1534/3).

These amoebae were maintained axenically in PPG at
20° (Band, 1959).   The cells thrived luxuriently in this
medium and no addition of killed bacteria was required.
Subculture was done every 3 weeks.

Other soil amoebae used in minor studies were:-

a)   Acanthamoeba sp. (Neff;   Cambridge collection of
protozoa and algae No. 1501/1).

This amoeba was grown in PPG in a manner described
for H. rhysodes.

b)   Schizopyrenus russelli.

c)   Didascalus thorntoni.

d)   Naegleria gruberi.

e)   Hartmanella glebae.

f)   Hartmanella rhysodes strain 15.

The last 5 species of amoebae were obtained through the courtesy of Dr. B. N. Singh, Central Drug Research Institute, Lucknow, India. The protozoa were grown at 20° on 0.1 per cent. yeast extract agar containing an overnight growth of an Aerobacter sp. (Bacterium 1912) (see below).

Bacterial strains used in Amoeba proteus studies.

1. Salmonella enteritidis Se 795: A smooth strain isolated from fur seals was obtained from Dr. E. Ribi, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana, U.S.A.

2. Salmonella typhimurium LT_2 (Lilleengen, 1948).

3. Bacillus subtilis Strain 841. This organism was kindly provided by Dr. N. Atkinson, Department of Microbiology, the University of Adelaide.

Bacteria used in soil amoebae studies.

Most of the work was done using the following bacteria:

1. Pseudomonas fluorescens. A strain maintained in the Department of Microbiology, the University of Adelaide. This organism was very actively motile. It grew well at 30° and did not grow at 37°.

2. Aerobacter sp. (Bacterium 1912). This organism was kindly provided by Dr. B. N. Singh, Central Drug Research Institute, Lucknow, India. This organism was non-motile and it was used in propagating many strains of soil amoebae

used in the study in Chapter VI.   Its biochemical characteristics have been described fully by Singh (1941).

3. Escherichia coli NCTC. 8196.   This organism was obtained from Mr.J.R. Harris, CSIRO Division of Soil Microbiology, South Australia.

4. Escherichia coli strain 812.   A strain maintained in Department of Microbiology, the University of Adelaide.

These last two organisms were originally non-motile. However, after a serial passage through semi-solid medium (Lederberg, 1956) at 30° three times, motile strains were obtained.   These organisms were actively motile only when they were grown at 30°.

5. Salmonella enteritidis.   A strain maintained in the Department of Microbiology, the University of Adelaide.

6. Other bacteria used in screening studies for a selection of appropriate species of bacteria to be used in the analysis of food choice mechanisms in soil amoebae.

a) Escherichia coli (Stoke-W)

b) Escherichia coli (Rechner)

c) Escherichia coli 1064

d) Escherichia coli (Edgar)

e) Salmonella derby

f) Salmonella cholerae-suis var. Kunzendorf

g) Salmonella anatum

h) Salmonella enteritidis Se 795

i) Pseudomonas chlororaphis

j) <u>Pseudomonas ovalis</u>

k) <u>Pseudomonas fluorescens</u>

l) <u>Aerobacter cloacae</u>

m) <u>Bacterium H.</u>   These were Gram negative non-motile cocco-bacilli which grew well at 25° but not at 37°. They did not ferment any of the sugars tested, viz. lactose, sucrose, glucose, manitol, dulcitol, and salicin.   Other reactions were: indol +, M.R. neg., V.P. slightly +, urea +, gelatine neg., H_2S neg., and citrate neg.

n) <u>Aerobacter aerogenes</u> (Routine).   This organism was maintained in the Department of Microbiology, the University of Adelaide.

The organisms from <u>a</u> to <u>g</u> were kindly provided by Dr. H. Atkinson, Department of Microbiology, the University of Adelaide, and the organisms from <u>i</u> to <u>m</u> were obtained from Mr.J.R. Harris, CSIRO Division of Soil Microbiology, Adelaide.

Maintenance of cultures.

Stock cultures were grown on nutrient agar and stored at 4° on slopes of this medium in 1 oz. screw-capped bottles, each of which was open no more than ten times.

Cultivation of bacteria.

Bacteria used in <u>Amoeba proteus</u> studies were sub-cultured into minimal medium and incubated on a reciprocating shaker at 37° for periods of either 6 or 18 hours, de-

pending on the type of experiments planned. Minimal medium
was made up according to the formula of Davis and Mingioli
(1950) as follows:-

Glucose	2 gm.
K_2HPO_4	7 gm.
KH_2PO_4	3 gm.
Sodium citrate, $2H_2O$	0.5 gm.
$MgSO_4.7H_2O$	0.1 gm.
$(NH_4)_2SO_4$	1 gm.
Glass distilled water	1 litre

Autoclaved at 121° at the pressure of 15 lbs. per
sq. in. for 15 minutes.

The organisms used in micro-injection studies
(Chapters III and V) were streptomycin sensitive S. enter-
itidis Sc 795 growing in logarithmic phase. This was pre-
pared by inoculating 0.5 ml. of overnight shaking culture
of this organism grown in minimal medium into 10 ml. of the
same medium and incubated further on a reciprocating shaker
at 37° for another 6 hours. After washing in minimal med-
ium by centrifugation at 3000 r.p.m. for 10 minutes, the
organisms were suspended in the injection medium and kept
in an ice bucket at 4°. For each injection, a sample was
taken from this stock bacterial suspension and transferred
on to the coverslip in an oil chamber in which a microdrop
of approximately 2 mm. was formed. Each batch of the stock

bacterial suspension was used no longer than 2 hours.

In all phagocytic experiments (Chapters IV and V), the organisms used were streptomycin resistant mutants of S. enteritidis Se 795 or S. typhimurium LT$_2$ growing in lag phase. Streptomycin marker was used to distinguish between the test organisms and the contaminants associated with amoebae in the culture dish. Minimal medium in which the organisms were grown contained 50 micrograms of streptomycin per ml. After incubation for 18 hours, the organisms were washed in minimal medium by centrifugation at 3000 r.p.m. for 10 minutes and finally resuspended in Chalkley's medium containing 0.1 per cent. glucose. All nutrient agar plates used in these experiments contained 50 micrograms of streptomycin per ml.

Organisms used in soil amoebae studies were grown in nutrient broth and incubated either at 30° or 37° depending on the optimal condition required by each organism. For chemotactic studies, however, the organisms were grown in PPG, since in this medium certain organisms especially the low temperature requiring bacteria gave much better growth.

Selection of streptomycin resistant strains.

Selection of a streptomycin resistant mutant was done by plating a dense suspension of an overnight broth culture of bacteria on to a nutrient agar plate containing a gradient concentration of streptomycin. This gradient

plate was prepared by overlaying warm nutrient agar in a
Petri dish in which a slant of nutrient agar containing a
known concentration of streptomycin was earlier made.
Colonies of bacteria were then selected from a portion of
the plate which had a higher gradient and inoculated into
broth.   After incubation at 37° overnight, a drop of this
culture was plated on to nutrient agar containing strepto-
mycin at a higher concentration gradient.   By this means,
mutants of <u>S. enteritidis</u> and <u>S. typhimurium</u> LT$_2$ resistant
to 200 micrograms of streptomycin per ml. were obtained.
The organisms were maintained at 4° on nutrient agar slopes
containing 50 micrograms of streptomycin per ml.

<u>Preparation of Bacillus subtilis spores.</u>

<u>B. subtilis</u> grown on nutrient agar for 48 hours at
37° was suspended in normal saline solution.   It was found
that in this suspension, spores tended to form aggregates
which could be conveniently separated from vegetative bac-
terial cells by centrifugation at 1000 r.p.m. for 5 minutes.
After twice more washing, these aggregates were resuspended
in physiological saline following by sonication in a MSE
ultrasonic disintegrator for 10 seconds to disaggregate the
spores.   The spore suspension was then heated at 80° for
10 minutes to kill residual vegetative bacteria after which
the spores were washed twice by centrifugation at 3000 r.p.m.
for 10 minutes and finally resuspended in the injection med-
ium.

Carbon.

A shellac free preparation of India Ink (Ink No. C11/1431a) prepared by Gunther Wagner, Hanover, Germany, was treated according to the method described by Biozzi, Benacerraf and Halpern (1953) giving a particle size of less than 500 Angstroms at a concentration of 32 mg. per ml. in 2 per cent. gelatin at pH 7.4. The carbon to be used for micro-injection was diluted 1:4 in distilled water to give a final concentration of 8 mg. carbon in 0.5 per cent. gelatin.

Polystyrene latex.

This material was obtained from the Dow Corning Corporation, Midland, Michigan, U.S.A. The particles were spherical of 2640 Angstroms. A stock suspension in physiological saline containing 106.5 mg. per ml. was kept at 4°.

Sera.

1. Antiserum against Hartmanella rhysodes.

A rabbit was given 5 intravenous injections of 1 ml. of saline suspension of washed H. rhysodes cells at intervals of 4 days and the total numbers of amoebae injected being 6×10^{6} cells. The rabbit was bled by cardiac puncture 7 days after the last injection.

2. Antisera against bacteria.

a) Specific antisera.

Antisera against S. enteritidis Se 795 and S. typhi-

murium LT$_2$ were prepared in rabbits. Bacteria used for immunisation were killed by exposure to 30 per cent. alcohol at 4° for 2 hours after which they were washed twice in physiological saline and finally resuspended in the same solution to give a final concentration of 10^9 organisms per ml. Animals were given a series of 4 intravenous injections consisting of 0.5, 1, 2 and 4 ml. of the bacterial suspension at intervals of 3 days. The animals were bled by cardiac puncture 7 days after the last injection, blood was allowed to clot at 37° for 1 hour followed by storage at 4° overnight. Serum was separated from the clot by centrifugation at 2000 r.p.m. for 10 minutes. Serum titres were determined by tube agglutination using a saline suspension of alcohol killed homologous bacterial cells as standard antigens. It was found that sera prepared against S. enteritidis and S. typhimurium LT$_2$ both had titres of 1:640.

b) Pig serum.

Serum was obtained, as described, from the blood of healthy pigs slaughtered at the Adelaide Metropolitan Abbatoirs.

All sera were stored at -20°, and heated at 56° for half an hour before use.

Source of complement.

Fresh normal rabbit serum was used as a source of complement. The animal was bled by cardiac puncture, the blood collected in 1 oz. siliconised bottles and allowed to

clot at 37° for 1 hour.   The serum was separated from the clot by centrifugation at 2000 r.p.m. for 10 minutes at 0°. To remove the 'natural' antibody against S. enteritidis, the serum was adsorbed at 0° for 30 minutes with homologous organisms killed by heat at 56° for half an hour.   The concentration of bacteria used in adsorption was 2 mg. dry weight per ml. of serum.   After adsorption the serum was centrifuged at 4000 r.p.m. for 10 minutes at 0° and filter- ed through a milipore membrane (pore size of 0.45μ) to remove any residual bacteria before being dispensed in 1 ml. volume in sterile Bijou bottles and stored at -20° for not more than a week.   The serum was thawed only once and used immediately.

Opsonisation of bacteria.

Specific antiserum was added to a culture of bac- teria containing 5×10^8 organisms per ml. to give a final concentration of serum as desired: unless otherwise stated this was equivalent to two-thirds of a minimal agglutinating dose per ml.   At this antibody concentration, the opsonised bacteria did not form any microscopic agglutination.   When serum antibody equivalent to one minimal agglutinating dose per ml. or more was employed, bacterial agglutination oc- curred.   This was undesirable, because the clump of bac- teria would block the micro-pipette or otherwise render difficulties in micro-injections.   No attempt was there- fore made to exceed this concentration of serum antibody.

When pig serum was used as a source of opsonins, equal volumes of serum and bacterial culture were mixed and allowed to stand for 20 minutes at 4°. After all opsonising procedures, the bacteria were washed with minimal medium by centrifugation at 3000 r.p.m. for 10 minutes and finally resuspended in an appropriate medium depending on the experiments planned.

In experiments where additional complement was needed, equal volume of bacterial suspension containing 10^9 organisms per ml. was mixed with adsorbed fresh rabbit serum to which specific antibody for the test bacteria had been added so that the final concentration of serum opsonin in this mixture was equivalent to two-thirds of a minimal agglutinating dose per ml. Opsonisation was allowed to occur at 30° for 20 minutes, after which the cells were washed at 4° and finally resuspended in the cold injection medium (4°) and used immediately.

Preparation of lytic phage.

A lytic mutant of phage P_{22} was propagated on S. typhimurium LT_2. High titre phage suspensions were prepared by an agar layer technique as described by Adams (1959a), and equilibrated with Chalkley's medium by dialysis against a large volume of the medium for 24 hours at 4°. The titre of phage was assayed also by an agar layer technique (Adams, 1959b).

Preparation of phage antiserum.

2.0 ml. of phage suspension in broth containing
2×10^{11} plaque forming units (PFU) was mixed with an equal
volume of Freund's complete adjuvant and injected subcutan-
eously into a rabbit, followed by 5 intravenous injections
of 10^{11} phage particles at intervals of 3 days. The an-
imal was bled by cardiac puncture 8 days after the last in-
jection. The velocity constant (K) of the antiserum was
11.5 as determined at 20° using Chalkley's medium as diluent
(Adams, 1959c).

Experimental methods for Amoeba proteus studies.

Inoculation of Amoeba proteus by a micro-injection
technique.

An oil chamber was made from a rectangular piece of
perspex measuring $2\frac{1}{2} \times 1\text{-}1/4 \times 1/8$ inches. A hollow re-
cess at the centre was sealed with a $2 \times 7/8$ inches glass
coverslip by means of silicone grease (Dow Corning, N.S.W.,
Australia). The coverslip was then overlaid with paraffin
oil (Townson and Mercer (S.A.) Pty. Ltd.). The appearance
of the oil chamber is shown in fig.1.

Several actively motile amoebae were placed in a
depression slide and washed while the cells were adhering
to glass by taking out the supernatant with a Pasteur pip-
ette and replaced by sterile Chalkley's medium. This pro-

TOP VIEW

SIDE VIEW
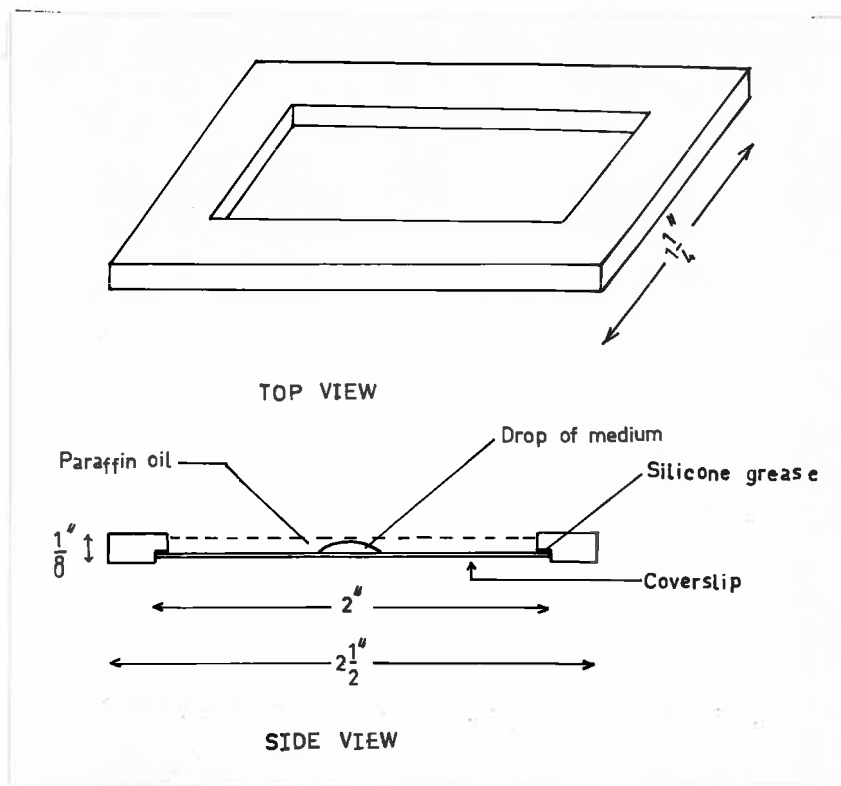
Fig. 1. Diagram showing an oil chamber.

Fig. 2. Photograph showing a micro-injecting device.

cess was repeated 10 times after which amoebae were removed individually by a micro-pipette controlled by mouth suction and washed twice more by passing through sterile Chalkley's medium in depression slides. Finally each cell was transferred, together with a small volume of Chalkley's medium, to a cover-slip in the oil chamber on which the medium spread out to form a droplet of approximately 3 mm. It was found that amoebae adhered satisfactorily to coverslips that had been cleaned by boiling in glass distilled water for 10 minutes.

Micropipettes used for micro-injections were drawn from soda glass tubing of 3.5 mm. external diameter, one end of which was tapered in a flame to 1 mm. diameter and finally shaped by means of a microforge (De Fonbrune, 1949). Internal diameters of the micropipettes ranged between 2 to 5 microns. The other end of the glass tube was also tapered in a flame to fit size 4 sterivac polythene tubing (Allen and Hanburys Ltd., London) connected to a 12 guage needle attached to a 2 ml. glass syringe. The micropipette, polythene tubing and syringe were then completely filled with paraffin oil (fig.2). This could be easily done by first filling the injecting device with paraffin oil up to the shaft, a few mm. away from the point where a micropipette was going to be made. Next, the micropipette was fabricated and the paraffin forced to pass through the small

orifice of the pipette. Since the distance between the tip of the pipette and the oil meniscus in the shaft was not great, only a gentle pressure was needed to achieve a complete filling. The entire procedure usually took no longer than 10 minutes.

Micro-injection of amoeba with bacteria was done on a platform so constructed that vibration transmitted from the surroundings was reduced to the minimum. This was achieved by placing instruments used for micrurgy on a wooden board resting on a rectangular dexion frame which was suspended with wires fastened to springs fixed to the bars at the roof of the dexion framework. The whole structure is shown in fig. 3.

Bacteria to be injected were suspended in Chalkley's medium containing 0.5 per cent gelatin (May and Baker gelatin sheet). Before using this medium in the test, it had been established that:-

1) The medium was not toxic to amoebae, since it was found that out of 13 amoebae which received injections with this medium with the amount approximately equivalent to half of the cell volume, eight cells divided by the third day and all remained actively motile.

2) Salmonella enteritidis cells suspended in this medium remained viable for a period of at least two hours. The viability of bacteria was determined by correlating the known numbers of the bacteria counted under phase contrast

with the numbers of colonies formed when these were plated
on nutrient agar.   The bacteria were counted under phase
contrast on a cover-slip overlaid with paraffin oil. This
was facilitated by transferring by means of micropipette,
a small number of bacteria from a 3 mm. drop of dense bac-
terial suspension in the injection medium on a coverslip,
to another part of the same coverslip, so that a micro-
droplet of a diameter of approximately 100 microns was
formed in which it was possible to count the bacteria ac-
curately (fig.4).   These bacteria were then transferred
by means of a micropipette to a drop of Chalkley's medium,
3 mm. in diameter, which was again removed by means of a
micropipette controlled by mouth suction and diluted in a
depression slide with 1.5 ml. of Chalkley's medium before
being incorporated into an agar pour plate for a viable
count.   The result was set out in Table I.   It can be
seen that average initial viability was not less than 90
per cent. and this did not alter appreciably during a 2
hour period in the injection medium.

Prior to injection into amoebae, the bacteria in a
micro-drop were counted as described above.   After rinsing
the micropipette with sterile Chalkley's medium, all the
bacteria in the micro-droplet were drawn into the pipette
again.   Before injection, the pressure inside the pipette
was equilibrated with another drop of the injection medium

TABLE I.

Correlation of the number of <u>Salmonella enteritidis</u> cells counted under phase contrast with the number of colonies resulting when these cells were plated out on nutrient agar

	Time of exposure to the injection medium (minutes)					
	20	40	50	70	95	135
Bacterial cell count under phase	49	54	56	69	64	51
Colony count on nutrient agar	53	52	59	72	66	57
Percentage viability of bacteria	108.2	96.3	101.8	104.3	103.1	111.7
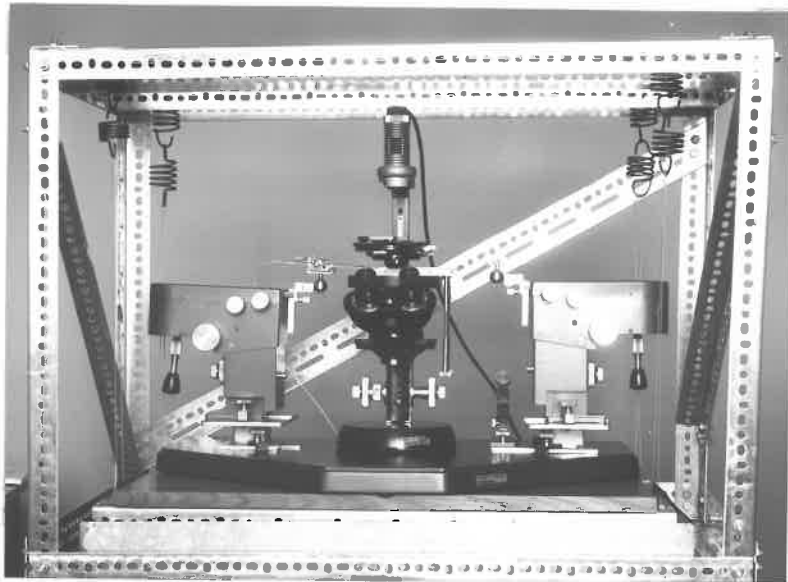
Fig. 3. Photograph showing apparatus used in micrurgy
and its supporting framework.
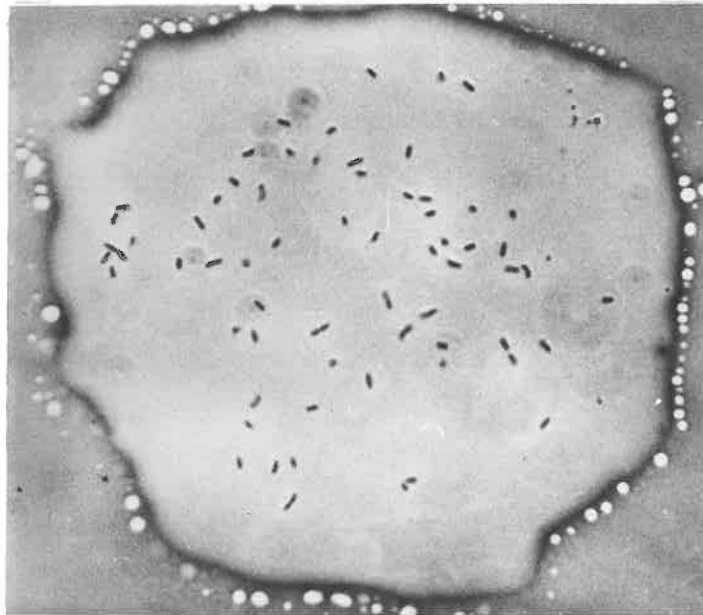
Fig. 4. Photograph showing a droplet of injection
medium containing Salmonella enteritidis
prepared for a direct count (phase contrast).

of 100-150 microns in diameter.   This precaution was taken
to prevent a premature leakage of the bacteria from the
pipette or to prevent the passive intake into the pipette
of the medium surrounding the amoeba in the microdrop.

The amoeba to be injected was viewed under phase
contrast with a Zeiss Plankton inverted microscope.   By
means of a Leitz micromanipulator, the micropipette con-
taining bacteria was introduced directly into the cytoplasm
and the organisms expelled.   The time taken to complete
one injection and the necessary washing of the amoebae sub-
sequent to this was not more than 5 minutes.   Stages of
this procedure are shown in fig.5a, b and c.

Precautions were taken to recover any bacteria that
might have leaked from the micropipette into the medium
surrounding the amoeba at the time of injection, or which
might have remained adherent to the wall of the pipette.
Any bacteria recovered from these situations were deducted
from the original count, thus giving the actual number of
organisms injected.   After being incubated in 1.5 ml. of
Chalkley's medium in a depression slide at 20° for a period
of time ranging up to 6 hours, the amoeba was again washed
and transferred to the surface of an agar plate where it
was crushed for a bacterial count.   Finally, the medium
from the depression slide, together with the medium used
for washing, were incorporated into an agar pour plate.   By
this means it was possible to recover any organisms which
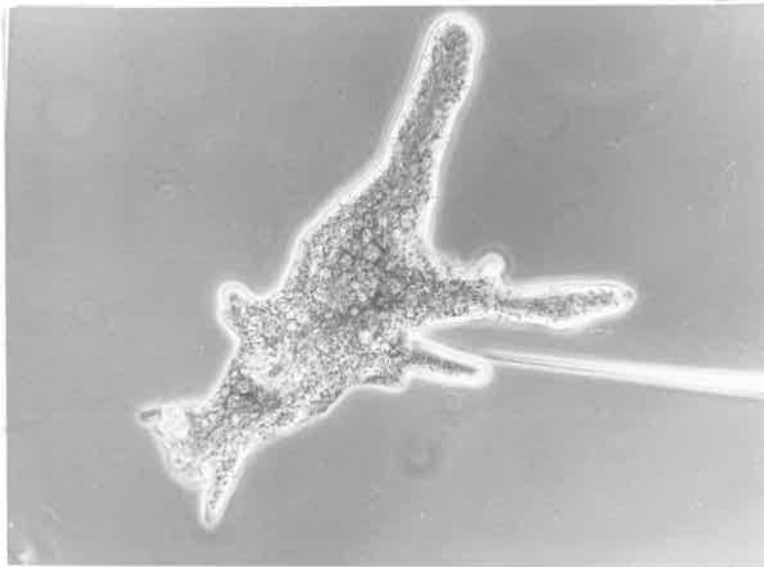
Fig.5a. Photograph showing a micropipette placed alongside
an amoeba adherent to a coverslip immediately prior to injection
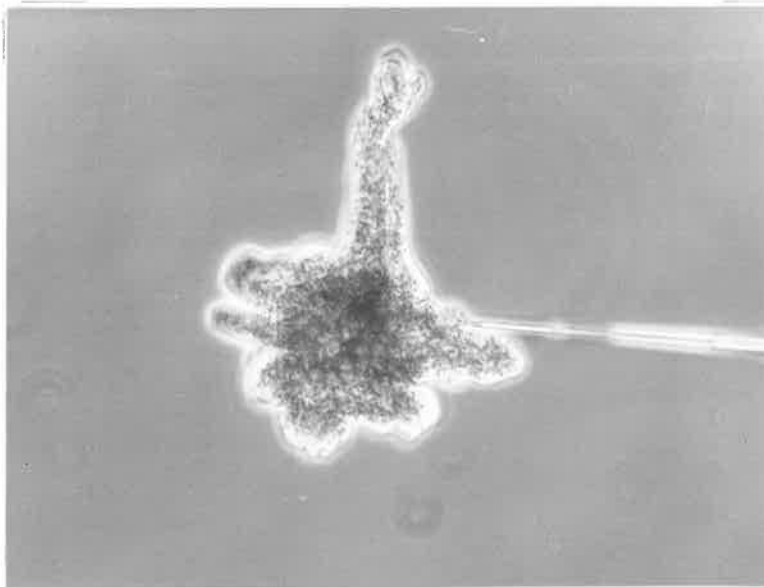
Fig.5b. The amoeba in a contracted state immediately after
        injection.
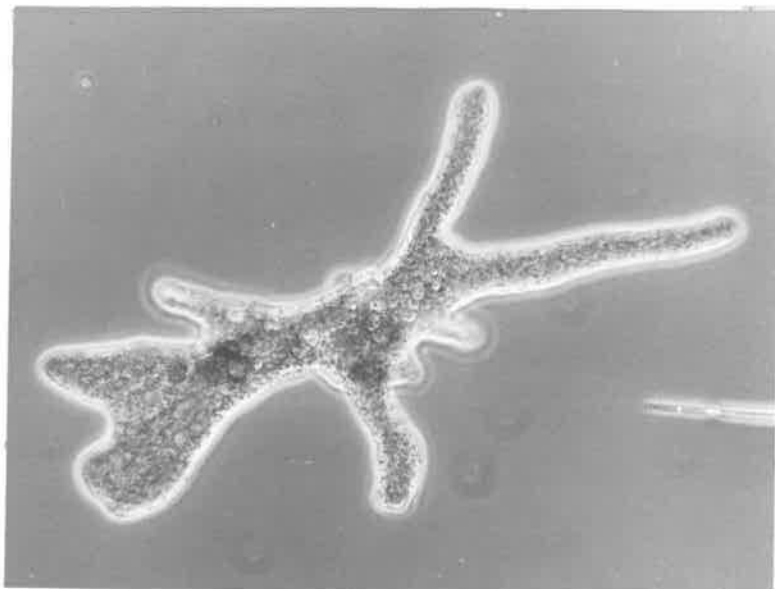
Fig. 5c.   Five minutes after injection, morphology and
activity have returned to normal.
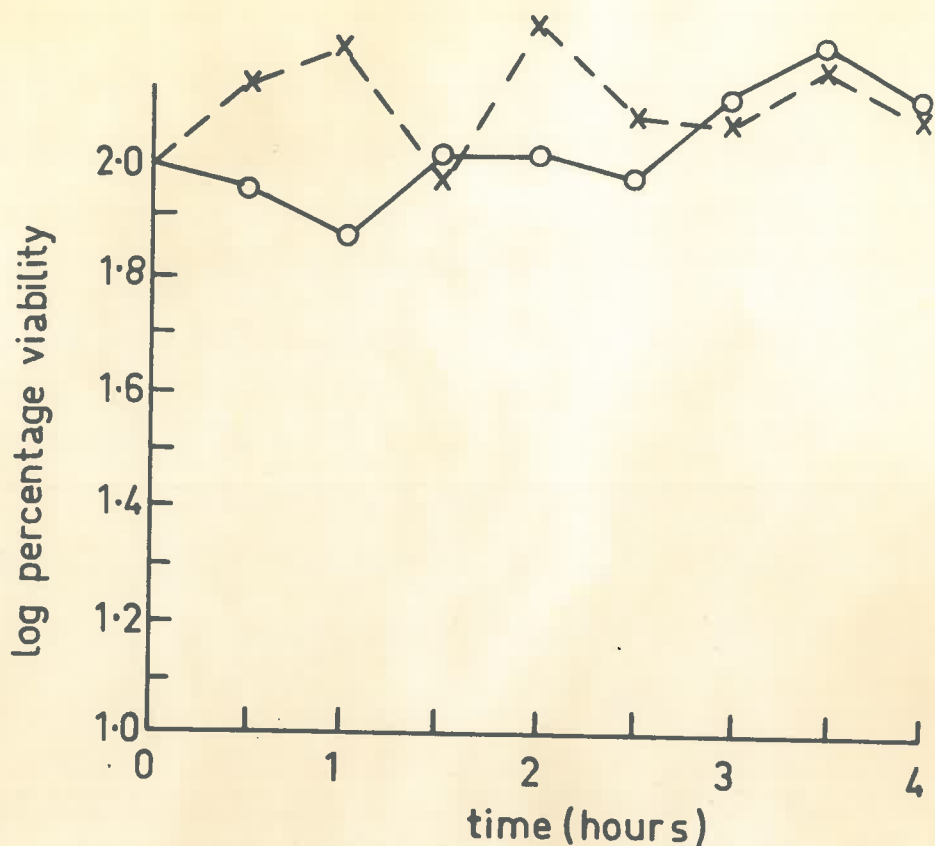
Text-fig. 1. Survival of unopsonised and opsonised
Salmonella enteritidis in Chalkley's medium.

o———————o   Unopsonised.

x—·——·—·—·x   Opsonised with pig serum.

Log. phase S. enteritidis cells used in this experi-
ment were grown in minimal medium (Davis and Mingioli)
and washed in the same medium by centrifugation at
3000 r.p.m. for 10 minutes and finally resuspended in
Chalkley's medium. After dilution in Chalkley's med-
ium to give a final concentration of approximately
2×10^3 organisms per ml., 0.1 ml. volume of this bac-
terial suspension was dispensed in a series of
depression slides each containing 1.5 ml. of Chalkley's
medium. At intervals, the total amount of the fluid
in the depression slide was incorporated into an agar
pour plate for a bacterial count.

might have been voided by the amoebae during the period of
incubation.   A control in which S. enteritidis unopson-
ised and opsonised with pig serum was exposed to Chalkley's
medium for 4 hours at 20° showed that the medium alone did
not have any bactericidal effect (text - fig.1).

Cell sectioning.

Many washed A. proteus cells were placed in Chalk-
ley's medium in a depression slide.   After the cells had
adhered to glass and become elongated, they were severed
under a dissecting microscope into two halves by means of
a small glass rod manipulated by free hand.   Both nuclear
and cytoplasmic halves were washed twice in Chalkley's
medium using a pipette controlled by mouth suction and fin-
ally each half was placed separately in a drop of 3 mm.
diameter on a coverslip in an oil chamber.

Micro-injection of bacteria into the cytoplasmic half of
A. proteus.

Since the cytoplasmic half does not adhere to glass,
a micro-cup was used to hold it in a fixed position.   The
cup was made from a tapering end of a soda glass tubing of
3.5 mm. external diameter using the technique described by
de Fonbrune (1949).   An isthmus was made at a distance
approximately 0.5 mm. from the tip of the cup to counteract
a rapid suction force when the pressure inside the cup was
suddenly released: and if this occurs the cell would be

Figs. 6. Photographs showing stages of micro-injection of bacteria into the cytoplasmic half of an _Amoeba proteus_.

a. A micro-pipette placed next to an enucleate amoeba held within a micro-cup prior to injection.

b. At the time of injection, the content in the micro-pipette was expelled into the cyto-plasmic matrix.

c. Immediately after injection, the enucleate amoeba was in a contracted state.

d. After injection, the enucleate amoeba was released from the micro-cup morphologically in-tact.

a
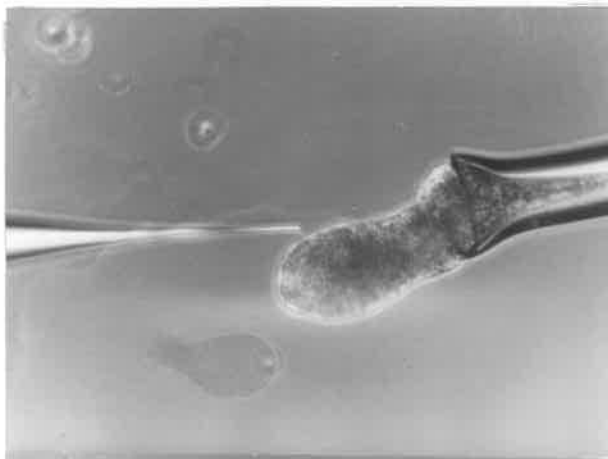
b
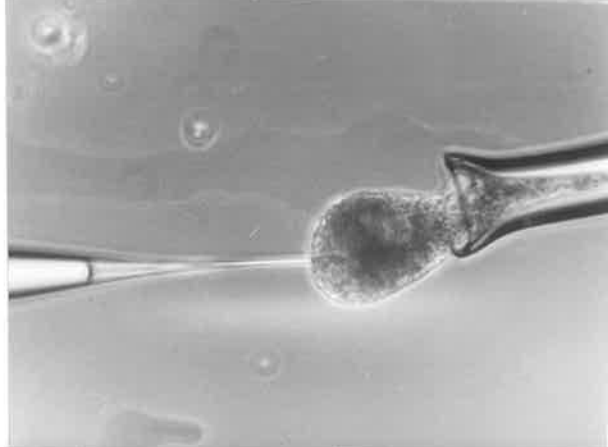
d
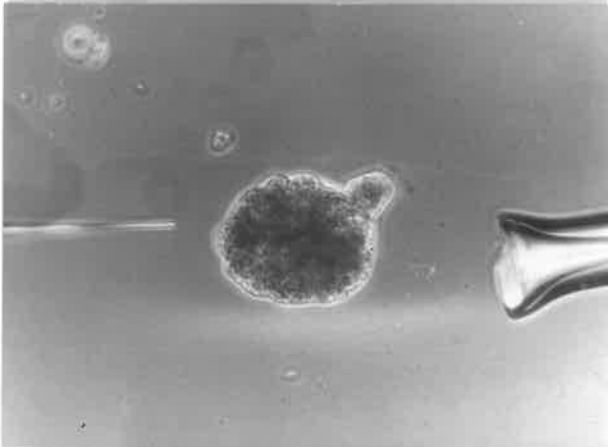
c
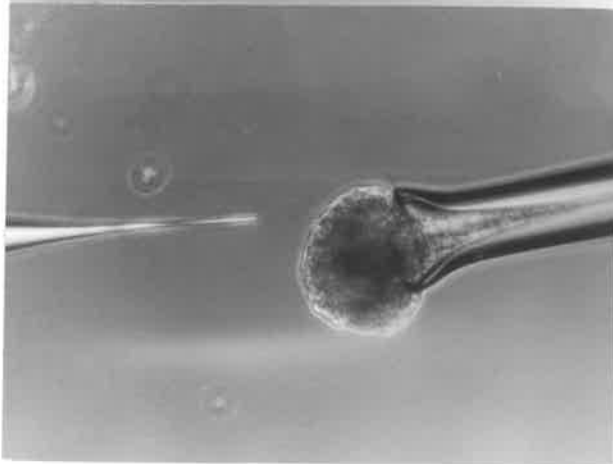
inevitably damaged. A known number of bacteria contained in a micropipette was then introduced into the cells by means of a micro-injection technique as shown in fig.6a, b, c and d.

Cytochemical determination of acid phosphatase.

A washed amoeba, together with Chalkley's medium, was transferred by means of a micropipette controlled by mouth suction on to a rectangular piece of coverslip placed on a wire gauze in a Petri dish containing few ml. of water. Having attached to the glass, the cell on the coverslip was fixed for 15 minutes in cold calcium formal fixative prepared as follows:-

40% formaldehyde previously neutralised with solid $CaCO_3$ and filtered	25 ml.
Glass distilled water	225 ml.
$CaCl_2$	1 gm.

The fixed specimen was then stained for acid phosphatase using a modified Gomori technique similar to that described by Birns (1960). The preparation was incubated at 37^0 for 45 minutes in a 2 inch diameter Petri dish containing Sodium beta glycerophosphate substrate pH 5. Following incubation the specimen was rinsed in water and immersed in 2 per cent. ammonium sulphide for 1 minute, dried, mounted in glycerogel and examined for acid phosphatase staining. It is noteworthy to mention that not very in-

frequently, the cell detached from the coverslip before
the whole process was accomplished.   In this case, the
cell was passed through the entire procedure using a pip-
ette controlled by mouth suction.   Before being treated
with ammonium sulphide, the cell was placed at the upper
part of a coverslip standing at an angle of approximately
60°, and its surface moistened with ammonium sulphide sol-
ution.   By this means, the cell became adherent again to
the glass and could be brought into contact with the re-
agent to allow a complete chemical reaction to occur.

Electron microscopy.

The amoebae were individually fixed with 2 per cent.
Osmium tetroxide in Caufield's (1957) solution at pH 7.5
for 10-45 minutes at 2°.   They were dehydrated through a
graded concentration of alcohol each for 5-10 minutes, and
embedded in a mixture of methyl and butyl methacrylate.
To facilitate locating the amoebae, a 1 mm. hole was drilled
1 mm. along the axis of an empty block of the resin.   The
amoeba was then pipetted into this, together with the em-
bedding mixture.   Trimming was aided by using both trans-
mitted and vertical illumination in a rotating block holder
(Casley-Smith, 1965, personal communication).   Sections
were cut on a Huxley Ultramicrotome (Cambridge Instrument
Corp.), mounted on carbon/collodion films and stained with
Lead citrate (Reynolds, 1963).   They were examined in a

Siemens Elmiskop I.

Phagocytosis experiments.

a) Preparation and washing of amoebae.

In order to free amoebae from excessive numbers of contaminating bacteria and debris, they were washed three times with sterile Chalkley's medium while still adherent to glass culture dishes, the cells were then transferred to siliconised roller tubes placed vertically in a rack. The cells were allowed to sediment to the bottom of the tube and as much as possible of the supernatant fluid was removed and replaced with sterile Chalkley's medium. The amoebae were washed in this manner five times and finally they were collected as a dense suspension containing approximately 5×10^4 cells per ml., 90 per cent. of which were actively motile. The number of amoebae was determined by pipetting 0.01 ml. of the dense cell suspension into 1.5 ml. of Chalkley's medium in a depression slide, the bottom of which was arbitrarily divided with marking ink into a number of squares. The cells were enumerated under a dissecting microscope. Damaged cells and cells which did not adhere satisfactorily to glass were not included in the count.

b) Phagocytosis by amoebae.

Aliquots of the washed amoebae suspension (0.1 ml.) containing approximately 5×10^3 cells, were placed in ser-

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b) Phagocytosis by amoebae.

Aliquots of the washed amoebae suspension (0.1 ml.) containing approximately 5×10^3 cells, were placed in ser-

ies of siliconised glass tubes, diameter 8 mm., each of
which contained 0.3 ml. of 2 per cent. agar in order to
reduce the curvature of the tube.   To each tube 0.02 ml.
of a bacterial suspension in Chalkley's medium was added;
this gave an average inoculum of 5×10^5 salmonellae per
tube.   Preliminary tests on the ability of amoebae to
phagocytose bacteria showed that the uptake of bacteria
was minimal unless the ratio of bacteria to amoebae was
high (in the order of 100 : 1).   After incubation at 20°
for one hour, the suspension was made up to 4 ml. with
fresh medium and transferred to a centrifuge tube where
the protozoa were deposited by centrifugation at 60 g for
3 minutes, after which a sample of the supernatant was
plated out for a bacterial count.   The remainder of the
supernatant was removed as completely as possible and the
amoebae were washed twice more with sterile Chalkley's
medium.   After washing, the cells were re-suspended in
0.4 ml. of fresh medium and transferred to a Leighton tube
having a well measuring 1.2 cm. x 0.75 cm. x 0.1 cm. (fig.7).
A series of 8 or 10 tubes was prepared in this standard
manner, so that the number of amoebae in all the tubes,
and the number of bacteria they had phagocytosed were com-
parable.   At the start of each experiment two tubes were
plated out for a total count of bacteria associated with
amoebae at time zero, thereafter tubes were sampled at var-
ious intervals after incubation at 20°.   Before each samp-
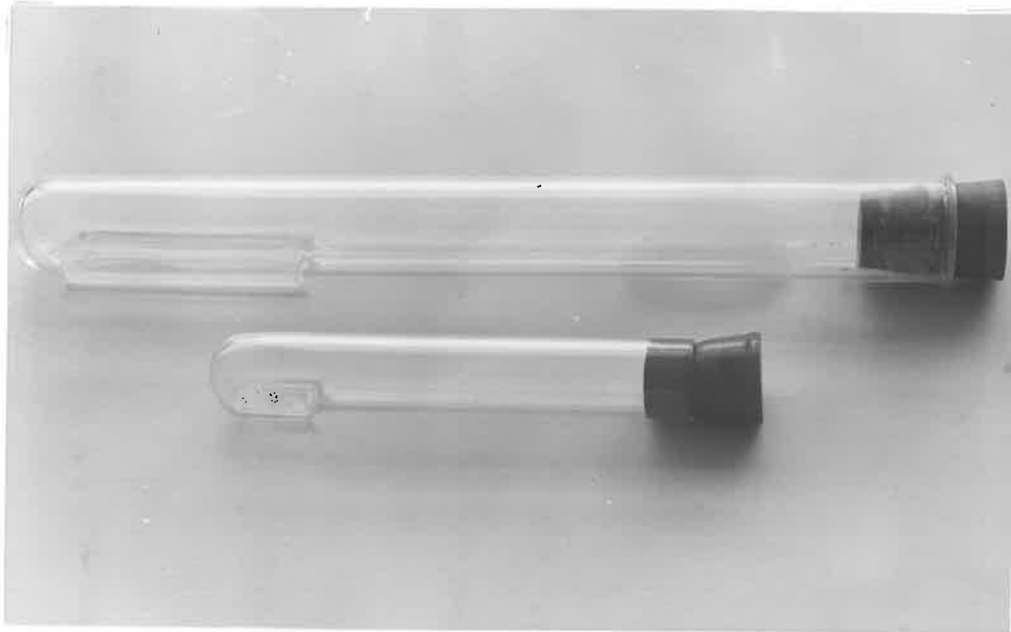
Fig. 7. Photograph showing a small Leighton tube
         having a well measuring 12 x 7.5 x 1 mm.
         and a standard Leighton tube having a
         well measuring 40 x 12 x 2 mm.

ling the amoebae were washed twice in Chalkley's medium
by centrifugation at 60 g for 3 minutes; this precaution
was possibly superfluous since not more than 3 per cent.
of the total number of bacteria associated with amoebae
at time zero could be recovered from the supernatant at
the four-, five- and six hour points. A control was set
up which consisted of a series of tubes containing in 0.4
ml. of Chalkley's medium the same number of amoebae as in
the test system and approximately 5×10^3 bacteria. Since
phagocytosis was negligible when the ratio of bacteria to
amoebae was as low as 1:1, the protozoa in these control
tubes served solely to condition the medium and thus allow
a growth curve to be plotted for extra-cellular bacteria
in the presence of amoebae.

Use of phage P_{22} to lyse extracellular S. typhimurium LT_2.

A phagocytosis experiment was set up in duplicate,
as described earlier in this chapter (page 68), with the
exception that Chalkley's medium used in suspending amoebae
after phagocytosis was supplemented with Calcium salt ac-
cording to the formula given on page 110. The reason for
this modification is also given on page 110.

Forty minutes prior to plating out the amoebae, 0.02
ml. of a phage suspension containing a total of 10^7 PFU was
added to the tube of a pair in order to eliminate extra-
cellular bacteria. Twenty minutes later the protozoa were

washed twice with Chalkley's medium by centrifugation at
60 g for 3 minutes and finally the total volume of the
cell suspension was restored to 0.4 ml. This was followed
by an addition of 0.3 ml. of phage antiserum diluted 1:5
in Chalkley's medium in order to inactivate phage which
remained free in the supernatant and thus prevent them
from interacting with intracellular bacteria to be liber-
ated from amoebae when the cells were disrupted on agar
plates. Phage inactivation was allowed to occur at 20°
for 10 minutes, after which the amoebae were deposited by
centrifugation at 60 g for 3 minutes and finally plated
out for a bacterial count. The tube which did not have
phage added to it was subjected to a similar series of
manipulations; the difference in the numbers of bacteria
recovered from amoebae in this tube and the tube in which
phage had been added would indicate the numbers of extra-
cellular bacteria. Another set of tubes was prepared in
duplicate to measure the rate of growth of extracellular
bacteria and to confirm that these bacteria were suscept-
ible to lysis by phage P_{22} in the presence of amoebae.
The details of this growth curve control have been des-
cribed earlier in this chapter (page 68). At intervals,
0.1 ml. of the appropriately diluted supernatant from one
tube was plated out directly for a bacterial count. To

the other tube, 0.02 ml. of phage suspension (containing 10^7 PFU) was added. After a period of 20 minutes to allow phage adsorption, 0.3 ml. of antiphage serum diluted 1:5 was added, and after an additional 10 minutes at 20° to allow phage neutralisation, 0.1 ml. of the appropriately diluted supernatant was plated out. Prior to employing phage P_{22} in these experiments, it was established that uptake of phage particles by amoebae was negligible, and that the lytic efficiency of the phage suspension was not reduced when the total bacterial numbers in the supernatant of each tube ranged between 5×10^4 and 5×10^2 organisms. It was also ascertained that the rabbit antiphage serum, as used in these experiments, was not toxic for amoebae (see Chapter IV).

The study of intracellular killing of Salmonella enteritidis ingested together with heat killed tetrahymena.

a) Preparation of a mixture of tetrahymena and bacteria.

The ciliates grown in 1 per cent. proteose-peptone solution at 20° were washed twice in Chalkley's medium by centrifugation at 1000 r.p.m. for 3 minutes. The cells were killed by immersing the tube containing them in boiling water for 1 minute. After two more washings in Chalkley's medium, the cell sediment was resuspended in the same

medium and a cell count was made using a haemocytometer.
The ciliate concentration was adjusted to 10^5 cells per
ml. Preliminary test showed that this ciliate suspension
was not bactericidal for S. enteritidis Se 795. To 3 ml.
of this suspension, 0.1 ml. of a washed bacterial suspen-
sion in Chalkley's medium containing 0.1 per cent. glucose
was added. This gave a final bacterial concentration of
approximately 2×10^6 cells per ml.

b) Phagocytosis experiment.

A volume of washed A. proteus (0.5 ml.) in Chalk-
ley's medium containing 6000 cells was dispensed in a
series of 10 standard Leighton tubes, each having a well
measuring 40 x 12 x 2 mm. (fig.7). After the cells had
settled to the bottom of the tube and become adherent to
the glass, 0.5 ml. of the mixture of bacteria and ciliates
was then added. Phagocytosis was allowed to occur at 20°
for 1 hour. It was found that during this period, con-
siderable numbers of ciliates were ingested. After in-
cubation, the amoebae were shaken gently off the glass and
transferred by means of siliconised Pasteur pipettes to a
series of siliconised conical centrifuge tubes. The cells

were washed 3 times in cool sterile Chalkley's medium by
centrifugation at 60 g for 2 minutes at 4°.   Finally the
cells, together with the suspending medium, were trans-
ferred into small Leighton tubes having a well measuring
1.2 cm. x 0.75 cm. x 0.1 cm., and incubated at 20° to
allow for killing of the phagocytosed organisms.   The
subsequent procedure in determining the numbers of bacter-
ia associated with amoebae at various intervals was the
same as that described on page 68.   The control for extra-
cellular multiplication of bacteria was also set up as des-
cribed on page 68.

Experimental methods for soil amoebae studies.
Determination of acceptability of bacteria as food for
  soil amoebae.

        This technique was essentially the same as that
described by Severtzova (1928).   Overnight broth cultures
of various organisms were streaked in the form of a radi-
ating star either on 0.1 per cent. yeast extract agar or
on non-nutrient agar plates (Singh, 1941).   The latter
was made up as follows:-

NaCl	5 gm.
Agar	20 gm.
Glass distilled water	1 litre.

Autoclaved at 15 lbs. per sq. in. at 121° for 10
   minutes.

After incubation at either 30° or 37° for 24 hours, one loop of amoebae suspension in PPG or in physiological saline containing approximately 300 amoebae were inoculated at the centre of the radiating streaks and the plate was incubated at 20° for 8 - 12 days. Acceptability of various bacteria as food for amoebae was compared with that of standard organisms by measuring along the bacterial streaks the clear zone formed as a result of ingestion. As a rule, the standard organism used in the study of food selection by H. astronyxis was Pseudomonas fluorescens and Bacterium 1912 in the study with other species of soil amoebae. The degree of edibility could be expressed arbitrarily as 1 to 4+. The organism which had edibility of 4+ means that the streak of this organism is cleared as fast as that of the standard, and conversely the organism which has edibility of 1+ means that the streak of that organism is cleared at the rate of only 1/4 of that of the standard.

Demonstration of food selection by soil amoebae.

The technique of demonstrating food selection by soil amoebae described by Singh (1941) was adopted. This was in effect very similar to that described above, except that two streaks of bacteria, one of which was a standard organism (see above), were prepared side by side on the same radius of a star dish. This was prepared by first arranging sterile glass capillary tubes of uniform length

and size in a sterile Petri dish in the form of a radiating
star.   Next, warm, sterile 0.1 per cent. yeast extract
agar was poured by means of a sterile Pasteur pipette along
each radius.   Preparation of bacterial streaks, inocula-
tion of amoebae and assessment of the degree of accept-
ability was the same as above.   The difference in the rate
of clearing of bacterial streaks under this condition would
be a good indication for food discrimination.

Demonstration of chemotaxis in soil amoebae.

The protozoa were washed while adherent to the
glass by two changes of sterile 0.06 M phosphate buffer
sodium chloride solution made up as follows:

5M NaCl	8.25 ml.
0.5 M Na$_2$HPO4	8.5 ml.
4M NaH$_2$PO$_4$	1.85 ml.

Glass distilled water added to make 1 litre.

Final pH 6.5.

Autoclaved at 15 lbs. per sq. in. at 121° for
10 minutes.

This medium was chosen for chemotactic study be-
cause in this solution amoebae appeared to be more active
and became adherent to the glass better than in PPG.
Washed amoebae were transferred together with the sus-
pending medium by a micropipette controlled by mouth suc-
tion on to a coverslip in an oil chamber within which the

fluid spread out and formed a drop of approximately 3 mm. in diameter.

Bacteria grown overnight in PPG were washed in physiological saline and the supernatant removed after centrifugation at 3000 r.p.m. for 10 minutes. One drop (0.02 ml.) of the remaining thick residue (containing approximately 10^{10} organisms in 0.1 ml.) was mixed in a depression slide with the same volume of warm 2.0 per cent. neutral agar. After solidification, the agar was broken into small pieces and a clump of bacteria in agar was selected, sucked into a micropipette controlled by mouth suction, washed three times by successive rinse in sterile 0.06 μ phosphate buffered NaCl solution and finally transferred into the drop of amoebae.

Migration of amoebae towards the clump of bacteria was recorded for a period of 30 minutes by means of a dark ground tracing technique using Ilford Micro-neg Pan film and an Olympus camera. This method was essentially similar to that described by Harris (1953).

Demonstration of selective phagocytosis of edible bacteria in colonies of mixed cultures.

Ps. fluorescens and two inedible bacteria, viz. S. enteritidis streptomycin resistant strain and E. coli 812 were used. It was found in the preliminary test that there was no distinguishable difference in acceptability of

streptomycin resistant S. enteritidis and the parental
strain as measured by Singh's method of study of food sel-
ection. All the organisms used in the test had been
passaged through semi-solid medium (Lederberg, 1956) twice
before use so that the bacterial cells were actively motile
before inoculation on agar.

The experimental procedures used for each of the
two inedible organisms differed slightly and, for the sake
of convenience, a full description will be given for the
method in which S. enteritidis was used. This organism
was grown in broth at 37° overnight and the bacterial con-
centration was adjusted to be approximately 2×10^8 cells
per ml. A suspension of Ps. fluorescens grown overnight
in broth at 30° was similarly prepared. Equal volumes of
these two bacterial suspensions were mixed and a standard
loopful of the mixture was inoculated on to dry 0.1% yeast
extract agar prepared on a 2 x 2 inch coverslip, after
which it was placed on top of a depression slide contain-
ing a drop of sterile distilled water and finally sealed
with petrolatum jelly. A series of 10 coverslips was
thus prepared. After incubation at 30° for 24 hours, 2
coverslips were taken out, each bacterial colony was remov-
ed, together with an underlying agar block, and suspended
in 5 ml. of physiological saline. The colony was scraped
off from agar with a loop followed by vigorous shaking so
that a homogenous suspension of bacteria was obtained.

This suspension was then appropriately diluted and plated
in duplicates on both nutrient agar and streptomycin agar
containing 50 micrograms of streptomycin per ml. After
incubation at 30° overnight, colony counts were made. By
this means it was possible to determine in a single mixed
colony the absolute number of each organism and the ratio
of them. The other coverslip preparations were divided
into two groups of 4, one to be used in the test series
and the other in the control. In the test series, each
colony was inoculated at the centre with a loopful of wash-
ed heavy suspension of H. astronyxis in physiological sal-
ine, but in the control series it was inoculated with a
loopful of physiological saline only. The preparations
were incubated further at 20° for either 48 or 96 hours.
During this period, it was found that amoebae had multiplied
and their growth covered the entire surface of the bacter-
ial colonies. At each time interval, duplicate prepara-
tions from each series were taken out and those from the
control series were treated as described earlier. In the
test series a similar procedure was carried out, but an
additional step was taken to measure the numbers of bacter-
ia associated with cells. This was done by washing the
cells in physiological saline by centrifugation at room
temperature at 1000 r.p.m. for 3 minutes three times, after
which they were resuspended in the same solution to give a
final volume of 10 ml. The cells were disrupted by son-

ication using MSE ultrasonic disintegrator and the cell suspension was appropriately diluted and plated out on both nutrient and streptomycin agar plates for a bacterial count.

When E. coli 812 was used in the test, similar procedures were carried out, except that plating was all done on duplicate series of plates, one of which was incubated at 30° to allow growth of both Ps. fluorescens and E. coli 812, whereas the other was incubated at 37° which allowed for the growth of the latter only. (It should be emphasised that Ps. fluorescens did not grow at 37°).

Phagocytosis of bacteria by soil amoebae.

The technique used was a modification of type I experiment described by Rowley and Whitby (1959).

Amoebae were washed twice with PPG while they were adherent to the culture vessel, after which the cells were pipetted off and transferred into a siliconised conical centrifuge (15 ml. capacity). The cells were washed once more in PPG by centrifugation at 1000 r.p.m. for 3 minutes and the cell sediment was resuspended in the same medium. After counting with haemocytometer, the cells were appropriately diluted to give a final concentration of 4×10^5 cells per ml. A volume of this cell suspension (1 ml.) was dispensed in a series of 4 siliconised 75 x 12 mm. tubes, and 0.1 ml. of washed bacterial suspension in PPG was added to each of them so that the ratio of bacteria and amoebae was

approximately 3:1.   A control of bacterial growth in PPG
alone was also set up by inoculating the same amount of
bacterial suspension into a series of tubes, each contain-
ing 1 ml. of the medium only.   All the tubes were rotated
end to end gently at room temperature in a drum moving at
the rate of 1 revolution per minute.   At 30 minute inter-
vals, one tube was taken from the test series and the super-
natant separated from the cells by centrifugation at 4° at
1000 r.p.m. for 2 minutes, after which it was appropriately
diluted in physiological saline and plated on nutrient agar
for a bacterial count.   The cells were washed further in
physiological saline by centrifugation at 1000 r.p.m. at 4°
for 2 minutes three times and the sediment was diluted with
the same medium to give the final volume of 10 ml. Follow-
ing disruption by sonication for 10 seconds using MSE ultra-
sonic disintegrator, 0.1 ml. of disrupted cell suspension
was plated out for a bacterial count.   At the same time
the supernatant count in the control series was similarly
determined.

The number of bacteria phagocytosed at a given time
could be calculated on the basis of the difference between
the numbers of bacteria recovered at the same time from the
control and the test supernate.   This would give only a
relative value, since some bacteria in the 'test' tube were
being phagocytosed, i.e. they were adherent to the surface
of amoebae.   The study in Chapter VI showed that a consider-

able period of time (approximately 4 minutes) was required for a complete engulfment to occur after bacteria had become adherent to the amoeban surface. Hence the actual numbers of bacteria ingested at a particular time would therefore be smaller than those implied by this calculation. If, however, a comparison has to be made between the rate of phagocytosis and killing of different organisms by amoebae using the same technique, the result obtained would be relatively valid.

The percentage phagocytosis that occurred at any time interval was given by the formula:-

$$\frac{A - B}{A} \times 100$$ where A = the supernatant count in the control tube at time t_x

and B = the supernatant count in the 'test' tube also at time t_x.

The percentage survival of phagocytosed bacteria inside amoebae was given by the formula:-

$$\frac{C}{A - B} \times 100$$ where C = the number of bacteria associated with amoebae at time t_x.

Killing of a bacterium by a single amoeba.

One motile bacterium from an overnight broth culture was brought by means of a micropipette into contact with an amoeba adhering to the glass coverslip in a drop of PPG in an oil chamber. Soon after the bacterium became adherent to the cell surface the inevitable engulfment by means of a food cup ensued. This process could be observ-

ed under phase contrast at 1000 times magnification. At
time intervals after engulfment, the amoeba was removed
from the microdrop, washed once in PPG and transferred
together with the suspending medium on to an agar plate
where it was crushed and spread by a glass rod for a bac-
terial count.

It is important in this type of study to be absol-
utely certain that each motile organism used in the test
was capable of forming a colony when it was plated. It
was essential therefore to make a correlation between the
bacterial count under phase and the colony count when this
was plated on nutrient agar. The technique used was almost
identical as that described in the work for <u>A. proteus</u>
(page 62), i.e. the bacteria were counted in a microdrop
of broth of approximately 100 μ in diameter: this was
transferred by means of a micropipette into a bigger drop
of broth of 3 mm. diameter which was again removed and
transferred into 1.5 ml. of broth in a depression slide be-
fore being incorporated into an agar pour plate for a bac-
terial count.

<u>Determination of the growth rates of amoebae feeding on
colonies of different bacteria.</u>

One small drop of an overnight broth culture of
bacteria was inoculated by means of a micropipette con-
trolled by mouth suction on to the surface of either 0.1

per cent. yeast extract agar or sodium chloride agar
(Singh, 1941) prepared on a glass coverslip of 2 x 2 in-
ches square.   This coverslip was placed on top of a
depression slide containing a drop of sterile distilled
water and sealed with petrolatum jelly.   Each preparation
contained 6 - 8 drops of different bacterial cultures.
After incubation at 30° or 37° for 24 hours, two amoebae
were inoculated on to each bacterial circle and incubated
further at 20°.   The number of amoebae in each bacterial
colony was counted daily.   By this means it was possible
to compare the growth rate of amoebae feeding on different
organisms under the same condition.

CHAPTER III.

FACTORS AFFECTING THE ABILITY OF AMOEBA PROTEUS TO KILL SALMONELLAE INTRODUCED DIRECTLY INTO THE CYTOPLASM BY MEANS OF A MICRO-INJECTION TECHNIQUE.

The enhancement of phagocytosis by serum opsonic factors is generally accepted (Wright and Douglas, 1903; Mudd et al., 1934; Rowley, 1962). However, there is much speculation as to whether the function of opsonin ceases once phagocytosis has been accomplished, or whether these serum factors continue to exert an influence by promoting intracellular killing mechanisms (Miya and Marcus, 1961). The question can only be answered by an experiment in which bacteria are introduced into phagocytic cells without prior opsonisation.

Jenkin (1963) has shown that mouse peritoneal macrophages could be induced to phagocytose Salmonella typhimurium in the absence of opsonins for the bacteria if the phage P_{22} was attached to the bacterial surface, thus allowing ingestion of the phage-bacterium complex in the presence of opsonins for the phage alone. By this means it was demonstrated that intracellular killing was poor if opsonins specific for the bacteria were lacking.

The main purpose of the work described in this chapter was to study this aspect of opsonic function using another approach whereby bacteria were introduced directly

into the cell by means of a micro-injection technique. With this advent, it is possible to study intracellular survival of a wide range of micro-organisms within phagocytic cells of various types.

Amoeba proteus was chosen as the test phagocyte, because this protozoon is relatively large compared with the vertebrate macrophage and thus suitable for micro-injection. If it is tentatively assumed that all phagocytic cells, mammalian or otherwise, share common bactericidal mechanisms, any factors which act to potentiate these processes in one situation should have similar actions in another. To test this idea, experiments were designed to study the ability of A. proteus to kill S. enteritidis both before and after opsonisation of the bacteria with serum. It is realised, of course, that opsonins of this type are not a feature of the natural environment of free living amoebae.

In this study two types of serum opsonins were used, a rabbit antiserum as a source of specific antibody and pig serum as a source of natural occurring antibody (Jenkin and Rowley, 1959). This work was also extended to include the influence of other factors, e.g. complement and the nucleus on intracellular bactericidal mechanisms. The exact location of the injected organisms was followed by an electron microscopic study.

Results.

1.   Study on the intracellular survival of S. enteritidis
in A. proteus and the influence of serum antibodies on intra-
cellular killing.

Salmonella enteritidis used in this study was in
logarithmic phase.   A known number of these bacteria
counted under phase contrast was introduced into the cyto-
plasm of A. proteus by means of a micro-injection technique.
After injection, the amoeba was washed in Chalkley's medium
and maintained in the same medium at 20° for a variable
period of time, after which it was washed and crushed on
the surface of nutrient agar for a bacterial count (Chapter
II, p.60).   The viability of bacteria was determined at
the end of each experiment and a good correlation between
the number of bacteria counted under phase contrast and the
number of the colonies formed was always obtained.   It is
reasonable therefore to regard that every bacterium used
in micro-injection was viable.

The percentage survival of S. enteritidis after
varying intervals of time within amoebae is shown in text-
fig.2.   It is clear that amoebae were able to kill the test
organisms, but only at a relatively slow rate since the
half-life of bacteria within the cells was approximately
5 hours.   It is also evident that pre-treatment of S. enter-
itidis with serum antibodies did not alter the rate of bac-
terial killing.   Data on which these results are based are
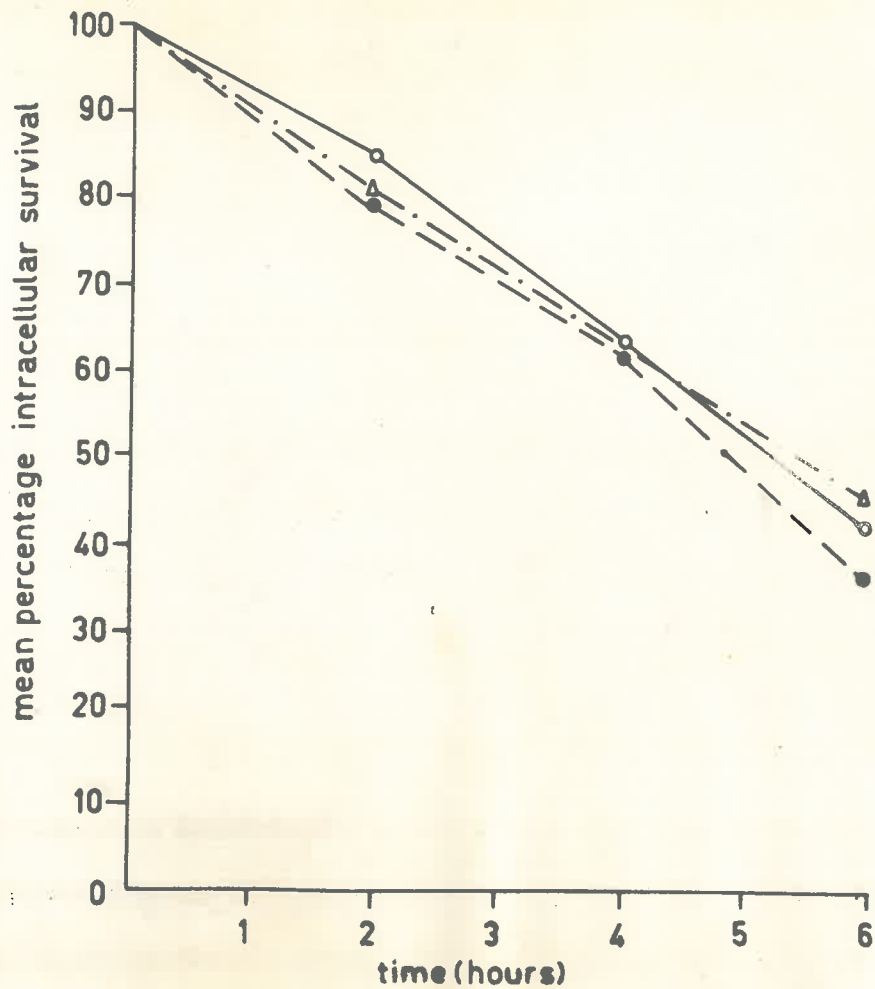
Text-fig. 2. Recovery of _Salmonella enteri-_
tidis after injection into the cytoplsam of
Amoeba proteus.

	Pre-treatment of bacteria
o———o	Unopsonised.
•– – –•	Opsonised with specific antiserum.
Δ–·—·—·Δ	Opsonised with pig serum.

TABLE IIa.

Recovery of S. enteritidis 2 hours after injection into the cytoplasm of A. proteus.

Pretreatment of bacteria	Unopsonised			Opsonised with specific antiserum			Opsonised with pig serum		
Amoeba No.	No.bac. inj.	No.bac. recovd.	% survival	No.bac. inj.	No.bac. recovd.	% survival	No.bac. inj.	No.bac. recovd.	% survival
1	35	31	88.6	30	18	60	48	49	102.1
2	27	23	85.2	72	78	108.3	30	32	106.6
3	32	26	81.2	32	28	87.5	44	38	86.4
4	27	23	85.2	67	59	88.1	37	26	70.3
5	40	38	95.0	38	28	73.7	40	27	67.5
6	40	34	85.0	38	32	84.2	30	24	80.0
7	37	30	81.1	47	19	40.4	46	34	73.9
8	41	38	92.6	54	35	64.8	49	41	83.7
9	34	26	76.5	26	26	100.0	37	30	63.8
10	42	35	83.3	52	48	92.3	36	25	69.4
Average	35.5	30.4	85.4	45.6	37.1	79.9	40.7	32.6	80.4
S.D.	5.5	5.7	5.5	12.7	19.1	20.1	5.7	7.9	14.7

TABLE IIb.

Recovery of S. enteritidis 4 hours after injection into the cytoplasm of A. proteus.

Pre-treat-ment of bacteria	Unopsonised			Opsonised with specific antiserum		
Amoeba No.	No. bact. inj.	No. bact. recovd.	% sur-vival	No. bact. inj.	No. bact. recovd.	% sur-vival
1	46	21	45.6	52	32	61.5
2	47	22	46.8	45	38	84.4
3	46	28	60.8	42	18	42.8
4	47	32	68.1	46	32	69.5
5	46	41	89.1	43	26	60.4
6	50	38	76.0	45	23	51.1
7	35	28	80.0	47	27	57.4
8	45	28	62.2	46	35	76.1
9	58	22	37.9	47	27	57.4
10	47	38	80.8	43	26	60.4
Average	47.3	29.8	63.7	46.4	28.4	62.0
S.D.	5.1	7.3	17.0	3.1	5.8	11.9

TABLE IIc.

Recovery of S. enteritidis 6 hours after injection into the cytoplasm of A. proteus.

Pretreatment of bacteria	Unopsonised			Opsonised with specific antiserum			Opsonised with pig serum		
Amoeba No.	No.bac. inj.	No.bac. recovd.	% survival	No.bac. inj.	No.bac. recovd.	% survival	No.bac. inj.	No.bac. recovd.	% survival
1	51	22	43.1	45	22	48.8	38	19	50.0
2	45	21	46.6	43	12	27.9	40	20	50.0
3	49	18	36.7	47	14	29.8	44	18	40.9
4	51	10	19.7	46	14	30.4	42	23	54.7
5	45	21	46.6	46	12	26.1	61	30	49.2
6	48	24	50.0	42	24	57.1	49	19	38.8
7	41	13	31.7	46	14	30.4	38	19	50.0
8	45	23	51.1	43	15	34.9	41	16	39.0
9	45	22	48.8	44	13	29.5	46	22	48.3
10	44	23	52.2	52	16	50.0	46	19	41.3
Average	46.4	19.7	42.7	45.4	16.6	36.4	44.1	20.5	46.2
S.D.	3.2	4.4	9.2	2.8	5.3	9.4	6.8	3.8	5.1

set out in Table IIa, b and c.   From these data it can
be seen that the rate at which individual amoebae killed
bacteria varied from one cell to another.   The majority
of reported quantitative studies on the effects of opsonins
in relation to the ability of mammalian phagocytes to in-
gest and kill bacteria have been done using populations
of phagocytic cells.   Data derived from such experiments
are, therefore, an expression of the functional capacity
of the average cell.   If useful comparisons are to be made
between these data and those derived from single cell stud-
ies, it is necessary to consider the mean of the values ob-
tained from a series of single cell experiments.

The washing procedures, used to free amoebae from
contaminating microflora present in the culture dish, were
judged to be efficient since the majority of amoebae used
in these experiments did not contain micro-organisms other
than those that had been injected into them.   However, it
was possible to recover a small number of contaminating
bacteria from some of the crushed amoebae;   these micro-
organisms did not confuse the assessment of salmonellae
survival, because their colonial morphology was usually
quite distinct.   If there was any doubt as to their origin,
colonies were checked individually for their ability to ag-
glutinate with specific antiserum for S. enteritidis Se 795.

In the course of this study, it was found that no
salmonella could be recovered from Chalkley's medium in

which the amoeba was maintained during the period of observation. In view of the fact that the medium alone was not bactericidal for the test organism (text - fig.1), it was concluded that no bacteria were voided in viable forms during this period.

2. Influence of complement on intracellular killing of S. enteritidis by A. proteus.

It is generally accepted that complement is a necessary requirement for the killing of bacteria by serum antibody in vitro (Maaløe, 1946; Osler, 1961). The intermediate steps of the bactericidal reaction were reported by Rother and co-workers (1964) to follow the same sequences as those in the haemolytic system. Recent studies of Miya and Marcus (1961), Wu and Marcus (1964), showed that complement also potentiated the enhanced effect of serum antibody in the digestion of P^{32} labelled Histoplasma capsulatum by normal and immune mouse peritoneal macrophages as measured by the release of labelled materials in the supernatant. Furthermore, Inoue et al. (1959) showed that in the presence of antibody, complement and Mg ions, E. coli was converted to a spheroplast by lysozyme. In view of the fact that spheroplast was not formed when lysozyme was lacking, these authors suggested that antibody and complement acted on the bacterial cell wall so as to uncover the substrate for lysozyme action.

As serum opsonins alone failed to enhance the intra-

cellular killing of S. enteritidis by A. proteus, it was
thought desirable to study whether complement and serum
opsonins exerted any influence on the intracellular bac-
tericidal mechanisms of these protozoa.

Salmonella enteritidis cells were opsonised with
specific antiserum in the presence of rabbit complement
according to the method described in Chapter II, p.59.
This treatment did not change the viability of bacteria
since there was a good correlation between the numbers of
opsonised bacteria counted under phase contrast and the
colony counts when these were plated on nutrient agar (Table
III). The percentage intracellular survival of these organ-
isms in A. proteus at 2 and 6 hours after injection is shown
in text - fig.3. Data on which these results are based are
given in Table IVa. It is clear that S. enteritidis opson-
ised at 30° with specific antibody plus fresh rabbit serum
was killed at a significantly faster rate than when organ-
isms were treated with serum opsonins alone (see text - fig.
2 for comparison). It is conceivable that this enhanced
killing was due to factors in the fresh rabbit serum and
these may be complement, since no definite increase in bac-
tericidal effectiveness was demonstrated when S. enteritidis
used in the test was opsonised with specific antibody in
the presence of fresh rabbit serum at 4° for 20° (Table IVb,
text - fig.3). (Under this condition, it is generally ac-
cepted that complement fixes poorly on to the bacterial

TABLE III.

Correlation between the number of S. enteritidis cells counted under phase contrast with the number of colonies resulting when these cells were plated out on nutrient agar. The cells were opsonised with specific antiserum in the amount equivalent to 2/3 of a minimal agglutinating dose plus complement from fresh rabbit serum.

	Time of exposure to the injection medium (minutes)							
	10	30	45	60	70	95	105	120
Bacterial cell count under phase	42	37	36	36	31	35	62	53
Colony count on nutrient agar	38	34	32	38	32	33	55	51
Percentage viability of bacteria	90.5	91.9	88.9	105.6	103.2	94.3	88.7	96.2
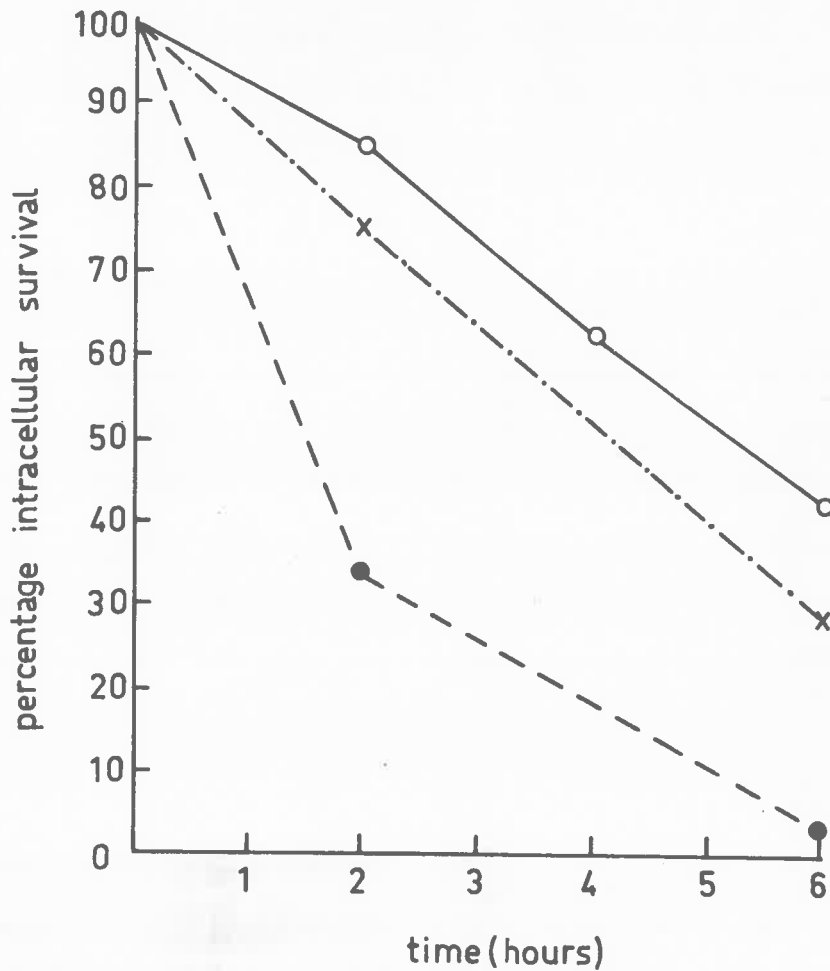
Text-fig. 3. Recovery of **Salmonella enteri-
tidis** opsonised with specific antiserum in the
presence of rabbit complement after injection into
the cytoplasm of **Amoeba proteus**.

	Pre-treatment of bacteria
o————o	Unopsonised.
•— — — •	Opsonised at 20° with speci-fic antiserum + complement.
x—•——•——•x	Opsonised at 4° with speci-fic antiserum + complement.

TABLE IVa.

Recovery of S. enteritidis opsonised at 30° with specific antiserum plus fresh rabbit serum at different time intervals after injection into the cytoplasm of A. proteus.

Amoeba number	Recovery at 2 hrs. after injection			Recovery at 6 hrs. after injection		
	No. bac. inj.	No. bac. recovd.	% survival	No. bac. inj.	No. bac. recovd.	% survival
1	42	6	14.3	46	0	0
2	46	4	8.7	52	1	1.9
3	57	21	36.8	40	1	2.5
4	38	6	15.8	41	1	2.4
5	51	12	23.5	37	5	13.5
6	45	21	46.6	43	5	11.6
7	55	37	67.2	46	0	0
8	56	22	39.2	47	4	8.5
9	45	12	26.6	44	1	2.3
10	52	28	53.8	43	1	2.3
11	-	-	-	51	0	0
12	-	-	-	55	0	0
13	-	-	-	49	0	0
	Average		33.3	Average		3.5
	S. D.		18.0	S.D.		5.6

TABLE IVb.

Recovery of S. enteritidis opsonised at 4° with specific antiserum plus complement at different time intervals after injection into the cytoplasm of A. proteus.

Time of recovery	2 hours			6 hours		
Amoeba number	No.bac. inj.	No.bac. recovd.	% survival	No.bac. inj.	No.bac. recovd.	% survival
1	52	44	84.6	51	9	17.6
2	56	29	51.8	53	13	24.5
3	55	52	94.5	51	14	27.5
4	47	37	78.7	46	17	36.9
5	49	17	34.7	48	24	50.0
6	55	32	58.2	49	14	28.6
7	50	37	74.0	45	9	20.0
8	56	50	89.3	47	26	55.3
9	54	47	87.0	51	9	17.6
10	45	33	73.3	50	10	20.0
11	51	49	96.1	52	14	26.9
12	47	41	87.2	46	8	17.4
13	48	35	72.9	51	15	29.4
14	-	-	-	54	18	33.3
		Average	75.6		Average	28.9
		S.D.	17.9		S.D.	11.8

cells).

3. The influence of the nucleus on intracellular killing.

The nucleus is generally regarded as the controlling centre of the cellular machinery. Comandon and de Fonbrune (1939a) showed that removal of the nucleus from Amoeba sphaeronucleus resulted in an immediate cessation of cellular co-ordinated movement and the cell became a globular mass which did not attach to the substratum. If, however, a homologous nucleus was reintroduced into the amoeba enucleated two days previously, normal function was restored within 2-4 minutes (Comandon and de Fonbrune, 1939b). Clark (1943) reported that an ingested rotifer remained alive in the cytoplasmic half of A. proteus for 2 days, although normally it would have died in 1-3 hours. In the cytoplasmic half of A. proteus, certain enzymes such as di-
/ acid
peptidase, esterase and phosphatase fell remarkably, especially after the fifth day, whereas others such as protease, aldolase and ATP-ase remained practically unchanged (Brachet, 1961). Goldstein and Plaut (1955) presented evidence demonstrating the nuclear secretion of cytoplasmic RNA in A. proteus. Since RNA metabolism and protein synthesis are closely linked (Goldstein, 1958), it is reasonable to assume that certain enzymes in the cytoplasmic half of an amoeba would be depleted resulting in a reduced ability to kill bacteria and degrade their substrates.

The immediate and delayed influence of the nucleus on intracellular bactericidal mechanisms was studied by injecting a known number of S. enteritidis into the nuclear and the cytoplasmic halves of the same amoeba which had been severed 2 or 24 hours previously. Since the cytoplasmic half does not feed, both halves to be used 24 hours after cutting were maintained in sterile Chalkley's medium free from food organisms, so that their nutritional state would be comparable. Introduction of bacteria into the cytoplasmic and nuclear portions of the cell was done using the technique described in Chapter II (p.65).

Unopsonised S. enteritidis from both cytoplasmic and nuclear halves of A. proteus were recovered 6 hours after injection. The choice of this time was based on the previous finding (Chapter III, text - fig.2), which showed that at this period an appreciable number of bacteria were killed. Preliminary experiments showed that if the recovery was delayed up to 24 hours, the cytoplasmic half would become very fragile and occasionally they were burst.

The results of the intracellular survival of injected S. enteritidis in cytoplasmic and nuclear halves of A. proteus at 20° are shown in text - fig.4, and in Table Va, b. It is clear that within 2 hours after cutting, both cytoplasmic and nuclear halves were equally effective in killing the injected organisms, but at 24 hours after cutting, the nuclear half was more capable in exerting its
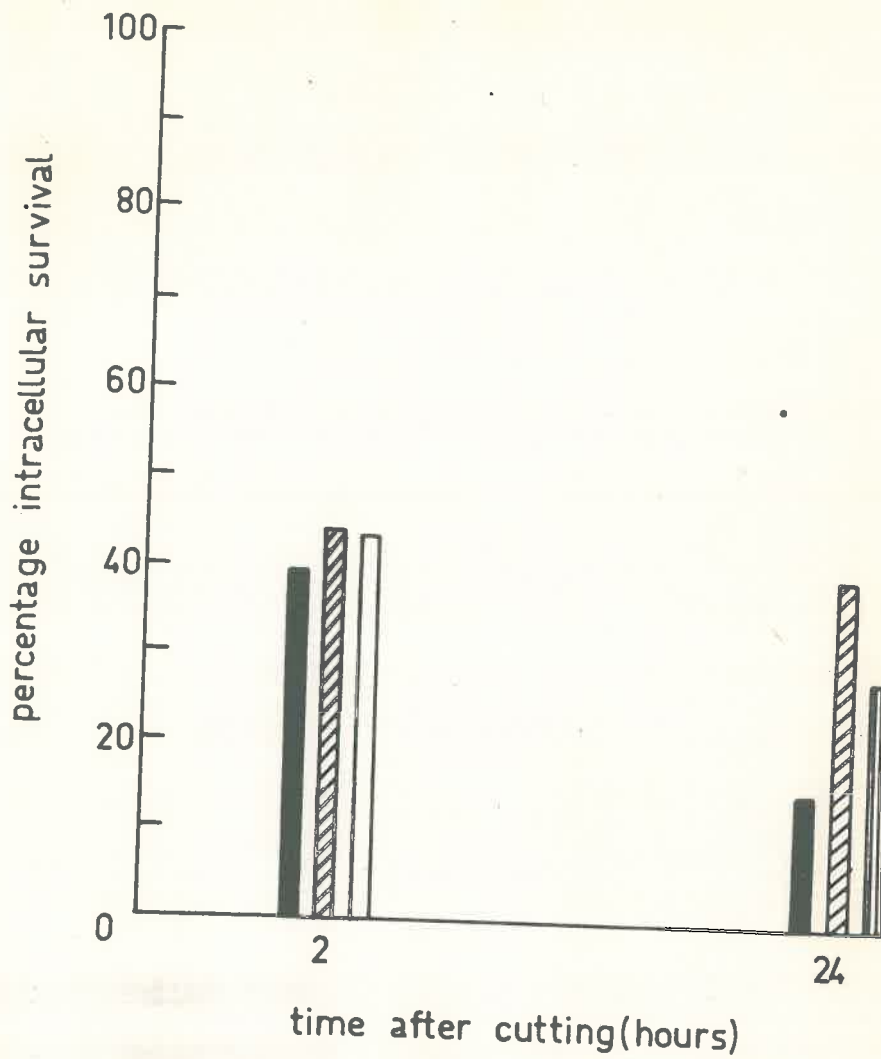
Text-fig. 4. Recovery of <u>Salmonella enteri-</u>
<u>tidis</u> six hours after injection into the cytoplasm
of the whole amoebae, the nuclear halves and the
cytoplasmic halves at 2 and 24 hours after cutting.

Nuclear halves.

Cytoplasmic halves.

Non-starved whole amoebae.

Whole amoebae starved for
24 hours.

TABLE Va.

Recovery of S. enteritidis 6 hours after injection into nuclear and cytoplasmic halves of the same A. proteus within 2 hours after cutting.

Amoeba number	Nuclear halves			Cytoplasmic halves		
	No.bac. inj.	No.bac. recovd.	% survival	No.bac. inj.	No.bac. recovd.	% survival
1	38	10	26.3	27	11	40.7
2	34	11	32.3	35	19	54.3
3	16	4	25.0	39	12	30.8
4	38	29	76.3	37	12	32.4
5	37	8	21.6	33	9	27.3
6	36	17	47.2	36	26	72.2
7	38	22	57.7	34	9	26.5
8	33	12	36.4	34	20	58.8
9	38	9	23.7	35	12	34.3
10	33	16	47.8	36	19	52.8
	Average		39.4	Average		43.0
	S.D.		17.4	S.D.		15.5

TABLE VII.

Recovery of S. enteritidis 6 hours after injection into nuclear and cytoplasmic halves of the same A. proteus severed 24 hours previously.

Amoeba number	Nuclear halves			Cytoplasmic halves		
	No. bac. inj.	No. bac. recovd.	% survival	No. bac. inj.	No. bac. recovd.	% survival
1	36	1	2.8	35	16	45.7
2	33	0	0	33	14	42.4
3	39	8	20.5	37	10	27.0
4	38	2	5.3	21	8	38.1
5	34	11	32.3	35	14	40.0
6	38	1	2.6	27	11	40.7
7	37	4	10.8	37	15	40.5
8	34	10	29.4	34	11	32.5
9	29	3	10.3	28	9	32.1
10	38	7	18.5	32	13	40.6
11	38	8	21.1	36	16	44.4
12	37	11	29.7	38	16	42.1
	Average		15.3	Average		38.8
	S.D.		11.5	S.D.		5.6

TABLE VI.

Recovery of S. enteritidis 6 hours after injection into whole amoebae starved for 24 hours.

Amoeba No.	No. bacteria injected	No. bacteria recovered	% intracellular survival
1	45	21	46.7
2	45	15	33.3
3	45	10	22.2
4	46	20	43.5
5	40	9	22.5
6	47	6	12.8
7	48	9	18.8
8	44	9	20.5
		Average	27.5
		S.D.	10.7

bactericidal effects.   Of particular interest is the find-
ing that killing in the nuclear half was more efficient than
that in the whole amoeba.   Since the cytoplasmic and the
nuclear halves were not fed during the 24 hour period after
cutting, it was thought that the enhanced killing in the
nuclear half might be due to starvation.   In order to check
this possibility, an experiment was done whereby S. enter-
itidis was injected into the whole amoeba starved for 24
hours.   The result is shown in text- fig.5, and in Table
VI.

     It can be seen that the intracellular survival of
S. enteritidis in starved whole amoeba was considerably re-
duced (Table VI), but this was still significantly longer
than that in the nuclear half.   It is considered that the
nuclear half 24 hours after cutting is likely to be in the
state of cellular regeneration in which the whole cell is
geared to develop increasing metabolic activities.   If
this is the case, one would expect an increase in some of
intracellular metabolic enzymes.   Acid phosphatase, for
instance, had been reported to decline in the enucleate half,
the reduction became pronounced after day 5 (Brachet, 1955).
It was thought desirable, therefore, to determine the level
of this enzyme using a cytochemical technique.

     4. Cytochemical demonstration of acid phosphatase in
nuclear and cytoplasmic halves.

   The Amoebae proteus used in this experiment were

Figs. 8. Photographs of the nuclear half and the cytoplasmic half of an _Amoeba proteus_ stained for acid phosphatase 36 hours after cutting.

a.   The nuclear half.

b.   The cytoplasmic half.

Fig. 9. Photograph showing typical acid phosphatase staining granules in an _Amoeba proteus_ immediately after injection with Chalkley's medium containing 0.5% gelatin.
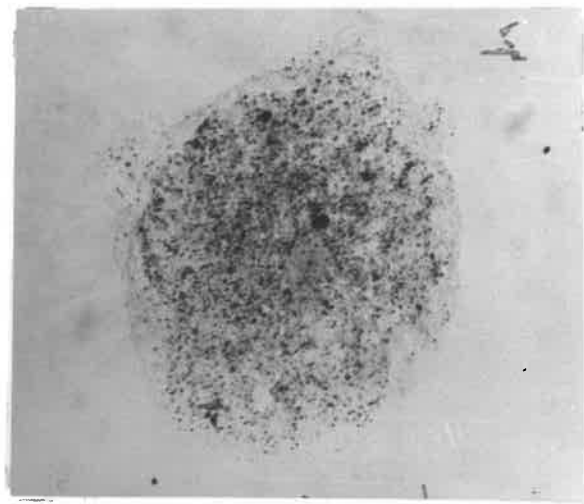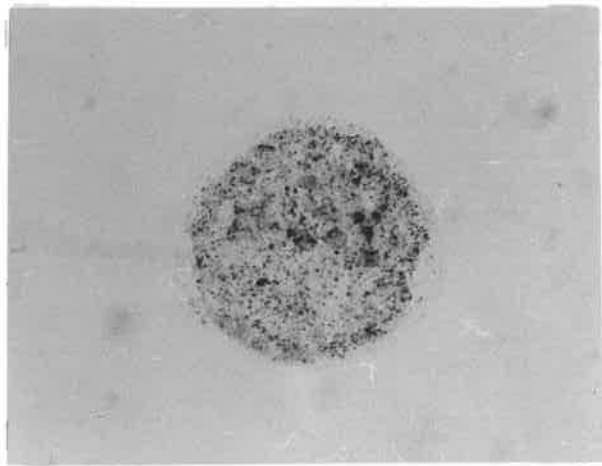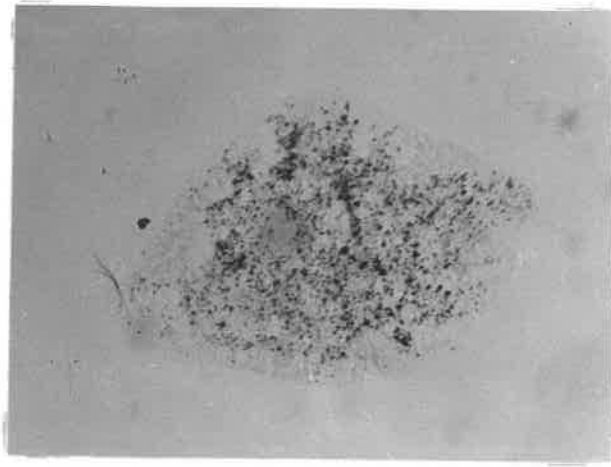
starved for 36 hours before cutting in order to deprive
them of food vacuoles. Acid phosphatase contents of
nuclear and cytoplasmic halves of the same amoeba were
compared at 2 and 24 hours after cutting. It was found
that at no stage was there a striking difference in the
acid phosphatase content between nucleate and enucleate
cells (fig.6a, b). This is not surprising, since the
technique employed was only qualitative and not sensitive
enough to detect a subtle difference in the cellular con-
tent of this enzyme. However, evidence was provided by
Brachet (1955) who showed by means of a quantitative assay
that at one day after cutting the nuclear half of A.proteus
contained more acid phosphatase than the cytoplasmic half,
the ratio being 1.23:1.

5. Determination of acid phosphatase in amoebae after
micro-injection.

A huge body of information has been accumulating in
recent years showing that during phagocytosis of bacteria
by mammalian cells, the enzymatic materials are released
from cytoplasmic granules into the cytoplasmic matrix (Hirsch
and Cohn, 1960). The degranulation usually occurs in the
vicinity of the phagocytic vacuole (Hirsch, 1962; Cohn and
Weiner, 1963). There is strong evidence from the electron
microscopic studies that fusion takes place between the
granule and the vacuolar membrane resulting in the discharge

of granule contents directly into the phagocytic vacuoles (Lockwood and Allison, 1963, 1964; North and Mackaness, 1963; Zucker-Franklin and Hirsch, 1964).

The purpose of this experiment was to investigate whether there was any change in the acid phosphatase content in A. proteus after injection with bacteria.

Non-starved amoebae and amoebae starved for 36 hours were injected with approximately 50 logarithmic phase S. enteritidis cells suspended in Chalkley's medium containing 0.5 per cent. gelatin. The control included normal untreated amoebae and amoebae injected with the injection medium only. The amoebae were stained for acid phosphatase at zero time, 2 and 6 hours after injection. It was found that at no stage was there any significant difference in these acid phosphatase staining granules in normal amoebae and in amoebae which received injections with either the injection medium alone or the injection medium plus bacteria. Figure 9 represents a typical appearance of acid phosphatase granules in an A. proteus.

6. The location within amoebae of bacteria or other particulate matters introduced into the cells by a microinjection technique.

Following injection of bacteria into the cytoplasm of A. proteus, it was not known whether the injected organ-

isms remained persistent in the cytoplasm or they were
enclosed in the vacuoles.   To elucidate this problem,
experiments were done whereby particulate matters, e.g.
carbon, polystyrene spheres, bacteria or bacterial spores
were injected into the cytoplasm of A. proteus and their
location was followed by means of light and electron mic-
roscopy.

1. Light microscopy.

Four amoebae starved for 24 hours were injected
with a suspension of carbon in sterile distilled water
containing 0.5 per cent. gelatin, the amount injected being
approximately equivalent to 1/4 of the cell volume.   Im-
mediately after injection, the cell was removed from the
cover glass in the oil chamber, washed in sterile Chalk-
ley's medium and finally transferred back on to a coverslip
in an oil chamber.   It was found that immediately after
injection, carbon particles were mixed thoroughly with the
cytoplasm and no aggregates were visible.   However, clumps
of carbon particles were seen in 2 amoebae as early as 30
minutes after injection, but in the other two they were
not apparent until after one hour.   Some clumps of carbon
were large and these could be easily seen even under low
power.   These aggregates became larger and larger and they
remained in the cell even at 24 hours after injection.   Of

Fig.10. Photograph showing aggregates of carbon in the vacuole of an _Amoeba proteus_ four hours after injection with colloidal suspension of this particle. C = carbon, V = vacuole.

particular interest is the finding that some carbon ag-
gregates were clearly seen in the vacuoles (fig.10). How-
ever, it was not known as to how this actually occurred.
Two possibilities exist - a) the formation of a membrane
around the carbon particles or b) the segregation of carbon
into pre-existing vacuoles. It was thought that this
problem could be solved if larger particles, e.g. bacteria
or bacterial spores were employed, since in this situation
segregation into pre-existing vacuoles is unlikely to occur.
It follows that any formation of vacuoles around them would
be evidence for de novo synthesis of the membrane. Since
by means of light microscopy, it is not possible to visual-
ise the bacterial cells in A. proteus, the location of
S. enteritidis inside the cell was followed by an electron
microscopic study.

2. Electron microscopy.

Amoebae proteus used in these studies were starved
for 24 hours. A mixture of S. enteritidis cells and carbon
or a mixture of polystyrene spheres and spores of B. subtil-
is was introduced into the cytoplasm of A. proteus using
the technique previously described in Chapter II, p.60.
Approximately 100 bacteria or 100 spores were injected into
each amoeba and the amount of the fluid injected did not
exceed 1/4 of the cell volume. After injection, the am-
oebae were maintained in Chalkley's medium at 20° and the

cells were processed for electron microscopy (Chapter II,
p.67) at 0, 2, 6 and 24 hours after injection. In certain
experiments, however, the cells were incubated at 4^0.

It was found that all the particulate matters used
in these studies behaved similarly. Since the spores were
the largest particles and were less likely to be confused
with the previously ingested bacteria, they were principally
used for illustrating the observations.

Initially the particles lie free in the cytoplasm
(fig.11). They are surrounded by a clear gap of ~ 1 µ
separating them from the cytoplasmic matrix, and which pre-
sumably represents the fluid injected with them. At 2
hours they are surrounded by many somewhat irregular part-
icles. These are 10 to 30 mµ in diameter, usually about
20 mµ. They are fairly electron dense stained with lead,
and greatly resemble ribosomes. In fact similar particles
are scattered throughout the cytoplasm, but in the region
of the particles they tend to aggregate into a rough shell
which surrounds the injected material (fig.12-15). As
time passes (2 to 6 hours) these particles become replaced
by a membrane (figs.14 - 16). Initially small areas of mem-
brane form (fig.14); later the particle is surrounded by a
membrane, with a few gaps containing the particles (fig.15);
finally the membrane is complete (fig.16). The replacement
of the particles by a membrane is most evident. The mem-
brane often has the two dark lines separated by a light one

of the typical unit-membrane (fig.16).   Even some of the
particles have dark rims and a pale centre - suggesting
their similarity to the membrane (fig.12).

In amoebae kept at 20°, almost all particles are
enclosed in membranes at the end of 6 hours, but only few
of them are in the vacuoles after 2 hours.   In contrast,
no membranes are formed in the amoebae which were kept at
4° after the injection (fig.19).   In this instance, the
small particles aggregate as in the amoebae kept at 20°
and a rough shell is formed around the particles, but they
are not replaced by membranes.   Some membranes are seen in
the neighbourhood of the particles, but if they are care-
fully followed they can all be seen to belong to adjacent
cytoplasmic organelles.

The size of the vacuole formed is initially just
larger than the size of the injected particles (fig.16).
Subsequently (6-24 hours), the injected material is seen in
typical food vacuoles (fig.17, 18).   Some of these food
vacuoles contain only the injected material - as if the
initial vacuoles enlarged to assume this form.   Other food
vacuoles contain much material, bacterial debris, etc.,
which has presumably been ingested in the normal manner
(fig.17, 18) - suggesting that the initial vacuoles united
with pre-existing food vacuoles.   The progression of size
of the vacuoles is from just larger than the particles
($\sim 1\,\mu$) to 20 - 50 μ of the old food vacuoles.   There is

no suggestion of an initial shrinkage, followed by a dilatation, followed by a further shrinkage as described by Mast (1942), and Roth (1960).

The digestion of the bacteria and spores can be followed with the electron microscope. The external membranes of the bacteria are the first to be attacked. These start to fragment even when the enclosing vacuole is far from complete (fig.14). At 6 hours the internal portion starts to be disorganised and this is nearly complete after 24 hours. The spores are rather more resistant. The spore wall is morphologically intact even after 6 to 24 hours (fig.17-18).

In the cytoplasm of normal amoebae and in the injected amoebae, there are many small (~100 mμ) vesicles containing materials having different electron density, i.e. some are electron transparent whereas others are finely granular and moderately electron dense (figs.12, 13, 15, 16, 19). Numerous granules containing electron dense materials lie near the injected particles at all times, thus suggesting their participation in the process of digestion. When the membranes are still far from complete, material closely resembling that contained in these vesicles comes to surround the injected particles (figs.14, 15). When the vacuole is finally formed it usually contains this material. Finally, when the food vacuole is formed, the outer layer of its contents also has this appearance (figs.17, 18).

Key to the abbreviation used in Figs. 11 - 19.

B	Bacterium
FV	Food vacuole
EDM	Electron-dense material
L	Lipid vacuole
MT	Mitochondrion
R	'Reticulosome'
SV	Small vesicle ($\sim$ 100 mμ)
V	Vacuole

The amoebae illustrated in figs. 11 - 18 were incubated at 20° and the amoeba in fig. 19 was incubated at 4°.

Fig. 11. The amoeba was fixed immediately
after the injection of bacteria. One of the
bacteria (B) is seen lying in the cytoplasmic
matrix, separated from this by a space (X) which
presumably represents the injected fluid. There
are many small particles (R) lying randomly in
the cytoplasmic matrix. At this stage it is
impossible to distinguish reticulosomes from
ribosomes. 80,000 X.

Fig. 12. Two hours after injection, a spore
(S) is surrounded by some particles (R) which,
for reasons given in the text, are identified as
reticulosomes. Some of these bodies seem to con-
sist of paler centres surrounded by electron-dense
exteriors. In the cytoplasmic matrix there are
numbers of small vesicles (SV), many of which con-
tain moderately electron-dense material (EDM).
55,000 X.
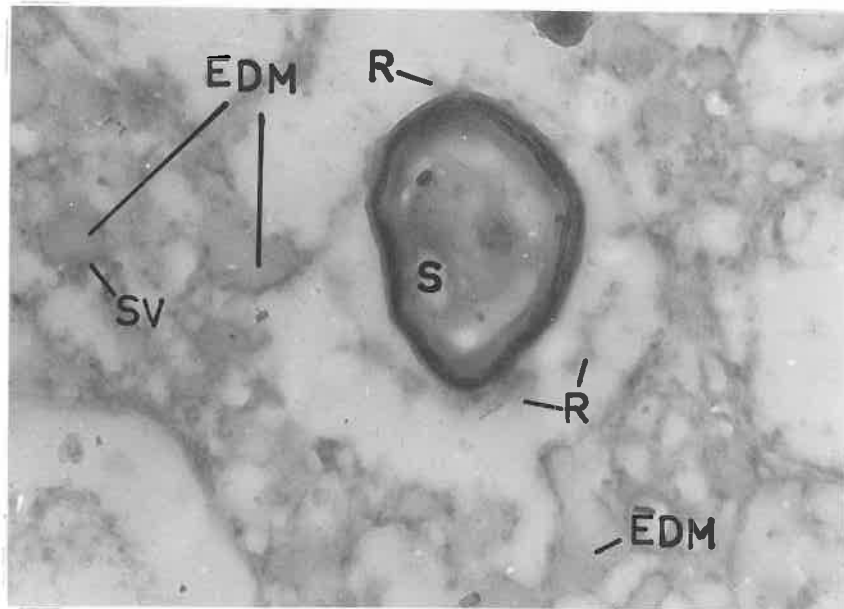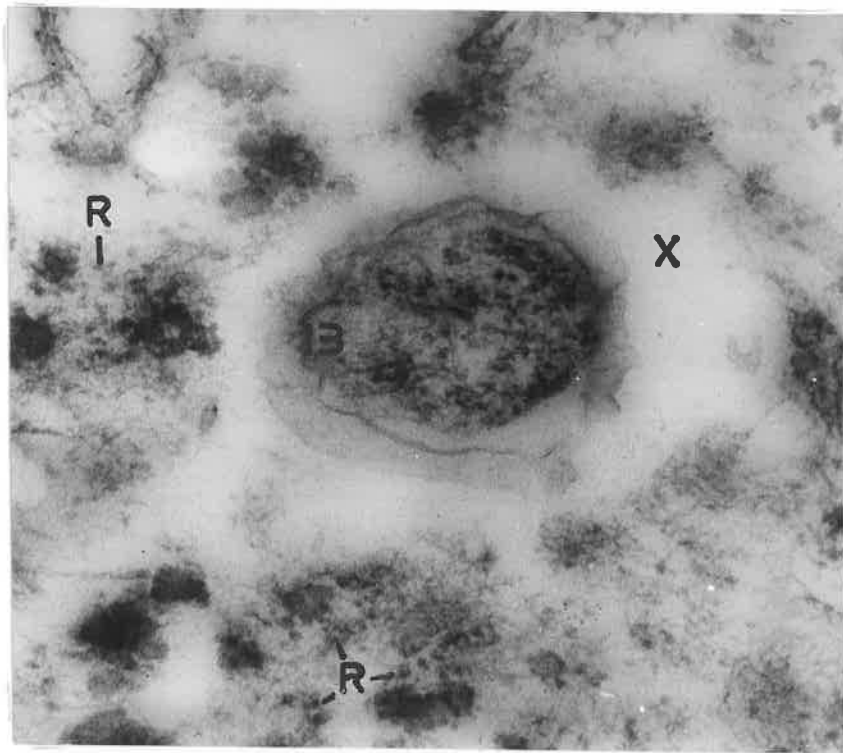
Fig. 13. Two hours after injection, a spore (S) is again surrounded by reticulosomes (R), which form an irregular shell around the particle. There are many similar particles lying randomly in the cytoplasm. Numbers of small vesicles containing moderately electron dense material (EDM) are visible. 100,000 X.

Fig. 14. Two hours after injection, two bacteria (B) lie in a space in the cytoplasm. Membranes (M) have appeared at some of the areas where the space meets the cytoplasmic matrix. Reticulosomes (R) are still very frequent around the space. Portions of the bacterial external membranes have been disrupted (arrows). 40,000 X.
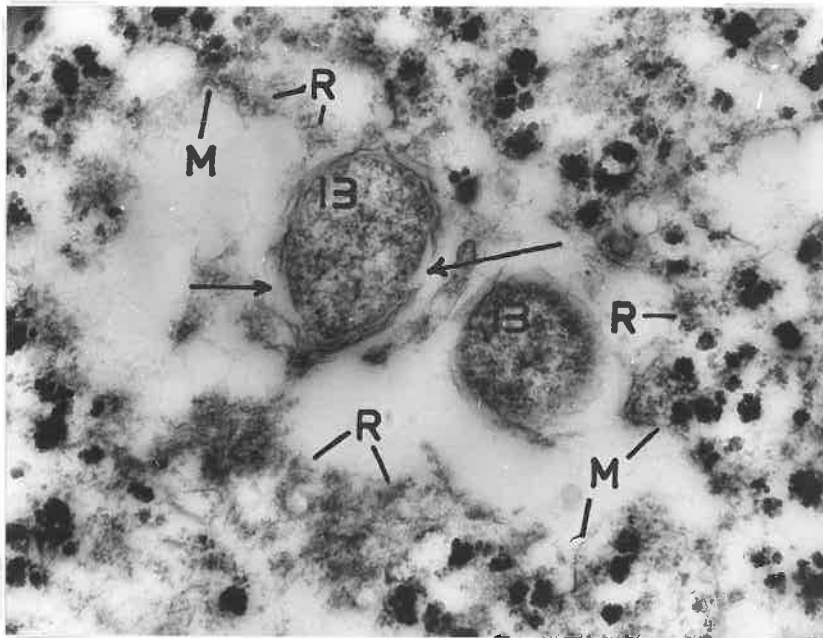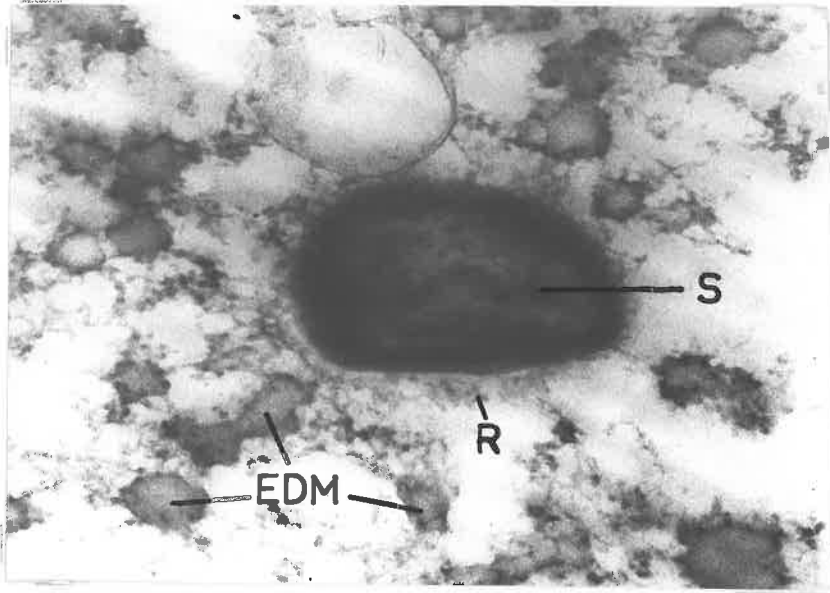
Fig. 15. Two hours after injection, a spore (S) is surrounded by much membrane (M). In the area where the membrane is discontinuous, there are numbers of reticulosomes (R), but these have been largely replaced in the areas where there is membrane. Small vesicles containing electron dense material (EDM) are visible and some of the material appears to be present in the forming vacuole (V) and in the surrounding cytoplasm. 120,000 X.

Fig. 16. Two hours after injection, a spore (S) is almost completely enclosed by membrane (M) to form a vacuole (V). The shell of reticulosomes appears to have given place to the membrane. 80,000 X.
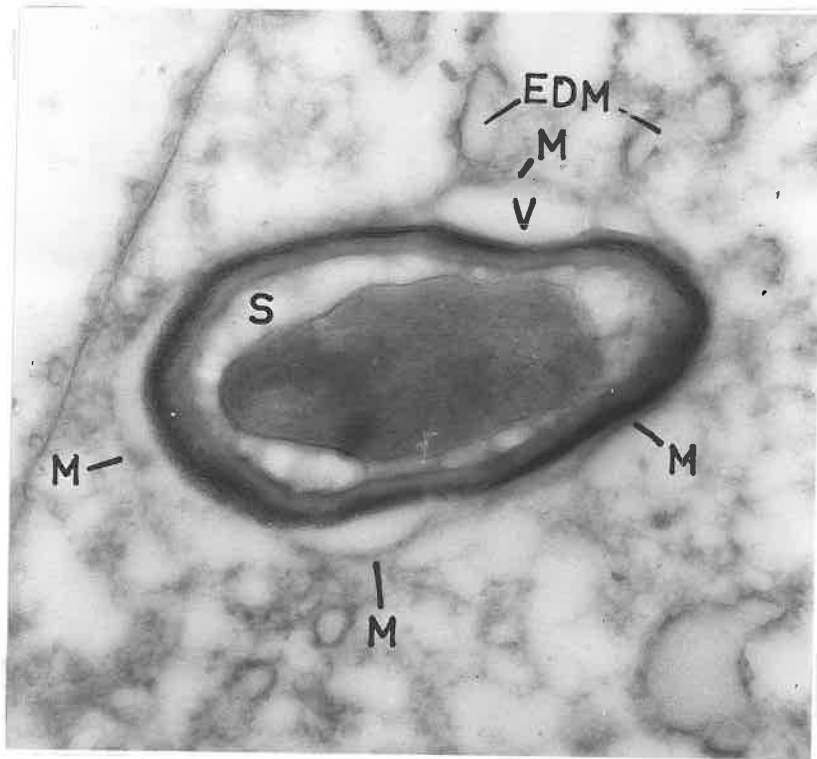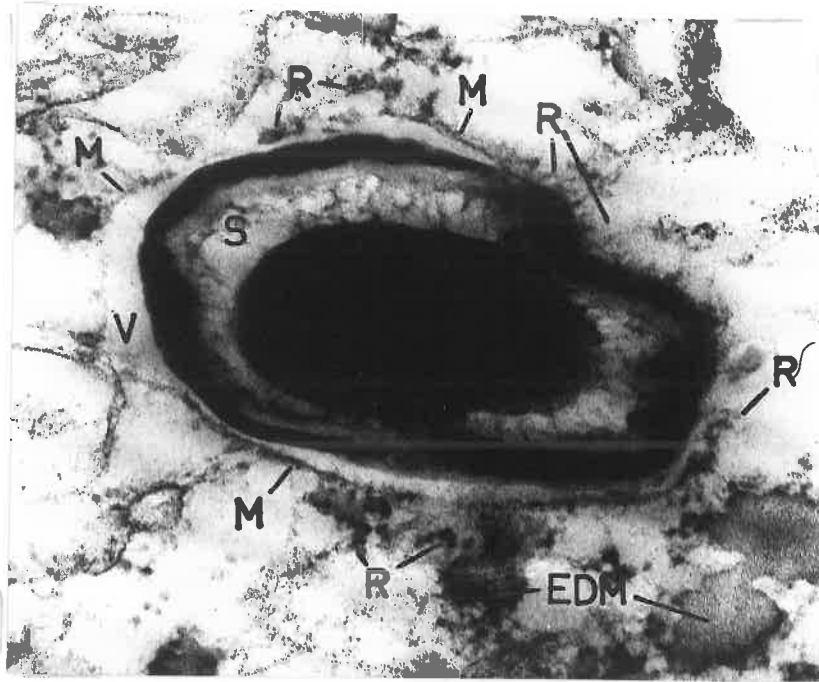
Fig. 17. Six hours after injection, an early
food vacuole (FV) contains a spore (S) which was
introduced by micro-injection and a bacterium
(B) which was not, and hence must have been phag-
ocytosed. The remaining contents of the food
vacuole consist of moderately electron-dense mat-
erial similar to that present in the small vesi-
cles of figs. 12 - 16. 70,000 X.

Fig. 18. Six hours after injection, food vac-
uoles (FV) contain numerous spores (S), the inner
portions of which are rather disrupted. Again
the outer portions of the contents of the vacuoles
consist of moderately electron dense material
(EDM), however these are more mature vacuoles than
in Fig. 17. Lipid vacuoles (L) and mitochondria
(MT) are also present. 8000 X.
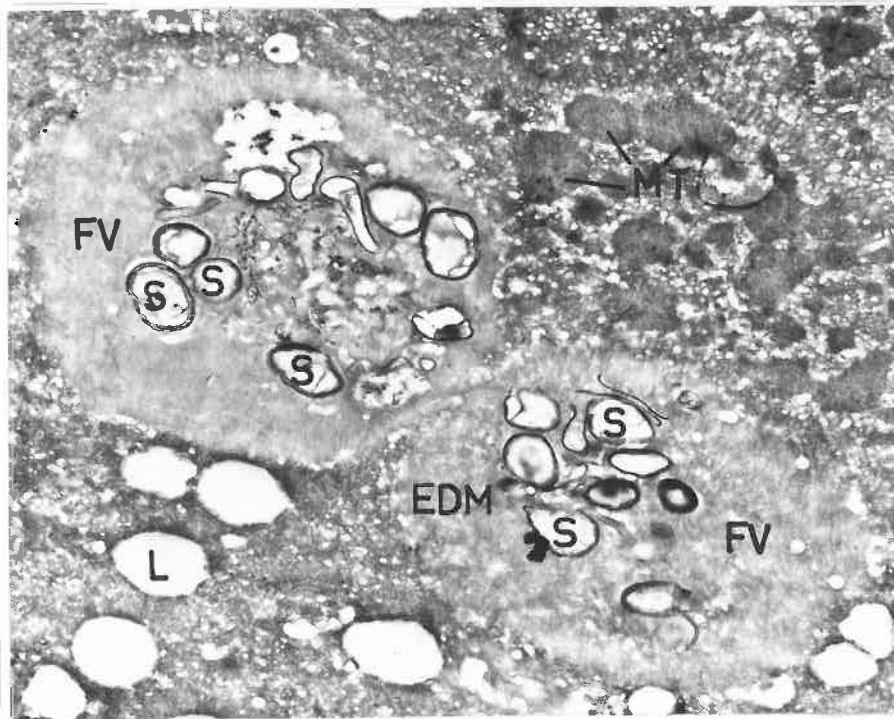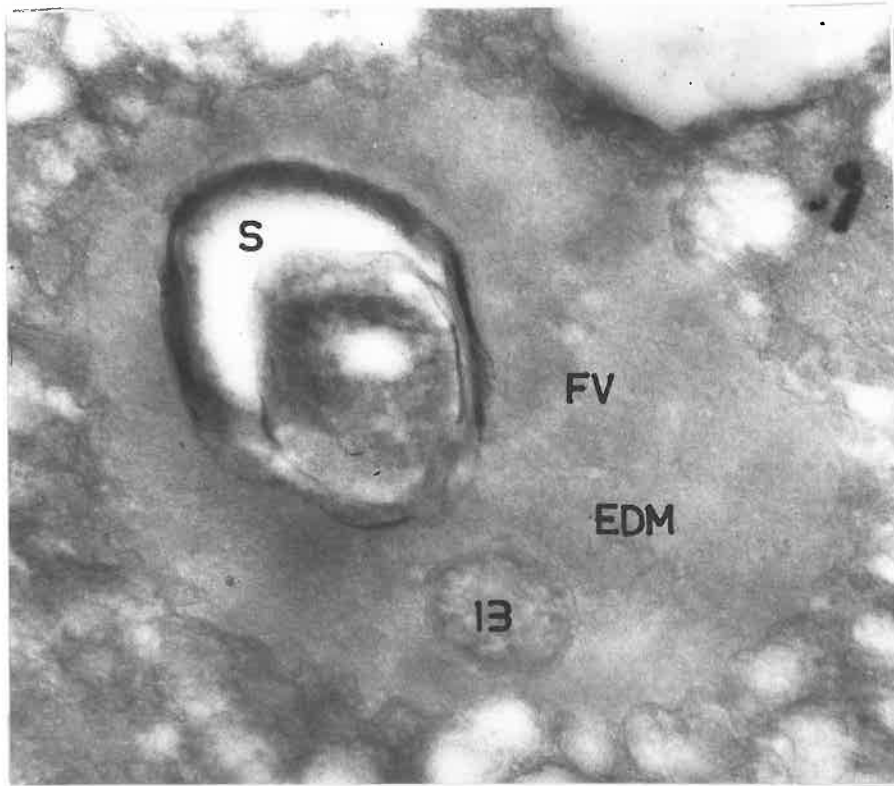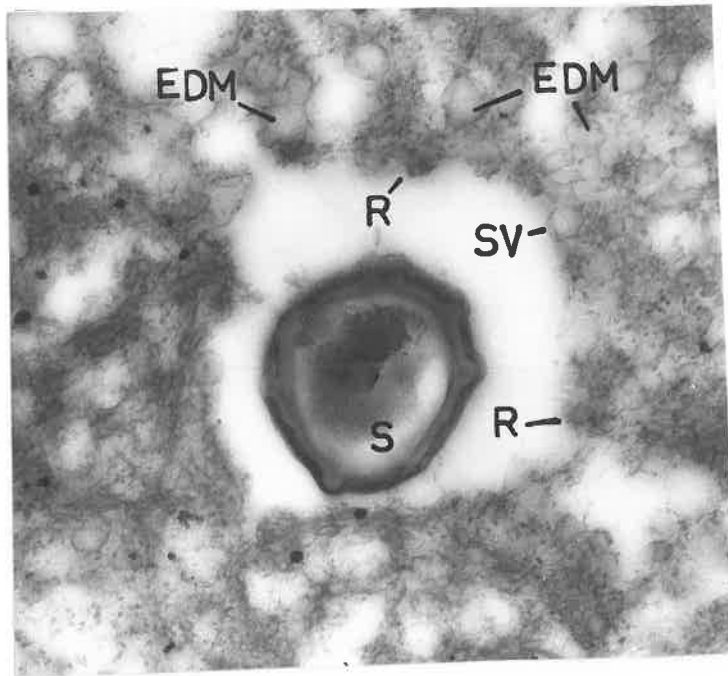
Fig. 19. Twenty hours after injection, the spore
(S) remains free in the cytoplasm of the Amoeba proteus
incubated at 4°. Some small particles (R) accumulate
around the injected particle, but these were not trans-
formed into membrane.

(This is in the early stages of the food vacuole, as described by Pappas, 1959).

Discussion.

To a micrurgist, the first thing that should be considered in any micro-injection study is possible injury to the cell as a result of the micro-manipulative procedure and the toxic effect of the injection medium. Fortunately, Amoeba proteus is one of the cells amenable to micro-injection. It has been claimed that injection of water or salt solution into A. proteus does not cause any harmful effect on the cell provided that the amount of the medium injected does not exceed half of the cell volume and the concentration of monovalent or divalent salt is not more than 0.01 M and 0.0025 M respectively (Chambers and Chambers, 1961). It is unlikely that the injection medium used in this study was toxic to amoebae, since the cells remained active and divided after injection with this medium even in the amount equivalent to half of the cell volume. Moreover, acid phosphatase associated granules did not appear to have any change at zero time, 2 and 6 hours after injection. Excess of water introduced into A. proteus would be eliminated through contractile vacuoles, the rate of output of which increased considerably in proportion to the amount of water injected (Howland and Pollack, 1927). Nevertheless, physiological or morphological disturbance

at a sub-microscopic level might have occurred.   Since the purpose of this study is to compare ability of A. proteus to kill the test organism treated in different manners using the same technique, the results obtained would be relatively valid.

These studies show that when S. enteritidis was injected into A. proteus, bacterial numbers declined at a rate which indicated they had a half-life of about 5 hours in this situation, the half-life was unaffected by pre-treating the bacteria with serum opsonin.   These findings are an interesting contrast to the fate of opsonised salmonellae within mammalian phagocytes, where the half-life is approximately 15 minutes (Rowley and Whitby, 1959). Moreover, the presence of opsonins on the bacterial surface is believed to be essential for killing to be efficient within mammalian phagocytes (Jenkin, 1963).   A possible reason why amoebae failed to show enhanced killing of bacteria in the presence of serum opsonins could be because these protozoa have not evolved in an environment where such an effect is likely to endow a selective advantage.

A consideration of possibly great significance in any micro-injection study is the fact that bacteria injected into the cytoplasm of a cell did not become immediately incorporated into the vacuoles.   The result from electron microscopic study showed that some bacteria remained free

in the cytoplasm even 6 hours after injection. It is
reasonable to suppose that these bacteria may not become
exposed to the same environment as those which have entered
the cell by the process of phagocytosis. In the case of
bacteria which were later incorporated in the vacuole, the
incorporation may be of slow process which allows the in-
jected organisms sufficient time to adjust themselves to
environmental changes. Since vacuoles are known to have
properties specific for them, it would be interesting to
know whether killing of S. enteritidis in the vacuole of
A. proteus differs from that in the cytoplasm and whether
serum opsonins enhance bactericidal mechanisms in the vac-
uolar environment. This work will be presented in Chapter
IV.

The enhanced killing of S. enteritidis opsonised
with specific antibody and fresh rabbit serum suggests that
complement plays an important part in promoting the bac-
tericidal effect. In order to establish this point, it
is necessary to repeat this type of experiment using fresh
rabbit serum which has been deprived of complement, either
by treatment with antigen-antibody aggregates or by heat
inactivation.

Electron microscopic studies of A. proteus injected
with either vegetative bacteria or bacterial spores showed
clearly the ability of these protozoa to synthesise de novo

the new membrane surrounding the injected micro-organisms.
It is possible that this may be the most efficient way by
which a cell combats any micro-organisms invading into the
cytoplasm.   It follows that recognition between self and
non-self in primitive cells may depend on this process.

Conclusion.

1.   When S. enteritidis was introduced into the cyto-
plasm of A. proteus, it was killed at a very slow rate
having an intracellular half-life of 5 hours.

2.   Serum antibody did not enhance a bactericidal ef-
fect.   In the presence of fresh rabbit serum, however,
significant enhancement was obtained and this was attrib-
uted to the potentiating action of complement.

3.   There was no significant difference in bactericidal
ability of nucleate and anucleate halves of A. proteus
which was merotomized 2 hours previously.   At 24 hours
after cutting, killing was significantly greater in the
nuclear half.   This enhancement of bactericidal mechanisms
could be ascribed in part to the effect of starvation and
in the other part to the changes associated with regener-
ative process.

4.   There was no change in cytochemical staining of
acid phosphatase in A. proteus after injection of the in-
jection medium with or without bacteria.

5.   Evidence for de novo synthesis of the membrane was
presented.

CHAPTER IV.

FACTORS AFFECTING THE ABILITY OF A. PROTEUS TO PHAGOCYTOSE

AND KILL INGESTED SALMONELLAE.

Introduction.

When S. enteritidis was introduced directly into
the cytoplasm of A. proteus by means of a micro-injection
technique, the bacteria were killed, and the rate of kill-
ing was not found to be altered by pre-treating the sal-
monellae with serum opsonins (Chapter III).   However, it
is possible that data obtained by means of micro-injection
experiments may not reflect the dynamics of bacterial kill-
ing as it occurs in the vacuoles of amoebae, i.e. after
micro-organisms have been ingested naturally.   The finding
that A. proteus could be induced to phagocytose moderate
numbers of either S. enteritidis or S. typhimurium provided
the ratio of bacteria to amoebae was sufficiently high,
makes it possible to measure the survival time of salmon-
ellae within vacuoles.   The enhancing effect of serum op-
sonins on the uptake of these organisms by amoebae has also
been studied, together with the possible contribution that
serum opsonins might make to intracellular killing.   When
survival of supposedly phagocytosed salmonellae is being
measured it may be difficult to decide whether bacteria
associated with amoebae are really in an intracellular pos-
ition, or merely adherent to the cell surface.   Since it

is important in a quantitative study to differentiate between these two possibilities, certain of the experiments described in this chapter have entailed the use of a lytic mutant of phage P_{22} to eliminate extracellular bacteria.

Results.

1. The effect of mammalian opsonins on phagocytosis.

The organisms used were streptomycin resistant strain of S. enteritidis Se 795. These organisms were opsonised according to a method which did not require bacteria to be washed by centrifugation after treatment, since such a procedure would facilitate agglutination. An 18 hour culture of this organism in minimal medium was washed by centrifugation at 3000 r.p.m. for 10 minutes and resuspended in Chalkley's medium containing 0.1 per cent. glucose to give a bacterial concentration of approximately 5×10^8 organisms per ml. Antiserum was then added to achieve a final concentration equivalent to 2/3, 1 and 4 of a minimal agglutinating dose. After incubating at 37° for 10 minutes, the bacterial suspension was diluted 1/20 before use.

The numbers of bacteria phagocytosed by A. proteus after one hour of contact were determined using the technique described in Chapter II, p.68. After phagocytosis, the amoebae were washed 4 times and then crushed on the surface of nutrient agar plate containing 50 micrograms of streptomycin per ml. for a bacterial count. Table VII shows that there was some increased uptake of bacteria with

TABLE VII.

Effect of serum opsonins on phagocytosis of S.enteritidis
by Amoeba proteus.

Dilution of specific antiserum used to pretreat bacteria. (Minimal bacterial agglutinating doses per ml.)	No. of bacteria associated with amoebae at various time intervals after initial contact	
	+ 15 minutes	+ 80 minutes
0	1022	2089
2/3	1075	3951
1	1052	4873
4	1771	9478

Number of amoebae per tube = approximately 5970.

Standard bacterial inoculum per tube = $4.0 \pm 0.2 \times 10^5$ organisms.

increasing degrees of opsonisation, but at no stage did the amount of phagocytosis approach that which would be expected if mammalian phagocytes had been used in these tests (Rowley and Whitby, 1959). Provided that salmonellae remain unagglutinated, it is likely that changes measured in bacterial uptake, although relatively small, are a true indication of opsonic effect, i.e. result from physico-chemical changes at the cell surface of individual organisms. However, in these experiments when one minimal agglutinating dose of antiserum was exceeded there was an increasing tendency for bacteria to agglutinate, as judged by their arrangement when viewed under phase contrast. It seems likely, therefore, that in the case of amoebae, increased phagocytosis of S. enteritidis in the presence of mammalian serum opsonins was in part due to ingestion of clumps of agglutinated bacteria.

2. Failure of serum opsonins to alter the rate of intracellular killing of salmonellae.

Survival of phagocytosed S. enteritidis and S. typhimurium M_2 with and without pre-treatment with serum antibodies is shown in text - fig.5, 6. The data on which this result is based are set out in Tables VIII and IX. These experiments showed that A. proteus was able to kill ingested organisms and that the rate of killing was not influenced when serum opsonins were adsorbed to the bacterial surface.
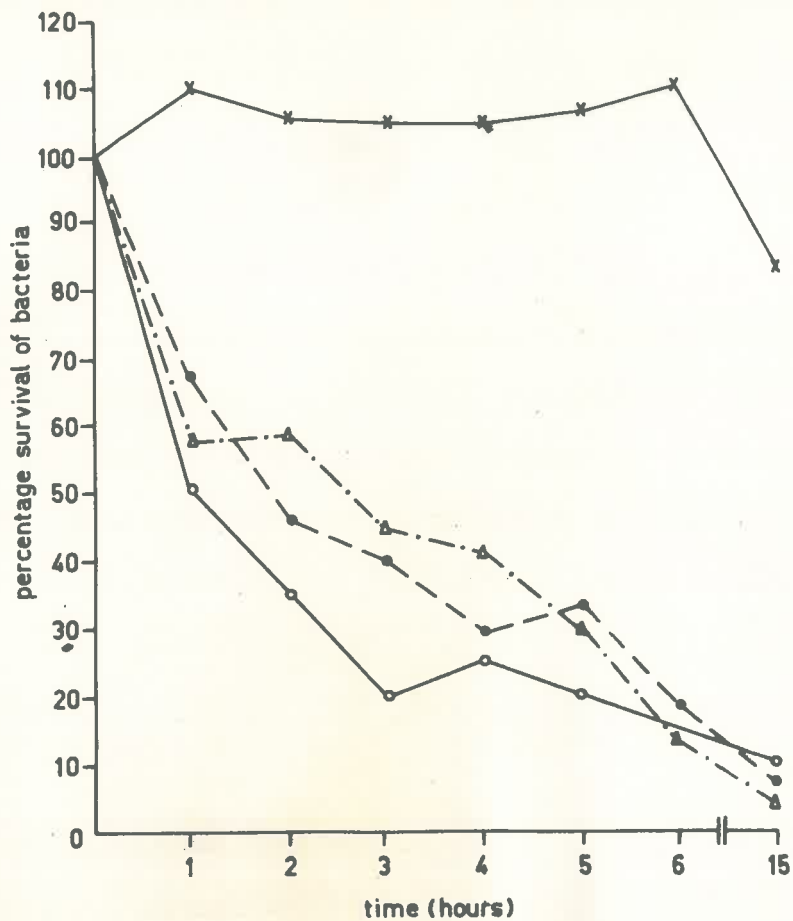
Text-fig. 5. Killing of opsonised and unop-
sonised Salmonella enteritidis after phagocytosis
by Amoeba proteus.

Pre-treatment of bacteria.

o————o   No opsonin.

●— — — ●   Opsonised with specific
antiserum.

Δ—·—·—·— Δ   Opsonised with pig serum.

x————x   Control growth curve.

Text-fig. 6. Killing of opsonised and unop-
sonised _Salmonella typhimurium_ LT$_2$ after phagocy-
tosis by _Amoeba proteus_.

Each slope represents the mean of two
experiments.

	Pre-treatment of bacteria.
o———o	No opsonin.
●- - - -●	Opsonised with specific antiserum.
x———x	Control growth curve for unopsonised bacteria.
Δ- - - -Δ	Control growth curve for bacteria opsonised with specific antiserum.

TABLE VIII.

Survival of Salmonella enteritidis Se 795 after phagocytosis by Amoeba proteus.

Time after 60 min. period allowed for phagocytosisT(hrs.)	Unopsonised bact.		Bact. opsonised with specific antiserum		Bact. opsonised with pig serum		Growth curve control for extracellular bacteria	
	Total recovery per tube	% survival	Total recovery per tube	% survival	Total recovery per tube	% survival	Total recovery per tube	% survival
Zero	4830	100	13281	100	9178	100	7055	100
1	2482	51.3	9108	68.6	5289	57.6	7841	111.1
2	1573	32.5	6392	48.1	5453	59.4	7610	107.8
3	1072	22.2	5364	40.4	4165	45.4	7538	106.8
4	1230	25.5	3959	29.8	3969	43.2	7476	105.9
5	961	19.9	4686	35.3	3018	32.9	7618	107.9
6	-	-	2412	18.2	1262	13.7	7875	111.6
15	491	10.2	1036	7.8	689	7.5	5847	82.9

Average number of amoebae per tube = 4300

* Percentage survival = $\frac{\text{Total bacteria recovered at T (hours)}}{\text{Total bacteria recovered at time zero}} \times 100$

TABLE IX.

Survival of *S. typhimurium* LT$_2$ after phagocytosis by *A. proteus*.

Time after 60 mins. period allowed for phagocytosis	Unopsonised bacteria				Opsonised with specific antiserum			
	Bacteria recovd. from amoebae		Growth curve control for extra-cellular bacteria		Bacteria recovd. from amoebae		Growth curve control for extra-cellular bacteria	
	Total recovery per tube	% survival	Total recovery per tube	% survival	Total recovery per tube	% survival	Total recovery per tube	% survival
Zero	5583	100	9925	100	6318	100	6970	100
1	3082	55.2	10442	105.2	2887	45.7	8042	115.4
2	2268	40.6	10241	103.2	2369	37.5	8170	117.2
3	1176	21.1	10678	107.6	1877	29.7	7836	112.4
4	639	11.4	10972	110.5	708	11.2	8120	116.4
5	559	10.0	11046	111.3	939	14.9	6780	97.3
6	542	9.7	13428	135.3	307	4.9	7642	109.6
15	159	2.8	9455	95.3	138	2.2	6042	86.7

Average number of amoebae per tube = 5635.

The fall in the number of micro-organisms recovered from amoebae could only be due to intracellular killing, since controls set up to measure the growth rate of bacteria free in supernatant fluid in the presence of amoebae showed that bacterial counts remained remarkably constant. Emphasis should be made that this growth curve control reproduces almost identical conditions to the test system. Determination of intracellular survival of micro-organisms in mammalian cells was dependent in part on the control growth of bacteria growing in a medium where phagocytic cells were absent. It is obvious that such a situation is not close to reality, since in the test system the medium is conditioned by certain cellular metabolic products which might exert influence on the growth rate of the bacteria it contains.

3. Lack of evidence for voiding of S. typhimurium by Amoeba proteus.

Since, in the aforementioned experiments, the amoebae had to be washed twice more before plating out, it is likely that bacteria which might be expelled from the cell during the period of incubation would be removed. It follows that the result obtained would not give a true dynamic of bacterial killing. In order to clarify this point, an experiment was done whereby the same technique of measuring phagocytosis and intracellular survival was employed, with

with the exception that the amoebae after being washed
three times were plated out without further washing. The
result is shown in text-fig.7, and Table X. It can be
seen that survival curve of S. typhimurium was very similar
to those obtained earlier. It is reasonable therefore to
assume that egestion of S. typhimurium by A. proteus was
unlikely to occur in this experimental condition and, if
it did, the extent of egestion was too small to interfere
with the assessment of intracellular killing.

4. Influence of temperature on intracellular killing
of S. enteritidis Se 795 by A. proteus.

The results from phagocytic studies showed that the
half-life of salmonellae in A. proteus was approximately
one hour, whereas the half-life of these organisms in mam-
malian phagocytes was only 15 minutes (Rowley and Whitby,
1959). It is thought that the discrepancy in bactericidal
ability between these two types of phagocytic cells could
be explained on the basis of the difference in temperature
in which the test was performed, e.g. 20° in A. proteus and
37° in mammalian cells. Madsen and Watabiki (1919) pres-
ented evidence showing that ingestion of staphylococci and
E. coli by mammalian leucocytes increased as the temperature
of incubation of bacterium-phagocyte mixture was raised.
Fenn (1922) re-calculated these results and found a surpris-
ingly uniform temperature co-efficient over the entire range
of 5° to 35°. He concluded that the rate of phagocytosis
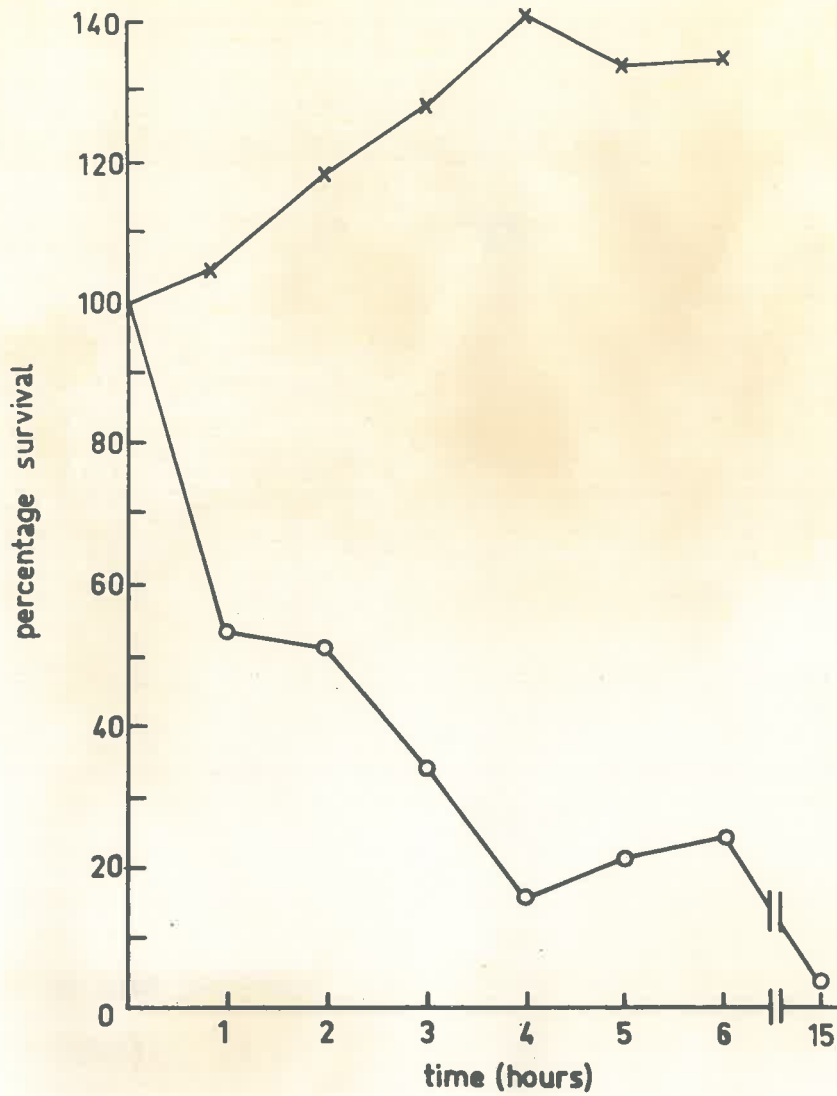
Text-fig. 7. Killing of unopsonised <u>Salmonella</u> <u>typhimurium</u> LT$_2$ by <u>Amoeba proteus</u>. In this experiment amoebae were washed only 3 times before plating out for a bacterial count.

o———o   Bacteria recovered from the amoebae.

x———x   Control growth curve.

TABLE X.

Survival of S. typhimurium LT₂ (unopsonised) after phag-
ocytosis by A. proteus.*

Time after 60 mins. period alld. for phago-cytosis (hr.)	Bacteria recovered from amoebae		Growth curve control for extracellular bact.	
	Total recov. per tube	Percent. survival	Total recov. per tube	Percent. survival
Zero	13914	100	7425	100
1	7494	53.8	7733	104.1
2	7014	50.4	8613	118.7
3	4800	34.5	9443	127.2
4	2142	15.4	10400	140.0
5	3020	21.8	9878	133.3
6	3394	24.7	9971	134.3
15	512	3.7	-	-

* Amoebae were washed only 3 times. No further
washing before the cells were plated out.
Average No. of amoeba per tube = 6170.
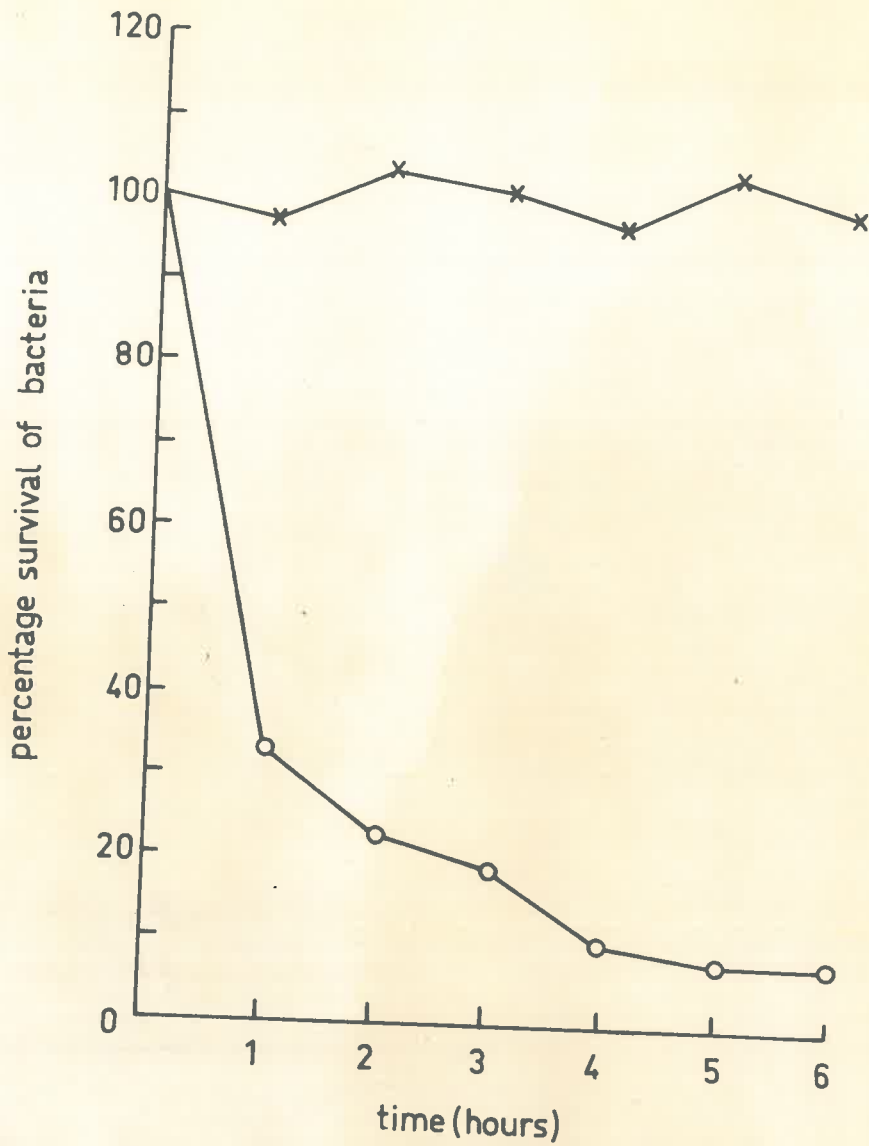
Text-fig. 8. Killing of unopsonised Salmonella enteritidis after phagocytosis by Amoeba proteus. The temperature of incubation of the amoebae after phagocytosis was 30°.

Each slope represents the mean of two experiments.

o————o   Bacteria recovered from amoebae.

x————x   Control growth curve.

TABLE XI.

Influence of temperature on killing of unopsonised
S. enteritidis Se 795 by A. proteus.x

Time after 60 mins. period allowed for phagocytosis (hours)	Bacteria recovered from amoebae		Growth curve control for extracellular bacteria	
	Total recov. per tube	Percent. survival	Total recov. per tube	Percent. survival
Zero	4143	100	4866	100
1	1354	32.7	4760	97.8
2	947	22.9	5030	103.3
3	784	18.9	4950	101.7
4	420	10.1	4749	97.5
5	340	8.2	5038	103.5
6	343	8.3	4824	99.1

x   The cells were incubated at 30°.

Average No. amoebae per tube = 5650.

was doubled by a rise in temperature of 10^0. If the Laws
of Thermodynamics can be applied to intracellular bacter-
icidal mechanisms, the half-life of salmonellae in A. proteus
should be reduced by half if the temperature of incubation
was raised up to 30^0.

To test this hypothesis, two experiments were done
whereby S. enteritidis was allowed to be phagocytosed by
A. proteus at 20^0 for 1 hour using the technique described
in Chapter II (p.68), with the exception that after washing
the cells were incubated at 30^0. The results in text -
fig.8 and Table XI show that the intracellular survival of
S. enteritidis in A. proteus was considerably reduced, i.e.
the half-life was approximately 40 minutes. Should the
test be performed at 37^0, the half-life of this organism
in A. proteus would probably be very similar to that in
mammalian phagocytes. In view of the fact that A. proteus
is damaged by a temperature as high as 37^0, it would be
interesting to determine the half-life of S. enteritidis
in mammalian macrophages at 30^0 and to compare this result
with that in A. proteus.

5. Use of phage P_{22} to eliminate extracellular S.typhi-
murium LT$_2$ from amoeba suspensions.

Investigators in the field of host-parasite rela-
tionship are frequently compelled to decide as to whether
the bacteria associated with phagocytes are inside the cells

or whether they remain adherent to the cell surface. To solve this problem, many techniques have been devised and most of them entail the use of antibiotics (Jenkin and Benaceraff, 1960; Pavillard, 1963). Rowley (1962) expressed concern on the use of antibiotics in phagocytic experiments on the ground that the drug might enter the cell and interact with the ingested organisms.

In this study, a new approach has been employed whereby phage P_{22} was used to eliminate extracellular bacteria. In the preliminary tests the following experiments were performed.

a) Demonstration of ability of phage P_{22} to lyse S. typhimurium LT_2 in the presence of amoebae.

In the light of a current notion that effective lysis of bacteria by phage require calcium ion (Adams, 1959d), it was thought desirable to study lysis of S. typhimurium by phage P_{22} in Chalkley's medium in which Ca ion was present. This medium was prepared as follows:

NaCl	80 mg.
$NaHCO_3$	4 mg.
$CaCl_2$	4 mg.
$CaH_4(PO_4)_2 \cdot H_2O$	1.6 mg.

Glass distilled water 1 L. (Livingstone E. 1962, personal communication).

A series of 7 tubes was prepared, each containing approximately 2×10^4 organisms and 5×10^3 amoebae in 0.4

ml.   Four of these tubes were each added with 0.02 ml. of phage suspension containing 10^7 PFU;   the other tubes served as controls.   After incubation at 20^0 for a variable period of time, 0.3 ml. of non-heated phage antiserum (diluted 1:5 in Chalkley's medium) was added to a tube in a test series to stop further phage reaction.   Phage inactivation was allowed to occur at 20^0 for 10 minutes after which 1.3 ml. of fresh Chalkley's medium was added;   this was followed by centrifugation at 60 g. for 2 minutes: the supernatant was removed and plated out on streptomycin agar plate for a bacterial count.   The tubes in the control series were similarly treated, but no phage was added.   The results in text - fig.9 and Table XII showed that phage P$_{22}$ was able to lyse S. typhimurium LT$_2$ in the presence of A. proteus and 90% of the organisms were eliminated in 20 minutes.   It also showed that phage antiserum used in this condition was not bactericidal for S. typhimurium since in the control series the number of the organisms treated with antiserum alone remained remarkably constant.

   b)   Demonstration of non-toxicity of phage antiserum for A. proteus.

   A group of 20 A. proteus washed 3 times in Chalkley's medium were exposed for a variable period of time to various twofold dilution of phage antiserum commencing from 1:10. It was found that serum dilution of 1:20 to 1:160 did not cause any morphological change during a period of half an
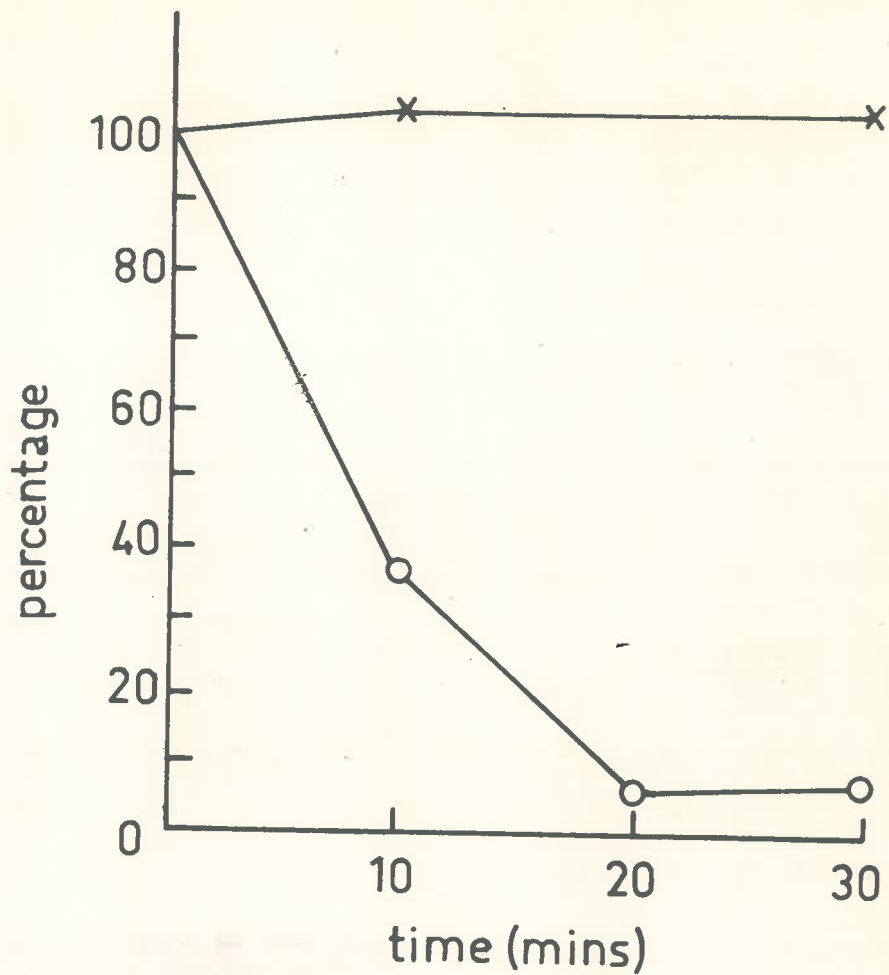
Text-fig. 9. Lytic activity of phage P_{22} for Salmonella typhimurium LT_2 in the presence of Amoeba proteus.

x————x   Bacteria recovered from the supernatant when no phage was added.

o————o   Bacteria recovered from the supernatant to which phage was added.

TABLE XII.

Lytic activity of phage P$_{22}$ for S. typhimurium LT$_2$ in the presence of amoebae.

Time (min.)	Bacteria recovered from the supernatant after phage treatment		Growth curve control (No phage treatment)	
	Total recov. per tube	Percent. survival	Total recov. per tube	Percent. survival
Zero	23240	100	24120	100
10	8660	37.3	24710	102.4
20	1580	6.8	-	-
30	1670	7.2	24920	103.3

hour of exposure.   At a dilution of 1:10, however, amoebae
were round up.   This change was reversible, since the am-
oebae exposed to the antiserum for 30 minutes became active
again after washing.   In view of the fact that serum con-
tains a high concentration of protein, it is reasonable to
suppose that the rounding up of amoebae is a pinocytotic
response to the protein inducer in the serum.   The finding
also suggests that serum up to 1:10 dilution was not toxic
to amoebae.

   c) <u>Phagocytosis of phage P$_{22}$ by A. proteus.</u>

   When phage was used to lyse extracellular salmon-
ellae in an amoebae suspension, it is not known as to wheth-
er there is any uptake of phage by amoebae.   In the light
of the likelihood that this phage might interact with bac-
terial cell inside the phagocyte, it is necessary to deter-
mine the number of phage associated with amoebae at a given
period of time after contact.

   0.02 ml. of phage suspension containing 10^7 PFU
was added to a series of 3 tubes, each containing approxim-
ately 5000 amoebae in 0.4 ml. Chalkley's medium.   After
incubation at 20^0 for a variable period of time up to 30
minutes, the amoebae were washed twice with fresh Chalkley's
medium by centrifugation at 60 g. for 2 minutes, the fluid
suspending the cell was then restored to 0.4 ml. to which
0.3 ml. of phage antiserum diluted 1:5 in Chalkley's medium

TABLE XIII.

Phagocytosis of phage P_{22} by A. proteus.

Time (min.)	Phage associated with amoebae	
	Plaque count per tube	Percentage of the inoculum
Zero	782	0.008
10	1150	0.012
30	2795	0.028

was added and the mixture incubated further at 20° for 10
minutes to allow phage inactivation to occur. After wash-
ing once more, the amoebae were plated on streptomycin agar
plate followed by overlaying with 5 ml. of melted agar prev-
iously inoculated with 4 drops of overnight broth culture
of S. typhimurium LT₂. Plaque counts were made after in-
cubation at 37° for 18 hours.

The result in Table XIII showed that the number of
phage particles which remained associated with amoebae,
even after 30 minutes contact, was very small (only one
phage particle per two amoebae). In phagocytosis exper-
iments, it was commonly found that there was on the average
only one bacterium in association with an amoeba; it is
highly unlikely, therefore, to visualise a single phage
particle to enter the cell and become incorporated in the
same vacuole as the ingested bacterium. It is reasonable
to suppose that uptake of phage particles by amoebae was
negligible with regard to the number of bacteria phagocytosed.

d) Demonstration of lytic ability of a constant number
of phage P₂₂ for a varying number of S. typhimurium LT₂.

The result from phagocytic experiments described
earlier in this chapter showed that the number of bacteria
associated with amoebae at different time of recovery varied;
it is important therefore to determine whether bacterial lysis
by phage occurs at the same degree when varying numbers of
bacteria are present.

0.02 ml. of phage suspension containing 10^7 PFU
was added to 4.0 ml. of amoeba-bacterium mixture contain-
ing 5000 amoebae and a varying number of bacteria ranging
from 5×10^2 to 5×10^4 organisms per tube.   The mixture
was incubated at $20°$ for 20 minutes, after which 0.3 ml. of
phage antiserum (diluted 1:5 in Chalkley's medium) was add-
ed to inactivate further phage lytic action.   After incub-
ation at $20°$ for 10 more minutes, an appropriate amount of
Chalkley's medium was added and the supernatant was plated
on streptomycin agar for a bacterial count.   Control tubes
were also set up, and these were treated similarly to those
in the test system with the exception that no phage was
added.   The result in Table XIV showed that phage lytic
activity was effective in eliminating a wide range of S.
typhimurium present in the supernatant.

d)   Elimination of extracellular S. typhimurium LT$_2$
from amoeba suspensions.

This experiment was done to demonstrate that the
majority of bacteria associated with washed amoebae after
a period of phagocytosis were in an intracellular location.
The detail of the experimental procedure has been described
in Chapter II (p. 69a.).   The result in text fig.10 and Table
XV shows that the number of bacteria recovered from phage
treated and untreated amoebae was comparable, and hence
indicated that very few salmonellae were situated outside
cells, i.e. where phage could adsorb to them.   The observ-

TABLE XIV.

Lytic ability of a constant number of phage P_{22} for a
varying number of S. typhimurium LT_2.

Recovery of bacteria from the supernatant	After phage treatment		No phage treatment	
No. bacteria in the inoculum	Total recov. per tube	Percent. survival	Total recov. per tube	Percent. survival
581	21	3.6	668	115
5494	148	2.7	5109	93
44971	4221	9.4	46000	102
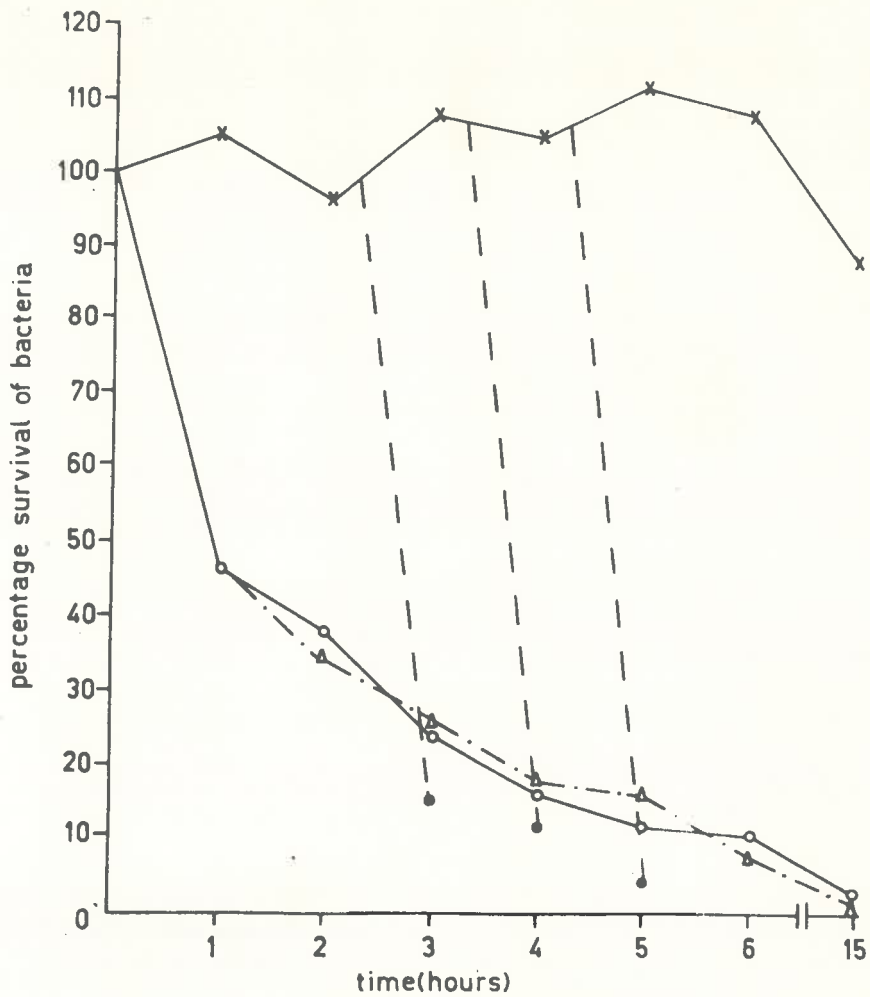
Text-fig. 10. Use of lytic phage P_{22} to demonstrate intracellular location of Salmonella typhimurium LT_2 after apparent phagocytosis by Amoeba proteus.

o————o   Bacteria recovered from amoebae.

Δ—·—·—·Δ   Bacteria recovered from amoebae
                  after phage treatment.

x————x   Control growth curve.

●— — — ●   Control growth curve after phage
                  treatment.

TABLE XV.

Survival of phagocytosed unopsonised S. typhimurium LT_2 in A. proteus with and without phage treatment.

Time after 60 min. period allowed for phagocytosis (hours)	Bacteria recovered from amoebae				Growth curve control for extracellular bacteria			
	No phage treatment		With phage treatment		No phage treatment		With phage treatment	
	Total recovery per tube	% survival	Total recovery per tube	% survival	Total recovery per tube	% survival	Total recovery per tube	% survival
Zero	6014	100	-	-	10896	100	-	-
1	2882	47.9	-	-	11495	106.5	-	-
2	2247	37.3	2122	35.3	10560	97.8	-	-
3	1504	24.8	1573	26.1	11660	108.0	1645	15.5
4	980	16.3	1040	17.3	11440	105.9	960	10.8
5	745	12.3	955	15.8	12039	111.9	25	3.9
6	704	11.5	503	8.3	11737	108.7	-	-
15	197	3.2	94	1.5	9460	87.7	-	-

Average number of amoebae per tube = 5920.

ation that phage P$_{22}$ was able to reduce the count of viable bacteria by as much as 90% in the control tubes, confirmed the phage to be a potent lytic agent for extracellular S. typhimurium under the conditions of these experiments.

6.   Phagocytosis of salmonella by A. proteus when the organisms were ingested in food vacuoles together with heat killed ciliates.

When bacteria and amoebae were mixed as described in the preceding section, it was found that appreciable numbers of the organisms were phagocytosed provided that the ratio of bacteria to amoebae is very high.   Killing of the ingested organisms was presumed to occur in the vacuoles free from food particles.   In view of the fact that A. proteus feeds mainly on ciliates, it may be reasonable to suppose that uptake of bacteria in its natural environment is an inevitable consequence of phagocytosis of ciliates. It would be interesting to know whether there is any quantitative difference between killing of salmonellae in ciliate-free vacuoles and vacuoles containing ciliates.

In order to be sure that bacteria phagocytosed together with heat killed ciliate was in fact located in food vacuoles, an experiment was performed whereby a constant number of S. enteritidis were allowed to be phagocytosed by A. proteus in the presence and absence of heat killed tetrahymena.   0.5 ml. aliquot of washed amoebae suspension containing 6000 amoebae was prepared in 4 standard Leighton

tubes.   Two of them were inoculated with 0.5 ml. of bac-
terium ciliate mixture containing 10^5 ciliates and 2×10^6
bacteria per ml. and the other two were added with the same
amount of bacterial suspension but without ciliates.   After
incubation at 20° for one hour, the amoebae were washed 5
times at 4° before plating out on streptomycin agar for a
bacterial count.   The average result was shown in Table
XVI.   It can be seen that the number of bacteria recovered
from the tubes containing heat killed tetrahymena was 4.3
times greater than that in which the ciliate was absent.
It follows that approximately 80 per cent. of bacteria phag-
ocytosed with ciliates was located in food vacuoles.

Survival in _A. proteus_ of _S. enteritidis_ phagocyt-
osed together with heat killed tetrahymenae was determined
using the technique described in Chapter II, p.71.   The
results given in text - fig.11 and Table XVII showed that
killing of _S. enteritidis_ by _A. proteus_ in the first 3 hours
was very similar to those reported earlier in this Chapter,
but after this period the number of bacteria associated
with amoebae remained exceedingly high.   Since the ingested
bacteria were presumed to be in the same vacuole as the in-
gested tetrahymena, it is likely that degradation product
from the ciliate might condition the vacuolar environment
so that it became less inimical to the bacterial cells.
Moreover, enzymes secreted into the vacuoles may be used

TABLE XVI.

Phagocytosis of S. enteritidis by A. proteus in the presence and absence of heat killed tetrahymena.

Number of bacteria ingested in the presence of ciliate	Number of bacteria ingested in the absence of ciliate
2795	648
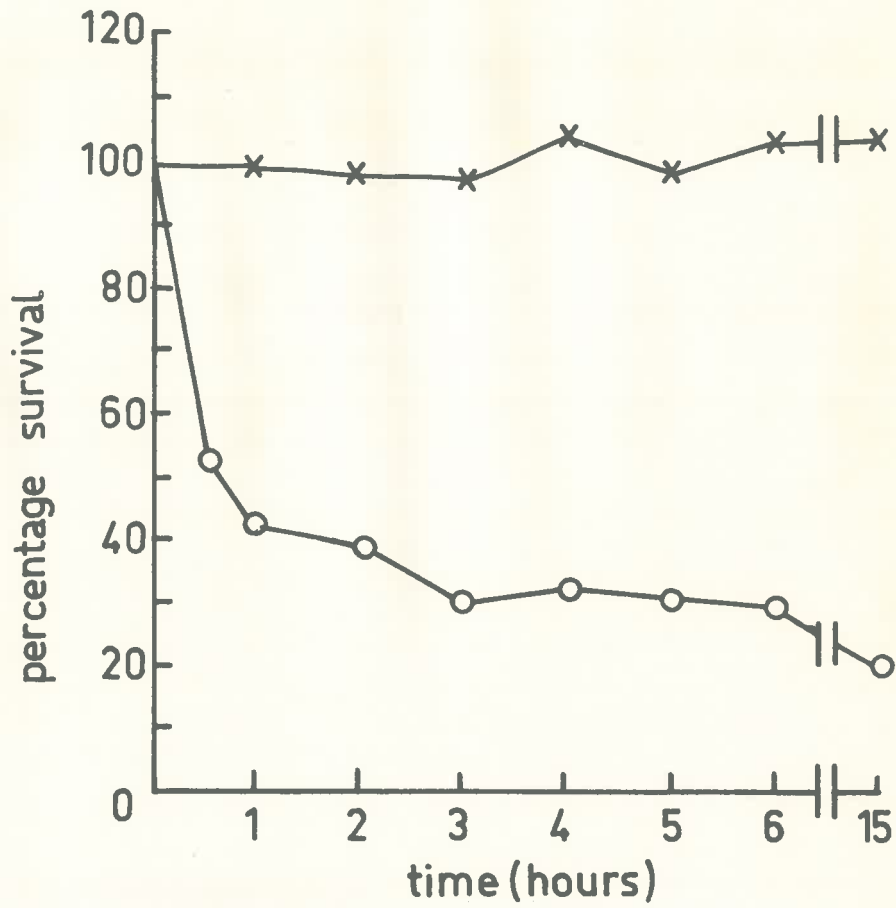
Text-fig. 11. Killing by **Amoeba proteus** of **Salmo-
nella enteritidis** phagocytosed together with heat
killed tetrahymenae.

Each slope represents the mean of three exper-
iments.

o———o   Bacteria recovered from amoebae.
x———x   Control growth curve.

TABLE XVII.

Survival in A. proteus of S. enteritidis Se 795 phago-cytosed together with heat killed tetrahymenae.

Time after 60 min. period allowed for phag-ocytosis (hours)	Bacteria recovered from amoebae		Growth curve control for extracellular bact.	
	Total recov. per tube	Percent. survival	Total recov. per tube	Percent. survival
Zero	5685	100	16317	100
$\frac{1}{2}$	3002	52.8	-	-
1	2496	43.9	16170	99.0
2	2266	39.8	16059	98.4
3	1750	30.8	15855	97.1
4	1829	32.2	17034	104.3
5	1809	31.8	16122	98.8
6	1685	29.6	16821	103.1
15	1089	19.2	17028	104.4

x This result was an average of 3 experiments.

Average No. amoebae per tube = 6000.

Average inoculum size per tube = 2.9 x 10^6 organisms.

for ciliate digestion. If these enzymes also function in intracellular killing, it is not difficult to conceive that its concentration would not be high enough to exert a bactericidal effect.

Discussion.

It is clear that *A. proteus* is able to phagocytose salmonellae and that mammalian serum opsonins increase the uptake of these bacteria, but only to a very limited extent. Such a finding contrasts strongly with the now generally accepted belief that serum opsonins are highly effective in promoting phagocytosis of bacteria by mammalian phago- cytes (Jenkin, 1963). These experiments also show that the presence of serum opsonins on the surface of *S. enter- itidis* or *S. typhimurium* does not influence their survival time within amoeba vacuoles.

It is evident from these studies that survival time of *S. enteritidis* within amoebae is related to the route by which the bacteria enter the cell, i.e. whether phagocytosed naturally or introduced directly into the cytoplasm by micro-injection. In view of the fact that the majority of injected bacteria were apparent in the vacuoles only at 6 hours after injection and only few of them were enclosed in vacuoles at 2 hours after injection, it is reasonable to suppose that the difference in survival time of *S. enter- itidis* was in part due to bacteria being in functionally

different location inside the cell, that is to say, in the
cytoplasm and in the vacuole. Ample evidence is available
to indicate that vacuolar environment is different from
that in the cytoplasm. The hydrogen ion concentration,
for example, was shown by Mast (1942) to vary between pH
5.6 to 7.3, whereas that in the cytoplasm was estimated to
be 7.4. Actual cytoplasmic pH as measured by injection of
indicator dyes into the cytoplasmic ground substance of A.
proteus was lower than that estimated by Mast, i.e. the
cytoplasmic pH was found to be 6.8 ± 0.2 instead of 7.4
(Wiercinski, 1944).

The study of Holter (1954) and Holter and Lowy
(1959), showed that A. proteus contained a variety of hydro-
lytic enzymes and at least one of them, i.e. acid phosphat-
ase was reported to be concentrated in pinocytotic and food
vacuoles (Novikoff, 1960; Birns, 1960; Muller et al.,
1962). It is also known that in mammalian phagocytes,
cytoplasmic granules rich in hydrolytic enzymes discharged
their contents into the vacuoles (Hirsch and Cohn, 1960;
Hirsch, 1962; Cohn and Weiner, 1963; North and Mackaness,
1963; Lockwood and Allison, 1963, 1964).

In the light of this evidence, it is conceivable
that the milieu within the vacuole is in a state of contin-
uous shifting in both the hydrogen ion concentration and
the enzyme contents: it is therefore not surprising if such

an environment is more detrimental to the test bacteria
than that in the cytoplasm. However, this view is only
speculative, since no causal relationship has been estab-
lished between these factors and cellular bactericidal
effectiveness.

The other explanation for an apparent slow rate
of killing of S. enteritidis introduced by micro-injection
would be a proportionately greater number of bacteria ent-
ering cells. In phagocytosis experiment, there was on
the average only one bacterial cell associated with one
amoeba; but in micro-injection study, one amoeba was
loaded with 30-40 bacteria. If cellular bactericidal
mechanisms could cope only with certain number of bacteria
at any particular time, it is conceivable that the effic-
iency in killing of only one organism would be far better
than killing of a greater number of bacteria. It is true
that in micro-injection there was no obvious morphological
change, nevertheless physiological disturbance in amoeba
as a result of this should not be overlooked. It might
be that after injection, amoebae have to readjust them-
selves and therefore no immediate action was directed against
the injected microbes: this would then allow the bacteria
sufficient time to adjust themselves in the new environment.

Conclusion.

1. Mammalian serum opsonins enhanced phagocytosis of
S. enteritidis by A. proteus, but the degree of enhancement

was very small as compared to that which would be expected
if mammalian phagocytes were used. The increase in uptake
was in part due to agglutination of bacteria which occurred
when the amount of opsonins used was in excess of one min-
imal agglutinating dose.

2. Serum opsonins had no measurable effect on the rate
of intracellular killing of S. enteritidis or S. typhimurium.

3. The survival time of S. enteritidis in A. proteus
appears to be related to the route by which the bacteria
entered the cells, and the faster rate of intracellular
killing was apparent when the organisms were phagocytosed
naturally.

4. Intracellular location of the phagocytosed S. typhi-
murium was demonstrated by using lytic phage P_{22} to elimin-
ate extracellular bacteria.

5. The rate of killing of S. enteritidis ingested to-
gether with heat killed ciliates in the first three hours
after phagocytosis was comparable to that in the absence of
ciliates, but the bacteria recovered subsequently remained
exceedingly high. This was attributed to modification of
vacuolar environment as a result of ciliate digestion.

CHAPTER V.

THE EFFECT OF PREVIOUS EXPERIENCE TO BACTERIAL SUBSTRATE ON THE ABILITY OF A. PROTEUS TO PHAGOCYTOSE AND KILL SALMONELLAE.

In higher vertebrates, immunity to infectious agents depends on both cellular and humoral factors, but this pattern of immunity is not clearly established in invertebrates. In fact, classical immune response has never been demonstrated in animals phylogenetically lower than a primitive cyclostome, the lamprey (Finstad and Good, 1964). Failure to produce antibody in the lowest form of vertebrates, e.g. the hagfish was found to be associated with the absence of proteins migrating as gamma globulins and the lack of lymphoid cells (Papermaster, Condie, Finstad and Good, 1964). Nevertheless, invertebrates such as insects could be artificially immunised against pathogenic organisms, but the pattern of immunological response was very distinct and it has been suggested that the concept of immunity in the lower forms of life should be considered separately from that in the vertebrates (Briggs, 1958).

In protozoa, reactions similar to immune response have been reported. Roux et al. (1964) showed that washed tetrahymenae which were previously grown with E. coli became resistant to an otherwise lethal action of this organism. When adapted tetrahymenae and E. coli were mixed,

agglutination occurred.   This reaction was specific, since
no clumping was apparent when S. paratyphi B was used in
the test.

In free living amoebae, responses to certain stim-
uli might probably have immunological basis.   For instance,
transfer of the nucleus or the cytoplasm from one individual
to the other was successful only when host and donor are
cells of the same or closely related species (Comandon and
de Fonbrune, 1939; Lorch and Danielli, 1953; Daniels, 1962).
Reynolds (1924) reported that a fragment of a pseudopod
severed from a shell amoeba, arcella, would be incorporated
into the other individual which was maintained in the same
environment.   Incompatibility occurred when the individual
tested was reared in a different environment.   Furthermore
the fragment was not incorporated into a descendant of the
same parental cell which had been living and dividing in
different environment as early as 7 days after separation.

The main purpose of the study described in this
chapter was to investigate whether A. proteus could be stim-
ulated to exert enhanced phagocytosis and bactericidal ef-
fectiveness after a prolonged period of contact with hom-
ologous organisms.   Two experimental approaches were at-
tempted, viz. by repeated injection of bacteria into the
same cells and by a prolonged exposure of amoebae in cult-
ure dishes to a high concentration of living organisms.
After a certain period of contact with bacterial substrates

these amoebae were challenged with homologous organisms
either by micro-injection or by a natural process of phago-
cytosis.

  1. Micro-injection studies.

    a) Immunisation in A. proteus by repeated injection
of living bacteria.

    Seventeen amoebae were used to initiate a population
of 'immunised' cells.   Each amoeba was injected daily with
approximately 60 to 80 log. phase S. enteritidis cells sus-
pended in Chalkley's medium plus 0.5% gelatin.   After in-
jection, the amoebae were washed and maintained at 20° in
Chalkley's medium in depression slides placed in moist cham-
bers.   The protozoa were fed on an alternate day with wash-
ed tetrahymenae and the medium was changed every 3 days.
When amoebae divided both daughter cells were given bacter-
ial injection.   The procedure was carried on for 7 days
and the total numbers of amoebae at 24 hours after the last
'immunising' injection rose up to 50.   This number did not
include the cells which were inactive or otherwise damaged
by the micro-manipulative procedures.

    b) Micro-injection of S. enteritidis in 'immunised'
A. proteus.

    The amoebae treated in the manner described above,
the 'immunised' cells, were challenged at 24 hours and 7
days after the last 'immunising' injection with a known num-
ber of log. phase S. enteritidis cells suspended in Chalk-

ley's medium containing 0.5% gelatin using the technique described in Chapter II (p.60). This interval of time was chosen basing on the assumption that any enhanced bactericidal ability in amoebae at 24 hours after injection would be due to physiological changes in response to repeated injections and that after 7 days due to immunological reactions. At 2 and 6 hours after challenge, the amoebae were disrupted on nutrient agar for a bacterial count. Since the organisms used in 'immunisation' and those used in the test were the same strain, it is exceedingly important to know the number of viable bacteria remaining in the cells at 24 hours after the last 'immunising' dose. This was done simply by plating out some of the 'immunised' amoebae on nutrient agar plate for a bacterial count. It was expected that this bacterial number would be negligible since, in a preliminary test, it was found that at 24 hours after injection only one bacterium was recovered from 3 amoebae sacrificed after having been 'immunised' for 2 or 3 days.

        c) <u>Results</u>.
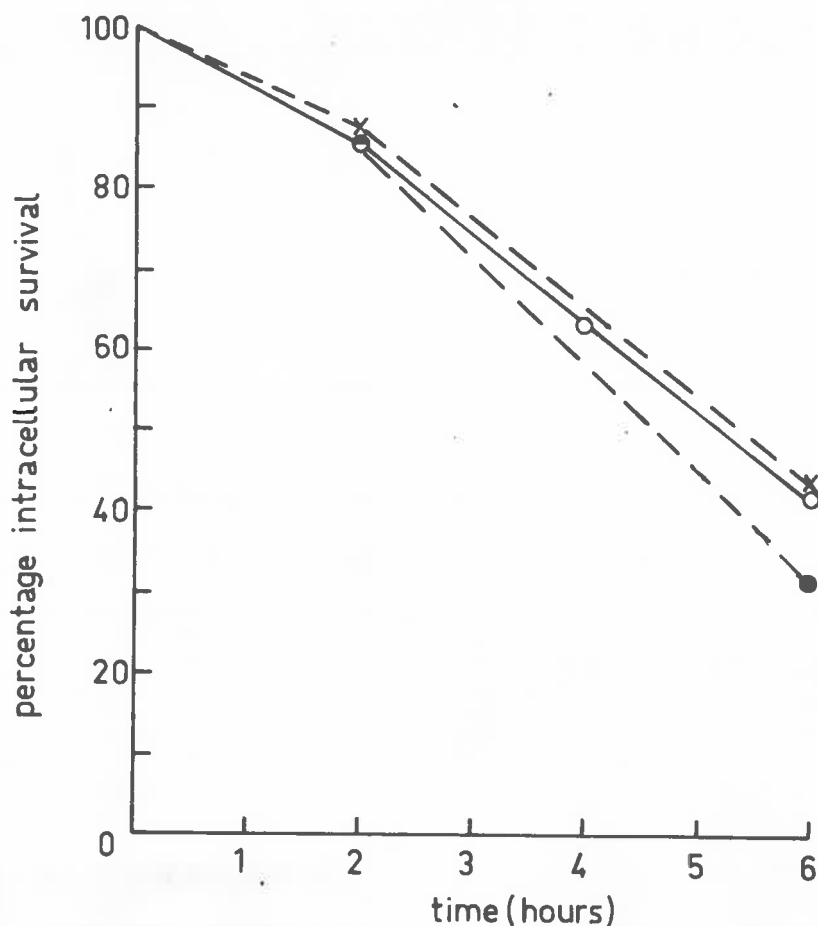
        1. <u>At 24 hours after 'immunisation'</u>.

The percentage survival of <u>S. enteritidis</u> in 'immunised' <u>A. proteus</u> at 2 and 6 hours after injection is shown in text - fig.12. The data on which these results are based are set out in Table XVIII. It is clear that the organisms injected into the cells were killed and the rate of killing

did not appear to be significantly different from that of normal amoebae. Despite the fact that the challenged organisms did not have any marker to be distinguished from those injected into the cells during the course of 'immunisation', the assessment of bactericidal effectiveness was still valid since the number of bacteria recovered from 10 untreated control amoebae was either nil or negligible. Furthermore, it was found that no bacteria could be recovered from the untreated daughter cells of amoebae no. 7 in the first column of Table XVIII and no. 3 in the second column of the same Table: these amoebae divided while they were in microdrops before challenge and this gave us an opportunity to use one daughter cell in the test and the other as a control. In view of the findings that only insignificant numbers of bacteria could be recovered from A. proteus at 24 hours after the last dose of a series of injections, it can be envisaged that in nature it is unlikely for S. enteritidis or related salmonellae to be able to be parasitised in the cytoplasm of A. proteus.

2. At 7 days after 'immunisation'.

The cells used in this study were descendants of 16 amoebae remaining from the preceding experiment. After successive multiplication, the total cell numbers at the beginning of the experiment rose up to 89. These 'immunised' amoebae were challenged with known numbers of S. enteritidis and the survival of bacteria in the cells at 2 and 6

Text-fig. 12. Recovery of <u>Salmonella enteriti</u>-<u>dis</u> after injection into the cytoplasm of 'immunised' <u>Amoeba proteus</u>. The cells were challenged at 24 hours and 7 days after the last 'immunising' injection.

o————o   Unimmunised control.

●– – – –●   Amoebae challenged at 24 hours
                after the last 'immunising'
                injection.

x– – – –x   Amoebae challenged at 7
                days after the last
                'immunising' injection.

TABLE XVIII.

Recovery of S. enteritidis at different time intervals from 'immunised' A. proteus challenged at 24 hours after the last 'immunising' injection.

Am-oeba No.	Recovery at 2 hrs. after injection			Recovery at 6 hrs. after injection			Recovery from un-treated control
	No. bac. inj.	No. bac. recov.	% sur-vival	No. bac. inj.	No. bac. recov.	% sur-vival	
1	53	45	84.9	58	17	29.3	0
2	52	43	82.7	50	1	2	0
3	48	44	91.7	56	18	32.1	0
4	48	46	95.8	54	25	46.3	0
5	50	49	98.0	52	10	19.2	0
6	49	29	59.2	47	21	44.7	1
7	46	46	100.0	50	11	22.0	0
8	52	43	82.7	48	35	72.9	0
9	41	30	73.1	48	2	4.2	0
10	50	44	88.0	52	22	42.3	1

	Average	85.6	Average	31.5
	S.D.	12.2	S.D.	21.1

TABLE XIX.

Recovery of S. enteritidis at different time intervals from 'immunised' A. proteus challenged at 7 days after the last 'immunising' injections.

Amoeba No.	Recovery at 2 hrs. after injection			Recovery at 6 hrs. after injection			Recovery from untreated control amoebae
	No. bac. inj.	No. bac. recov.	% survival	No. bac. inj.	No. bac. recov.	% survival	
1	45	38	84.4	57	29	50.9	0
2	22	19	86.4	51	23	45.1	0
3	51	48	94.1	49	21	42.9	0
4	45	38	84.4	48	29	60.4	0
5	45	37	82.2	53	20	37.7	0
6	43	35	81.4	47	17	36.2	0
7	52	51	98.1	41	13	31.7	0
8	57	54	94.7	49	20	40.8	0
9	46	42	91.3	47	17	36.2	0
10	45	39	86.7	48	25	52.1	0
11	42	38	90.5	48	20	41.7	0
12	46	37	80.4	-	-	-	0
13	48	43	89.6	-	-	-	-

Average 82.0   Average 43.2

S.D. 5.7   S.D. 8.8

hours after injection was measured.   The results in text -
fig.12 and Table XIX show that the rate of intracellular
killing of the test salmonella was comparable to that of
normal amoebae or 'immunised' amoebae at 24 hours after the
last 'immunising' injection.

2.   Phagocytosis experiments.

In the preceding experiments, attempts to enhance
bactericidal ability of A. proteus by means of repeated
micro-injection of bacterial substrate were not successful.
It was thought that the failure to respond could be due to
the way in which bacteria entered the cells.   It may be
possible that acquisition of enhanced intracellular bacter-
icidal effectiveness could be achieved only when the bacter-
ia enter cells by a natural process of phagocytosis.   In
order to study this possibility, experiments were performed
by which phagocytosis and bactericidal ability of A. proteus
was measured after the cells had been cultured in the pres-
ence of streptomycin sensitive S. typhimurium LT_2 for a
given period of time.

a)   Preparation of 'experienced' amoebae.

Washed overnight minimal medium culture of strepto-
mycin sensitive strain of S. typhimurium LT_2 suspended in
Chalkley's medium was added to cultures of A. proteus twice
weekly, so that the bacterial concentration in the culture
vessels was maintained between 10^5 to 10^6 organisms per ml.

These amoebae were used after having been exposed to the
bacterial substrate for 3 or 6 months.

b) Phagocytosis of S. typhimurium LT$_2$ by 'normal'
and 'experienced' A. proteus.

'Experienced' amoebae used in this experiment had
been cultured in the presence of streptomycin sensitive S.
typhimurium LT$_2$ for 3 months. Washed 'normal' and 'ex-
perienced' cells were dispensed in 0.1 volume in two series
of tubes each containing 6000 amoebae. It is essential for
this type of experiment that the number of normal and 'ex-
perienced' cells in each tube should be comparable. In
preparing cell suspensions containing a standard number of
amoebae, 0.05 ml. volume was taken from 10 ml. suspension of
thoroughly washed amoebae in siliconised graduated centri-
fuge tube and transferred into an appropriate amount of
Chalkley's medium in a depression slide which had been arb-
itrarily divided with marking ink into a number of squares.
The cells were then counted under a dissecting microscope
and finally the supernatant was appropriately removed from
the stocked amoeba suspensions so that the cell concentration
would be 6000 cells per 0.1 ml. 0.02 ml. of washed lag.
phase culture of streptomycin resistant S. typhimurium LT$_2$
suspended in Chalkley's medium containing 0.1% glucose was
then added to each tube. After incubation at 20° for either
15 or 70 minutes, duplicate tubes in each series were taken
out, the cells were washed 4 times at 4° and finally plated

out on streptomycin agar.

The result in Table XX shows that the numbers of
S. typhimurium associated with 'normal' and 'experienced'
A. proteus were almost comparable. This finding was inter-
preted to mean that A. proteus could not be induced to exert
more efficient phagocytic ability for salmonella by means
of a continuous exposure to the homologous organism for a
period as long as 3 months.

3.   Intracellular survival of S. typhimurium LT$_2$ in
'experienced' A. proteus.

A. proteus used in this study had been grown in the
presence of streptomycin sensitive S. typhimurium LT$_2$ for 3
or 6 months.   Survival of streptomycin resistant S. typhi-
murium LT$_2$ in 'experienced' amoebae was determined using
the technique described in Chapter II, p.68.   0.02 ml. of
washed lag. phase culture of this organism suspended in
Chalkley's medium containing 0.1% glucose was added to a
series of tubes each containing approximately 6000 cells in
0.1 ml.   After incubation at 20° for 1 hour, the amoebae
were washed and finally transferred into a series of Leigh-
ton tubes within which the cells were incubated further for
a given period of time.   Recovery of bacteria from 'exper-
ienced' amoebae was done at intervals and the rate of intra-
cellular survival of the test salmonella determined.

The rates of intracellular killing of S. typhimurium
LT$_2$ by 'experienced' A. proteus which had been exposed to

TABLE XX.

Phagocytosis of _S. typhimurium_ LT_2 by 'normal' and 'experienced' _A. proteus_.

Time of recovery (min.)	No. of bacteria associated with amoebae at various time intervals after initial contact	
	Normal amoebae	Experienced amoebae
15	1666	1741
70	6486	7632

No. of amoebae per tube = 6000

Inoculum size per tube = 10^6
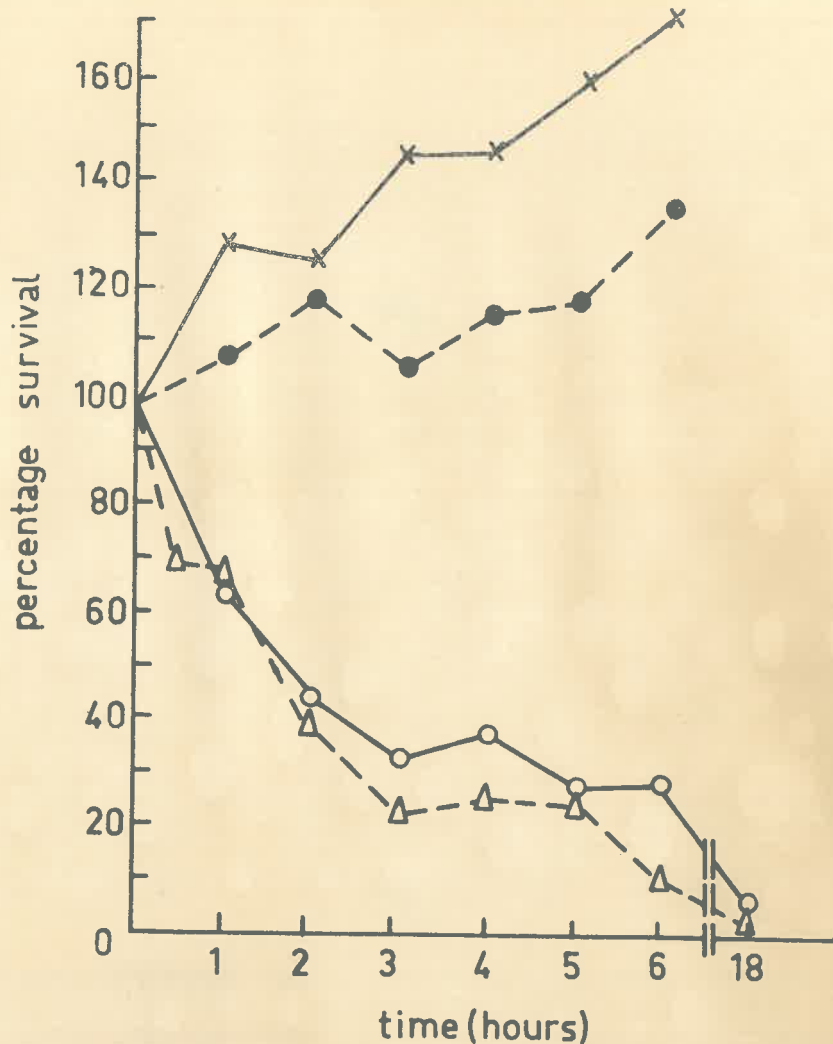
Text-fig. 13. Killing of phagocytosed <u>Salmonella</u> <u>typhimurium</u> LT$_2$ by 'experienced' <u>Amoeba proteus</u>.
Each slope represents the mean of two experiments.

	Amoebae exposed to the bacterial substrate for 3 months.
o————o	Bacteria recovered from amoebae.
x————x	Control growth curve.

	Amoebae exposed to the bacterial substrate for 6 months.
△— — —△	Bacteria recovered from amoebae.
●— — —●	Control growth curve.

TABLE XII.

Survival of S. typhimurium LT$_2$ after phagocytosis by A. proteus which had been cultured in the presence of streptomycin sensitive strain of this organism for 3 months.

Time after phago- cytosis (hours)	Bacteria recovered from amoebae		Growth curve control for extracellular bact.	
	Total recov. per tube	Percentage survival	Total recov. per tube	Percentage survival
Zero	7525	100	6300	100
1	4748	62.9	8151	129.3
2	3859	43.1	7920	125.7
3	3122	32.6	9152	145.2
4	3632	37.3	9251	146.8
5	2718	26.5	10131	160.8
6	3055	27.6	10945	173.7
18	658	7.6	-	

Average inoculum per tube = 6.4 x 10^5 organisms.

Average number of amoebae per tube = 6217.

This result was an average of two experiments.

TABLE XXII.

Survival of **S. typhimurium** LT$_2$ after phagocytosis by
A. proteus which had been cultured in the presence of
streptomycin sensitive strain of this organism for 6
months.

Time after phago-cytosis (hours)	Bacteria recovered from amoebae		Growth curve control for extracellular bact.	
	Total recov. per tube	Percentage survival	Total recov. per tube	Percent. survival
Zero	5835	100	7233	100
¼	4002	68.5	-	-
1	3717	63.7	7782	107.6
2	2258	38.6	8629	119.3
3	1386	23.7	7658	105.9
4	1467	25.1	8341	115.3
5	1457	24.9	8585	118.7
6	647	11.1	9889	136.7
18	176	3.0	-	-

Average inoculum per tube = 7.5 x 10^5 organisms.

Average number of amoebae per tube = 6000

This result was an average of two experiments.

the bacterial substrate for 3 and 6 months are shown in text - fig.13. The data on which these results are based are set out in Table XXI and Table XXII. It can be seen that these were not significantly different from that of 'normal' amoebae (Chapter IV, p.106). The apparent faster rate of extracellular multiplication of the test salmonella in the control in Table XXI should not have any interference with the assessment of intracellular killing, since the test amoebae were washed twice more before plating and the small number of bacteria which might have multiplied would also be removed.

Discussion.

It is clear from this study that A. proteus could not be induced to acquire enhanced bactericidal effectiveness, either by repeated injections or by prolonged exposure to bacterial substrates. It follows that development of immune response to microbial infection does not seem to exist in these free living amoebae, and this supports the contention expressed by Papermaster et al. (1964) that host resistance in the lower forms of life appears to be a natural, non-induced capacity. Failure of amoebae to respond to a continuous stimulation by bacterial substrates could be due to the inherent characters of these protozoa. It is known that A. proteus feeds mainly on ciliates and not bacteria. When the bacteria are killed either in the cell

cytoplasm or in the vacuole, their substrates may be degraded in such an extent that they are no longer antigenic; and in this respect amoebae resemble mammalian polymorphonuclear leucocytes but not peritoneal macrophages, since it has been reported by Cohn (1964) that no antibody against E. coli was produced in mice after this organism had been killed and digested by rabbit polymorphonuclear cells for a given period of time. On the other hand, it is possible that antigenicity of ingested salmonella in A. proteus is retained, but these protozoa by nature are refractory to the stimulation by bacterial antigens. As a result, no specific immunological response is directed against bacteria which enter the cells. The other possibility which merits consideration is that pertaining to the nutritional state of A. proteus used in the test. In view of the fact that the protozoa were not starved during the entire period of exposure to bacterial substrates, it is conceivable that in the continued presence of abundant supply of ciliates, amoebae do not have to adjust themselves to feed on bacteria, since such an adaptation would not provide cells of any survival advantage.

Conclusion.

1. Repeated daily injections of living S. enteritidis into the cytoplasm of A. proteus for 1 week did not stimulate these cells to develop enhanced bactericidal mechanisms for homologous organisms challenged at 24 hours and 7 days

after the last 'immunising' injection.

2.   Continued exposure of $A.$ $proteus$ in culture dishes to living $S.$ $typhimurium$ LT_2 for a period of 3 and 6 months did not endow these protozoa in acquiring more efficient phagocytosis ability or enhanced bactericidal effectiveness.

CHAPTER VI.

SELECTION OF BACTERIAL FOOD BY SOIL AMOEBAE.

Evidence is available which indicates that soil amoebae do not feed indiscriminately, i.e. they ingest preferentially certain species of bacteria and disregard others (Severtzova, 1928; Singh, 1941, 1942, 1945). However, the mechanisms whereby selection is achieved are not entirely understood. The works of Singh (1941, 1942, 1945) and Severtzova (1928) showed that there was a parallelism between selection and edibility of bacterial food, but criteria for edibility were not related to any taxonomic characters, e.g. Gram staining, motility, slime production, pigmentation, proteolytic power and ability to nitrify and fix nitrogen. Ray (1951) showed that the growth rates of a limax amoeba in 0.1 per cent. yeast extract solution to which bacteria had been added could be correlated with the degree of bacterial motility. This is perhaps not surprising, since motile bacteria are known to become agglutinated on the amoeba surface after contact (Mouton, 1902; Ray, 1951), and it is conceivable that far greater numbers of actively motile organisms would be made available to the amoebae. However, the possession of flagella per se seems likely to act through purely for mechanical reasons, since Ray (1951) showed that on a solid medium, streaks of motile and non-motile strains were cleared by soil amoebae with

the same avidity.

Failure of amoebae to grow in the presence of certain bacteria may be due to toxic properties of microorganisms (Cutler and Crump, 1928; Van Rooyen, 1932; Singh, 1945; Dudziak, 1962).   The mechanisms by which these toxic substances act are not known.   Singh (1945) reported that amoebae would die or encyst within 7 days if they were grown in the presence of diffusible products of Ps. pyocyaneus or non-diffusible substances from certain species of chromobacteria.   Dudziak (1962) on the other hand, maintained that in the case of mycobacteria, the toxic substances acted in such a way that amoebae could neither multiply nor encyst.

Drozański (1963) presented data which indicated that selection in soil amoebae might operate at the level of food ingestion.   He showed that intact aerobacter killed by boiling at 100° did not support the growth of certain species of limax amoebae maintained in normal saline, but an aqueous extract of boiled organisms acted as an adequate source of nutritive material when added to the amoebae in normal saline.

The main purpose of the studies described in this chapter was to re-investigate the problem of food choice mechanisms in soil amoebae.   It was hoped that this study would provide some information concerning the level at which selection is operated.   In the proposed scheme of analysis

the following items were included:-

1. Chemotaxis.

2. Selective ingestion when bacteria of different degrees of acceptability were present together in a colony of mixed cultures.

3. The rate of intracellular killing.

4. The growth rate of amoebae feeding on colonies of various microbial species.

1. <u>Selection of suitable species of soil amoebae and bacteria for a study of food selection in these protozoa.</u>

For rapid selection of suitable species of soil amoebae and bacteria to be used in the analysis of the mechanisms of recognition of bacterial food by these protozoa, the method described by Severtzova (1928) was used (Chapter II, p.73). Overnight broth cultures of bacteria were inoculated either on 0.1 per cent. yeast extract agar or on non-nutrient agar (Singh, 1941) in a form of a radiating star, so that each radius would be consisting of only one bacterial strain. After incubation to allow bacterial colonies to be formed, soil amoebae were inoculated at the centre of the star, and the plates were incubated further at 20^{0} for 8-10 days. Acceptability of the bacteria was assessed according to the method described in Chapter II, p.73.

<u>Result</u>.

The result in Table XXIII shows that different spec-

TABLE XXIII.

Acceptability of bacterial food by various species of soil amoebae.

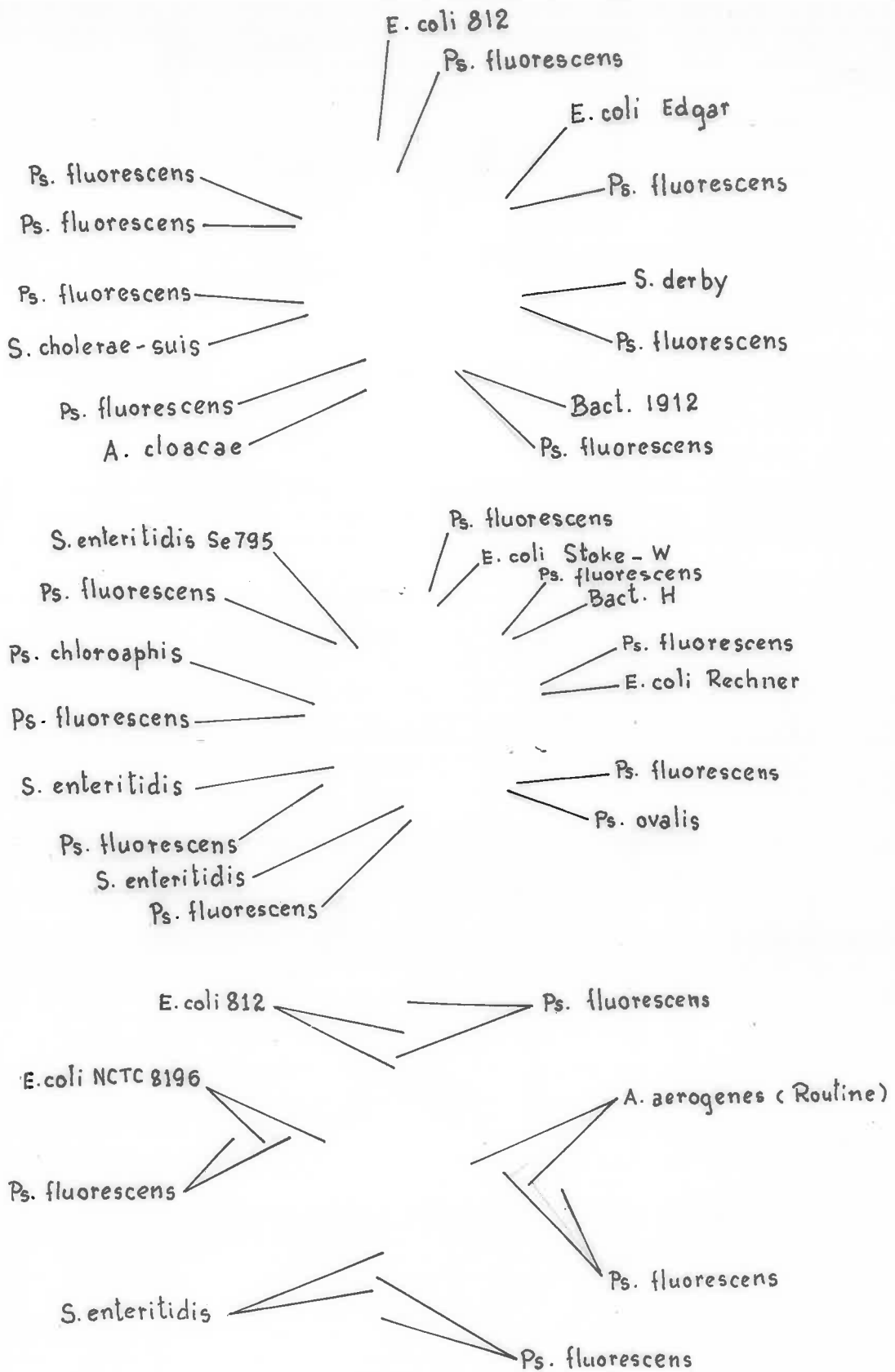|	H.astro-nyxis	H. rhys-odes	Acantham-oeba (Neff)	H. glebae	N. gruberi	Sch. russelli	D.thontoni
Ps. fluorescens	4 +	4 +	4 +	4 +	4 +	4 +	4 +
Ps. ovalis	4 +	4 +	4 +	3 +	4n+	4 +	4 +
Ps. chloroaphis	3 +	4 +	2 +	3 +	4 +	4 +	1 +
Bacterium 1912	3 +	4 +	4 +	4 +	4 +	4 +	4 +
A. cloacae	3 +	4 +	3 +	4 +	4 +	4 +	4 +
S. enteritidis	1 +	4 +	4 +	4 +	4 +	4 +	4 +
E. coli (Rechner)	2 +	2 +	2 +	4 +	4 +	3 +	4 +
E. coli (Stoke-W)	3 +	2 +	2 +	4 +	4 +	3 +	4 +
E. coli (Edgar)	2 +	2 +	2 +	4 +	4 +	3 +	4 +
E. coli 1064	2 +	2 +	3 +	4 +	4 +	4 +	4 +
E. coli 8196	1 +	2 +	3 +	-	0	-	-
E. coli 812	1 +	1 +	1 +	2 +	0	1 +	0
Bacterium H	0	0	0	0	0	0	0
A. aerogenes (routine)	2 +						

ies of bacteria behaved differently with respect to their acceptability as food for soil amoebae. This was not influenced by the medium on which the bacteria were grown, i.e. on 0.1 per cent. yeast extract agar or on non-nutrient agar. It was also observed that H. astronyxis exhibited a sharper zone of clearing and a broader spectrum of selection than other species of amoebae. Furthermore, this amoeba could be grown in both liquid medium and on solid medium, whereas some others can be grown only on solid medium. In the light of these observations, it was decided to use mainly H. astronyxis in the study described thereafter. As regards the bacteria, the following organisms were chosen, viz. S. enteritidis, E. coli 812, E. coli 8196 and bacterium H, since these were not accepted by H. astronyxis.
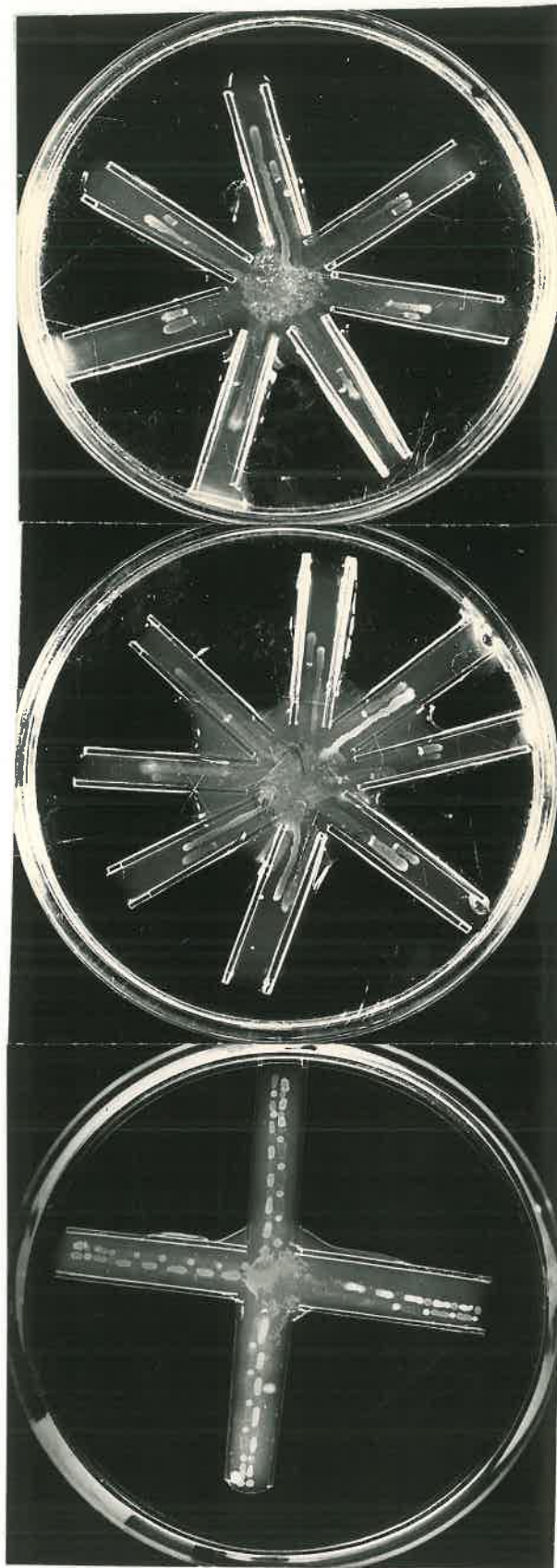
2. Demonstration of food selection.

For a more precise demonstration of food selection, the technique described by Singh (1941) was used (Chapter II, p.74). H. astronyxis used was grown axenically in 3 per cent. yeast extract medium. The result is shown in fig.20, 21. It can be seen that S. enteritidis, E. coli 812 and Bacterium H were not acceptable, thus confirming the result obtained in the preceding section. Similar results were obtained when H. astronyxis used were grown in PPG together with Ps. fluorescens, S. enteritidis, E. coli 812 or E. coli 8196, all of which had been killed by heating at 56° for $1\frac{1}{2}$ hours.

Figs. 20 and 21. Photographs showing select-
ive ingestion of edible bacteria by Hartmanella
astronyxis.

Fig. 22. Photograph showing selective feeding
of bacterial food by Hartmanella astronyxis.
The bacterial colonies were placed alternately
along two lines parallel to each other. The
amoebae migrated in a zigzag manner along the
tract formed by colonies of edible organisms
and left the colonies of inedible organisms
quite untouched.

E. coli 812

Ps. fluorescens

E. coli Edgar

Ps. fluorescens

Ps. fluorescens

Ps. fluorescens

S. derby

Ps. fluorescens

S. cholerae - suis

Ps. fluorescens

Ps. fluorescens

Bact. 1912

A. cloacae

Ps. fluorescens

S. enteritidis Se795

Ps. fluorescens

E. coli Stoke - W

Ps. fluorescens

Ps. fluorescens

Bact. H

Ps. chloroaphis

Ps. fluorescens

Ps. fluorescens

E. coli Rechner

S. enteritidis

Ps. fluorescens

Ps. fluorescens

Ps. ovalis

S. enteritidis

Ps. fluorescens

E. coli 812

Ps. fluorescens

E. coli NCTC 8196

A. aerogenes (Routine)

Ps. fluorescens

Ps. fluorescens

S. enteritidis

Ps. fluorescens

E. coli 812
Ps. fluorescens
E. coli Edgar
Ps. fluorescens
Ps. fluorescens
Ps. fluorescens
Ps. fluorescens
S. derby
S. cholerae - suis
Ps. fluorescens
Ps. fluorescens
Bact. 1912
A. cloacae
Ps. fluorescens

S. enteritidis Se 795
Ps. fluorescens
Ps. fluorescens
E. coli Stoke - W
Ps. fluorescens
Ps. chloroaphis
Bact. H
Ps. fluorescens
Ps. fluorescens
E. coli Rechner
S. enteritidis
Ps. fluorescens
Ps. fluorescens
Ps. ovalis
S. enteritidis
Ps. fluorescens

E. coli 812
Ps. fluorescens
E.coli NCTC 8196
A. aerogenes (Routine)
Ps. fluorescens
Ps. fluorescens
S. enteritidis
Ps. fluorescens

The fineness of selection of bacterial food by soil amoebae was shown in an experiment in which H. astron-yxis grown in PPG in the presence of heat killed Ps. fluor-escens were inoculated on to 0.1 per cent. yeast extract agar plate in which overnight colonies of edible bacteria (Ps. fluorescens) and inedible bacteria (E. coli 812 and E. coli 8196) or less acceptable bacteria (Aerbacter aer-ogenes routine) were placed alternately in two lines paral-lel to each other. The result in fig.22 shows that the amoebae were able to recognise the edible organisms (Ps. fluorescens) and migrated in a zigzag manner along the tract formed by colonies of these organisms.

3. Chemotaxis studies.

Directional migration of mammalian phagocytic cells towards clumps of bacteria has been unequivocally demonstrat-ed (McCutcheon, 1946, 1955; Harris, 1954, 1960). On the contrary, little is known about chemical attraction in am-oebae. The work of Schaeffer in 1916 and 1917 showed that within a certain range from the cells A. proteus could sense certain particles or substances and moved towards them in a definite direction. Recently, it has been reported that A. proteus could be induced to form pseudopods and food cups by extracts of Tetrahymena pyriformis. Hydra viridans and by heparin (Bingley et al. 1962; Jeon and Bell, 1962). There is good reason to believe that these inducers acted by de-

polarising the surface membrane (Bell, 1963), but the act-
ual mechanisms of membrane depolarisation is not known.
Chemotaxis in soil amoebae has never been demonstrated.
In view of the fact that in nature these protozoa feed
mainly on bacteria, it is reasonable to believe that the
ability to locate the whereabouts of bacterial food would
endow the cells with survival advantage.   It was thought
desirable, therefore, to investigate chemotaxis in soil am-
oebae and try to find out whether this has any bearing on
the mechanisms of food selection.

Unless otherwise stated, H. astronyxis used in
these experiments were grown in PPG, together with heat
killed Ps. fluorescens, whereas H. rhysodes and Acanthan-
oeba Sp. (Neff) were grown in PPG alone.

a) Attraction of H. astronyxis by Ps. fluorescens.

A clump of washed Ps. fluorescens incorporated in
agar-agar was added to amoebae attached to a glass cover-
slip in a drop of 0.06 μ PO_4 buffer NaCl solution and the
migration of the cells towards the bacterial clump was re-
corded by a dark ground tracing technique (Chapter II, p.75).
The result was shown in fig.23, and the movement of indiv-
idual amoebae was depicted sequentially in fig.24a, b, c
and d.   It can be seen in these photographs that amoebae
were attracted by the bacterial clump, the range of attrac-
tion being 100-200 microns.   It was also observed that in-
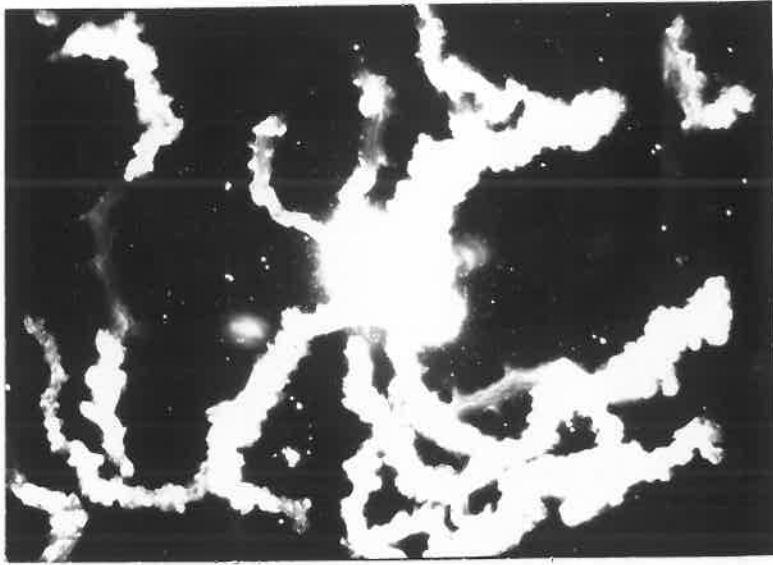gestion of bacteria occurred almost immediately after the

Fig. 23.   The pattern traced out by Hartmanella
astronyxis moving directionally towards a clump of
Ps. fluorescens.   (Exposure 30 minutes, 70 X).

Figs. 24. Serial photographic records show-
ing directional movement of Hartmanella astronyxis
towards a clump of Ps. fluorescens (B). (Phase
contrast).

a. At zero time.

b. Five minutes. The amoeba had moved half-
way towards the clump.

c. Twelve minutes. The amoeba arrived at
the target.

d. Three hours. Numerous amoebae accum-
ulated at the bacterial clump and remained at
this spot for many hours.
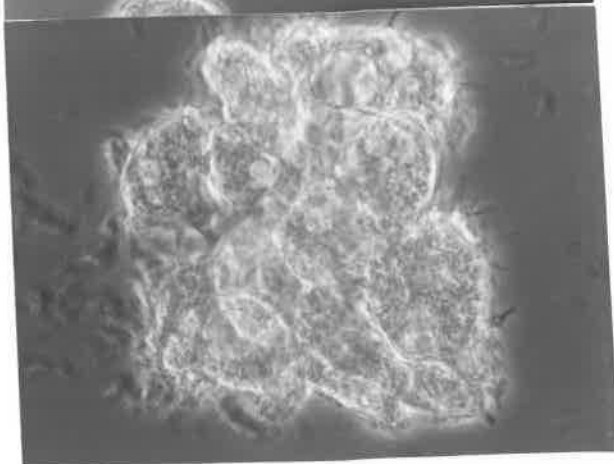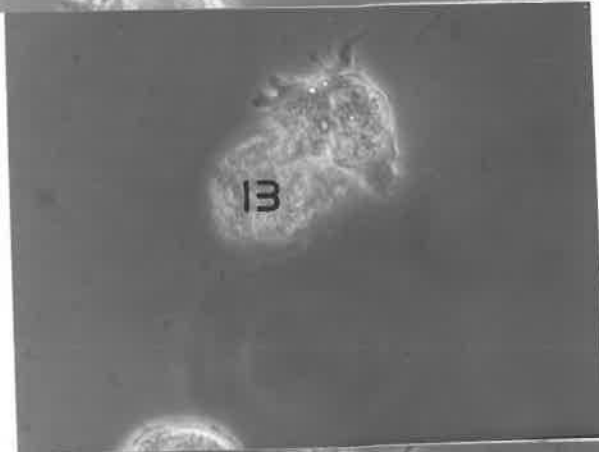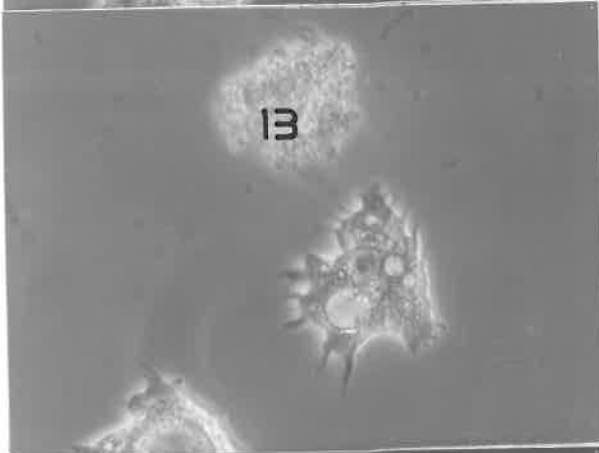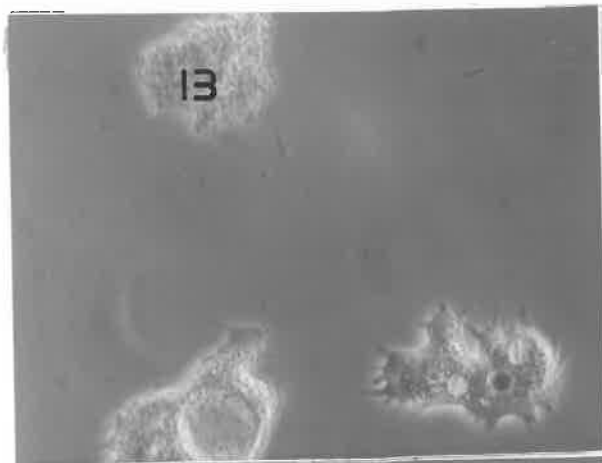
amoebae had arrived at the target, and these cells remained at that spot for many hours. The possibility that attraction of amoebae was due to agar could be ruled out, since movement of these protozoa was randomised when agar alone was present. There is good reason to believe that chemotactic substances were inherently associated with the bacterial cells and not due to acquisition of any factors present in the medium in which bacteria were grown, i.e. PPG, since chemotaxis still occurred when Ps. fluorescens was grown in minimal medium of Davis and Mingioli (1950).

b) Factors affecting chemotactic properties of Ps. fluorescens.

1. Heat inactivation.

An overnight culture of Ps. fluorescens was washed in physiological saline by centrifugation at 3000 r.p.m. for 10 minutes and finally resuspended in the same solution. After heating at either 56° or 100° for half an hour, the cells were washed twice more in physiological saline and finally incorporated in agar to be used in the test. It was found that this organism no longer exerted chemotaxis (see fig.25). Since the bacteria were washed again after heating, it can be argued that heating may dissociate from the cells the chemotactic substances which would be removed by washing. To exclude this possibility, an experiment was done whereby the washed thick residue of Ps. fluorescens was heated for half an hour at 56° and finally incorporated

in agar without further washing.   Here again no chemotaxis
was demonstrated.

2. Killing by alcohol.

Washed Ps. fluorescens suspended in physiological
saline was exposed to 30% alcohol at 4° for 2 hours, after
which the cells were washed and finally incorporated in
agar before being used in the test.   Again it was found
that chemotactic properties were lost.

3. Killing by streptomycin (STM).

Saline suspension of washed Ps. fluorescens was ex-
posed to STM at a concentration of 5000 micrograms per ml.
at 30° for periods of either $1\frac{1}{2}$ or 7 hours.   This was fol-
lowed by further washing and finally the cells were incorp-
orated in an agar block.   It was found that chemotactic
properties of Ps. fluorescens exposed to STM for $1\frac{1}{2}$ hours
were profoundly diminished and at 7 hours after exposure
they were completely lost.   This result could be interpret-
ed to mean that at $1\frac{1}{2}$ hours after exposure the majority of
bacteria are dying but not dead and some enzymes may be
functioning but to a very much less extent.   In this sit-
uation, certain metabolic product will be produced just
enough to exhibit chemotaxis.   On the contrary, the bac-
terial cells after 7 hours of exposure to STM may be all
dead and consequently no metabolic product is available to
cause chemotaxis.

4. The effect of sonication.

Ps. fluorescens cells were sonicated for 10 seconds using a MSE ultrasonic disintegrator followed by washing twice in physiological saline and finally incorporated in agar to be used in the test. It was found that chemotaxis was still positive. In view of the notion currently held that sonication strips off flagellae from the cells, it is reasonable to suppose that possession of flagellae per se is not a determining factor for chemotaxis.

c) Failure of diffusible and dialysable product of Ps. fluorescens to exert chemotaxis.

1. Diffusible product.

Ps. fluorescens were grown over the entire surface of 0.1% yeast extract agar prepared on a glass coverslip placed and sealed on top of a depression slide containing a drop of sterile distilled water. After incubation at 30° for 24 hours, a bacteria free lump of agar was cut with a razor blade from the main block and this was added to amoebae whose migration was recorded by a dark ground tracing technique. It was found that no chemotaxis was demonstrable.

2. Dialysable product.

It has been shown by Shaffer (1956) that acrasin, a chemotactic substance from a slime mould, Dictyostelium discoidenum, lost its activity very rapidly at room temperature and this was attributed to destruction by enzymatic action of another slime mould product. Being a small mol-

ecular weight compound, acrasin could be removed from the inhibiting substance by dialysis through a semi-permeable membrane. If this relationship could be applied to chemical attraction of soil amoebae by Ps. fluorescens, it is possible that dialysable products of this organism are chemotactic. To test this possibility, the organisms were grown on a thin layer of 0.1% yeast extract agar separated from the other layer of this agar medium by a cellophane membrane. After incubation at 30° for 24 hours, a block of agar from the bottom layer was cut and used in the test for chemotaxis. Here again, it was found that this was not chemotactic.

d) Lack of evidence for chemical attraction of H. astronyxis by inedible organisms.

Inedible bacteria used in this test were S. enteritidis, E. coli 8196 and E. coli 812. It was found that the protozoa reacted with indifference to the presence of clumps of these bacteria. Fig.26 depicts random movement of amoebae when E. coli 812 was used. Occasionally, however, it was observed that amoebae moved towards clumps of these organisms in such a way that suggested that they were chemically attracted: but on arriving at the target, the cells did not show any sign of active ingestion and sooner or later they moved out from the scene. Movement of a single amoeba to and away from a clump of E. coli 8196 was illustrated in fig.27. This finding is of interesting
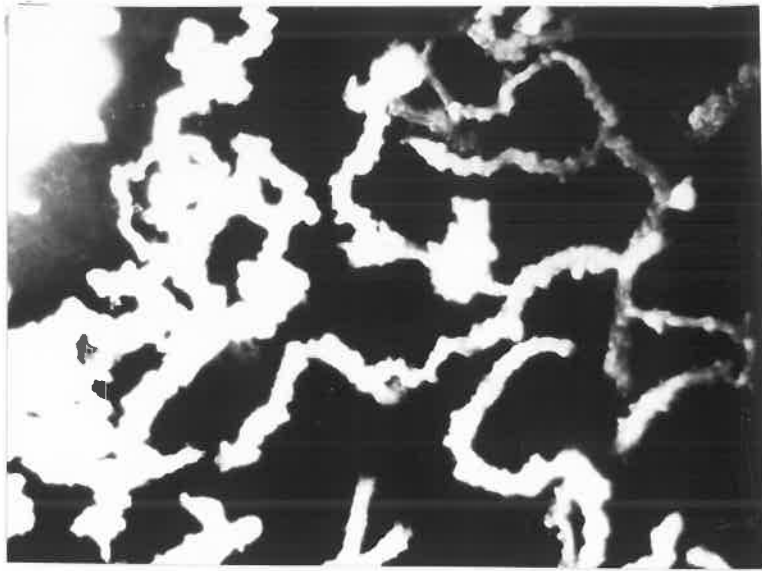
Fig. 25. The pattern traced out by Hartmanella astronyxis moving at random in the presence of a clump of Ps. fluorescens killed by heat at 56° for 30 minutes. (Exposure 30 minutes, 70 X).

Fig. 26. Dark ground tracing pattern showing randomised movement of Hartmanella astronyxis in the presence of a clump of E. coli 812. (Exposure 30 minutes, 70 X).

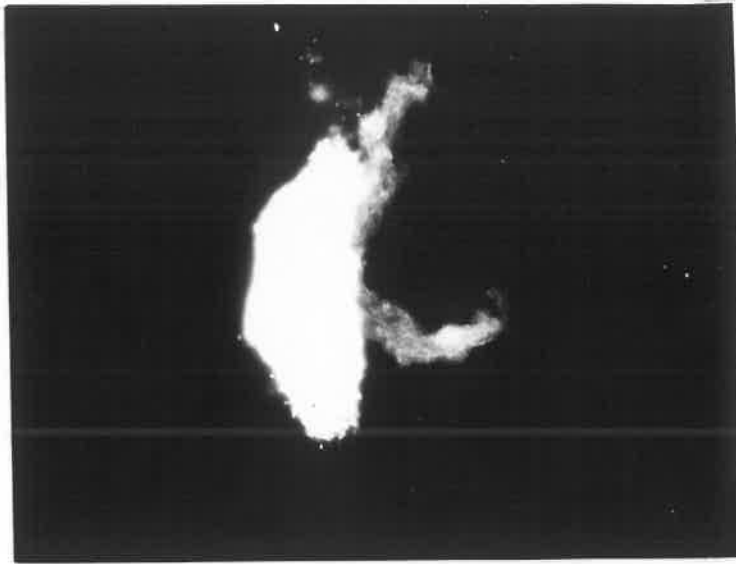Fig. 27. The pattern traced out by a single
Hartmanella astronyxis migrating to and away from
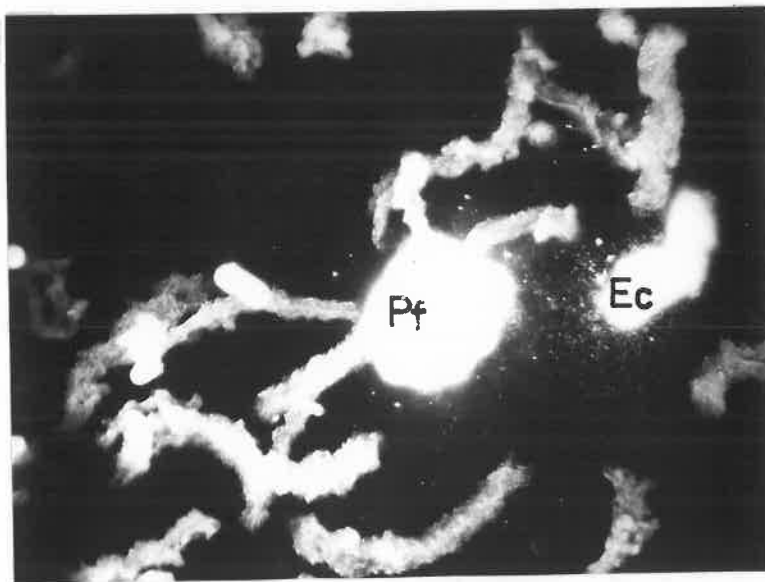a clump of E. coli 8196. (Exposure 30 minutes,
250 X).

Fig. 28. The pattern traced out by Hartmanella
astronyxis in the presence of the clumps of edible
and inedible bacteria. The amoebae ignored the
clump of E. coli 8196 (Ec) and moved selectively to-
wards the clump of Ps. fluorescens (Pf).(Exposure 30 min.)

contrast to that known to occur when Ps. fluorescens was used: in this instance the amoebae stayed at the clump for a very long period of time.

e) Failure of H. astronyxis 'adapted' to grow with inedible bacteria to react chemotactically to homologous organisms.

H. astronyxis which had been grown with either E. coli 8196 or S. enteritidis on a solid medium for a period of 4 months were used. The protozoa were scraped from the culture plate, washed in 0.06 μ PO$_4$ buffer saline and finally starved for 24 hours to allow for digestion of the organisms previously ingested. Again, it was found that these amoebae were not attracted chemotactically by these inedible bacteria. It is clear that by virtue of growing with homologous inedible bacteria, amoebae did not learn to recognise them chemotactically.

f) Selective chemotaxis.

These experiments were designed to determine whether H. astronyxis was able to recognise by means of chemical perception a clump of edible bacteria when another clump of inedible organism was also present. The results in fig.28 show that when a clump of Ps. fluorescens and a clump of E. coli 8196 were placed near to each other, amoebae were able to recognise the edible organisms and the cells progressed towards them as though the other organisms were not

present.   Similar result was obtained when Ps. fluorescens
and E. coli 812 were used in the test.

  g) Chemotaxis of H. astronyxis by other edible bacteria.

   Three species of edible bacteria, Ps. chloroaphis,
S. cholerae-suis and Bacterium 1912 were used.   It was
found that whereas Ps. chloroaphis and S. cholerae-suis were
chemotactic, Bacterium 1912 was not.   It is clear, there-
fore, that chemotaxis alone is not the sole factor determin-
ing acceptability of food organisms.

  h) Chemotaxis of Ps. fluorescens for other soil amoebae.

   This experiment was done to find out whether chemo-
tactic action of Ps. fluorescens is limited only for H. ast-
ronyxis or whether it is able to attract other species of
soil amoebae as well.   A clump of this organism was added
to either Acanthamoeb sp. (Neff) or H. rhysodes prepared in
a microdrop of 0.06 µ phosphate buffered NaCl solution and
the migration of the protozoa was recorded by means of a
dark ground tracing technique.   It was found that Ps. flu-
orescens was chemotactic for both species of amoebae.   It
follows that different species of amoebae have a feature in
common in response to chemotactic substances associated with
some edible bacteria.

  i) Induction of chemotaxis in inedible bacteria by
serum antibodies.

   It is known that many changes on the surface of bac-
teria occur as a result of serum treatment (Mudd et al. 1934;

Berry and Spies, 1949). Furthermore, activation of heat labile components of normal serum by an antigen-antibody complex results in liberation of substances chemotactic for mammalian polymorphonuclear leucocytes (Boyden, 1962). It would be interesting to know whether inedible bacteria could be rendered chemotactic for soil amoebae after treatment with antiserum. S. enteritidis grown overnight in PPG was opsonised with specific antiserum to give a final antibody concentration equivalent to 4 minimal agglutinating dose. After incubation at 37° for 10 minutes, the bacterial cells were washed and incorporated in agar to be used in chemotaxis experiments. The result in fig.29 shows that chemotaxis was positive. This chemotactic response was unlikely to be due to any factors present in S. enteritidis antiserum, since chemotaxis could not be demonstrated when either undiluted serum or serum at the concentration of 4 minimal agglutinating dose was incorporated into agar and used in the test. Furthermore, Bacterium 1912 cells treated with this serum in the amount of 4 minimal agglutinating dose and finally sedimented by centrifugation without further washing did not elicit chemotaxis.

Similar chemotactic response was obtained when E. coli 812 used in the test was opsonised with pig serum at 4° for 20 minutes and then washed. On the contrary, chemotaxis did not occur when pig serum used in opsonisation was adsorbed twice at 4° with homologous organisms killed

Fig. 29. The pattern traced out by <u>Hartmanella</u> <u>astronyxis</u> in the presence of the clump of <u>S. enter-itidis</u> after treatment with specific antiserum at a concentration of 4 minimal agglutinating doses per ml. Within certain range of the clump, all the traces converge directly upon it. (Exposure 30 minutes, 100 X).

by heat at 56° for half an hour at the concentration of
2 mg. dry weight per ml. of the serum.

j) Discussion.

It is clear from this study that Ps. fluorescens
and some other edible bacteria were chemotactic for H. ast-
ronyxis, but the mechanisms whereby this was achieved were
not clearly understood.   There is good reason to believe
that chemotactic principles were associated with living
bacterial cells only, since chemotaxis was lost when the
organisms were killed either by heat, alcohol or strepto-
mycin.   The results also suggest that possession of flag-
ellae per se is unlikely to be a determinant for chemotaxis
since this was not affected when Ps. fluorescens was de-
flagellated by sonication.   As regards the possibility of
a metabolic product from Ps. fluorescens acting as a potent-
ial chemotactic substance, our results failed to support
this, since agar containing diffusible or dialysable prod-
ucts of Ps. fluorescens was not chemotactic for H. astron-
yxis.   This is not at all surprising, because it is likely
that the concentration of chemotactic substances in the
agar block was not high enough to produce a chemotactic
effect.   It is also possible that chemotactic substances
may be only intermediary products of cellular metabolism
which have a very short half-life.   In order to elicit
chemotaxis, these substances may have to be constantly
manufactured and released.

One of the interesting findings which came out as a result of this study is the fact that inedible bacteria failed to excite chemotaxis. Nevertheless it was occasionally observed that few amoebae moved towards clumps of these bacteria in the manner similar to chemotactic response: but the cells did not stay at the clump. When inedible bacteria were treated with antiserum they became chemotactic. The significance of this finding will be discussed again in Chapter VII.

In the light of the present results, it is clear that chemotaxis plays some part in the recognition of bacterial food, but this should not be the sole factor since certain acceptable organisms, e.g. _Bacterium 1912_ failed to elicit chemotaxis.

As regards the fact that not only _H. astronyxis_ but also _H. rhysodes_ and _Acanthamoebae sp._ responded chemotactically to the same stimuli, it is reasonable to postulate that chemotaxis is a non-specific reaction shared by many species of amoebae in response to a common source of chemotactic stimuli.

4. _Selective phagocytosis of edible bacteria in a colony of mixed cultures._

Demonstration of food selection in soil amoebae has been done so far by measuring the ability of these protozoa to clear streaks of bacterial colonies on solid medium. It

is clear, however, that such a technique does not give a true measurement of selective feeding in a strict sense that the term implies, since the organisms were grown in separate colonies. It is likely that the rate of multiplication of amoebae in colonies of different bacterial species may not be the same and this was supported by experiments described later in this chapter. It follows that the numbers of predators in certain streaks of bacteria would be greater than others even though the numbers of bacteria ingested per amoeba may be comparable. In order to prove unequivocally selective feeding in soil amoebae, experiments should be done whereby protozoa are allowed to phagocytose bacteria present together in a colony of mixed cultures. However, evidence along this line has never been convincingly demonstrated. Severtzova (1928) claimed that a strain of soil amoeba was able to ingest selectively E. coli in confluent colonies containing also B. ellenbachensis, but her result was not reproducible and the reason for this was not known. It was necessary to repeat this type of experiment hoping that the results obtained would give some indication as to whether discrimination operates at the level of food ingestion.

H. astronyxis cells were inoculated to colonies of mixed cultures containing either Ps. fluorescens and S. enteritidis or Ps. fluorescens and E. coli 812 according to the technique described in Chapter II, p.76.
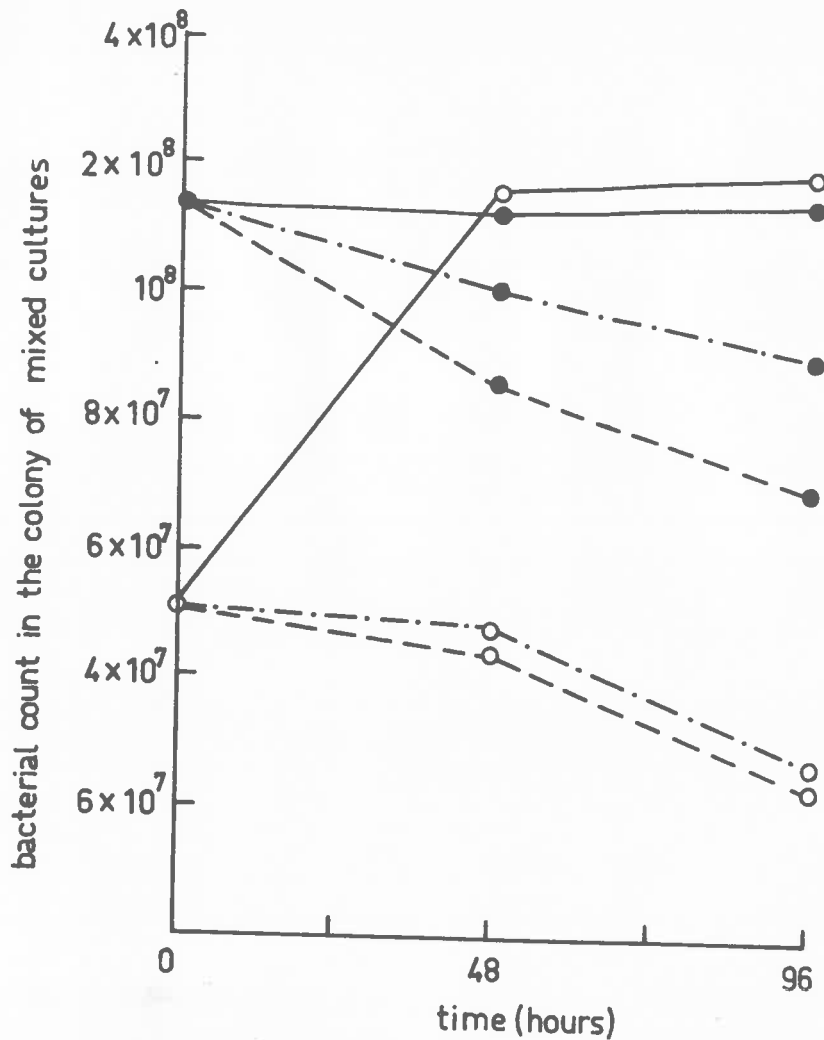
Results.

The results were illustrated in text - fig.14 and 15, and the data on which these results are based are given in Table XXIVa and b.   It can be seen that in mixed colonies uninoculated with amoebae, both Ps. fluorescens and two inedible organisms were able to multiply but the rate of multiplication of the former was much faster. This could, perhaps, be attributable to the inherent character of Ps. fluorescens in being a low temperature requiring organism. In the test series, however, there was a reduction in total counts of edible and inedible bacteria, but the degree of reduction was remarkably greater in the case of Ps. fluorescens.   Should Ps. fluorescens be ingested to the same extent as S. enteritidis or E. coli 812, one would expect that the numbers of this organism in the supernatant in the test series should be much higher than that actually obtained. It follows that H. astronyxis was able to feed preferentially on edible organisms.

Another finding which came out from this study was that much greater numbers of inedible bacteria remained associated with amoebae.   If it is tentatively assumed that the majority of them were in an intracellular location, this result could be interpreted to mean that the rate of intracellular killing of the inedible organisms was comparatively much slower than that of Ps. fluorescens.
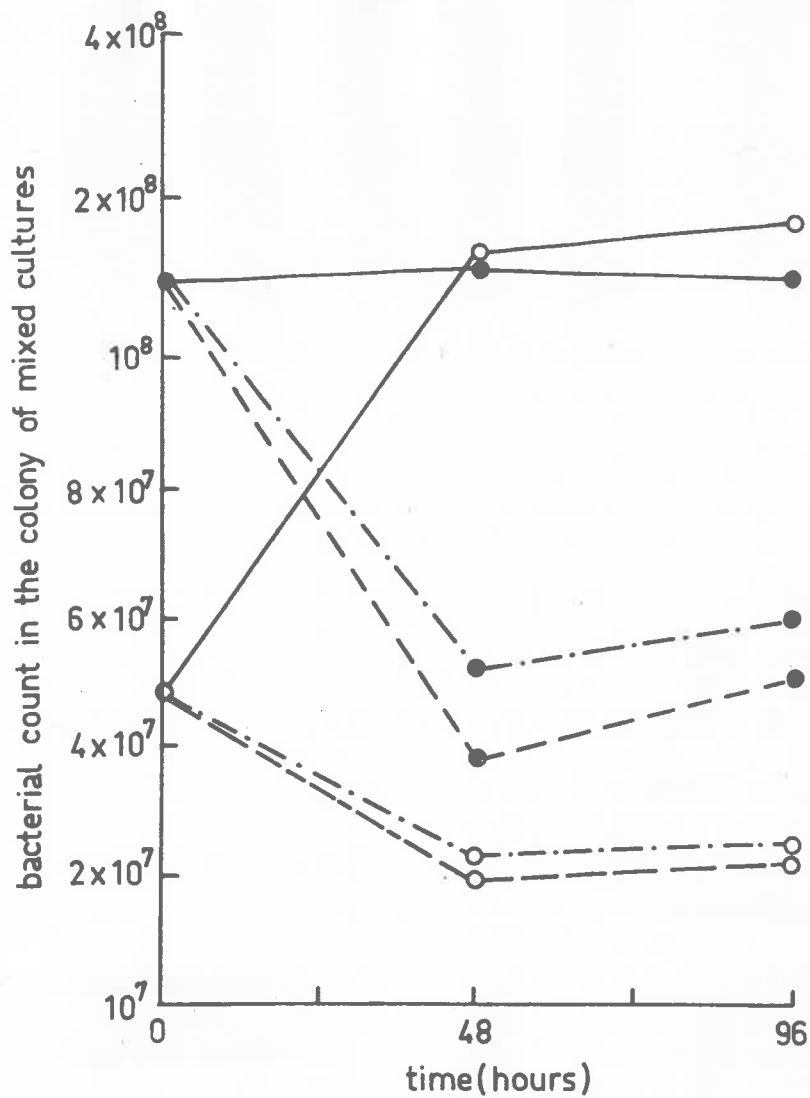
Text-fig. 14. Phagocytosis by Hartmanella astronyxis of Pseudomonas fluorescens and Salmonella enteritidis in colonies of mixed cultures.

Ps. fluorescens   S. enteritidis

o————o   •————•   Total recovery from colonies without amoebae.

o—·—·—·—o   •—·—·—·—•   Total recovery from colonies inoculated with amoebae.

o— — — —o   •— — — —•   Extracellular bacteria from colonies inoculated with amoebae.

Text-fig. 15. Phagocytosis by Hartmanella astronyxis of Pseudomonas fluorescens and Escherichia coli 812 in colonies of mixed cultures.

Ps. fluorescens   E. coli 812

o————o   ●————●   Total recovery from colonies without amoebae.

o—·—·—·o   ●—·—·—·●   Total recovery from colonies inoculated with amoebae.

o – – – o   ●– – – ●   Extracellular bacteria from colonies inoculated with amoebae.

Recovery of Ps. fluorescens and S. enteritidis from colonies of mixed cultures.

Time (hours)	Average number of bacteria in the colony of mixed cultures.											
	In the absence of amoebae			In the presence of amoebae								
				Average number of bacteria outside amoebae			Average number of bacteria associated with amoebae			Total number of bacteria in the colony		
	Pf*	Se	Pf:Se	Pf	Se	Pf:Se	Pf	Se	Pf:Se	Pf	Se	Pf:Se
Zero	4.78×10^7	1.25×10^8	1:26									
48	1.65×10^8	1.47×10^8	1:0.89	1.94×10^7	4.07×10^7	1:2.1	3.55×10^6	1.17×10^7	1:3.3	2.29×10^7	5.24×10^7	1:2.3
96	2.16×10^8	1.26×10^8	1:0.58	2.22×10^7	5.05×10^7	1:2.3	1.39×10^6	6.93×10^6	1:4.98	2.36×10^7	5.71×10^7	1:2.4

* Pf = Ps. fluorescens

Se = S. enteritidis.

TABLE XXIVb.

Recovery of Ps. fluorescens and E. coli 812 from colonies of mixed cultures.

	Average number of bacteria in the colony of mixed cultures											
	In the absence of amoebae			In the presence of amoebae								
				Average number of bacteria outside amoebae			Average number of bacteria associated with amoebae			Total number of bacteria in the mixed colony		
	Pf*	E.coli	Pf:E.c.	Pf.	E.coli	Pf:E.c.	Pf.	E.coli	Pf:E.c.	Pf.	E.coli	Pf:E.c.
Zero	5.06×10^7	1.35×10^8	1:2.7									
48	1.55×10^8	1.29×10^8	1:0.83	4.45×10^7	8.65×10^7	1:1.94	2.74×10^6	1.52×10^7	1:5.5	4.72×10^7	1.02×10^8	1:2.2
96	1.78×10^8	1.44×10^8	1:0.81	2.45×10^7	6.94×10^7	1:2.8	2.80×10^6	2.15×10^7	1:7.7	2.73×10^7	9.09×10^7	1:3.3

Pf = Ps. fluorescens
E.coli = E. coli 812.

Discussion.

The first point which merits consideration is that pertaining to the technique employed. It is obvious that this technique was crude and the results obtained were based on many assumptions. Firstly, it was assumed that the total numbers of organisms in each colony were comparable, though it was realised of course that this could not be true. Surprisingly enough, results of bacterial recovery from a duplicate pair showed that this variation was not too great since this ranged between 10-40%. Since the main aim of this study was to measure only a gross difference in the numbers of bacteria phagocytosed probably in the order of one log scale, the small variation in the numbers of bacteria in each colony should not interfere with the assessment of the degree of phagocytosis and intracellular killing. Secondly, determination of the numbers of bacteria phagocytosed by soil amoebae was based on the difference between the numbers of organisms recovered from the colony in the control and the test series. It is obvious that the rate of multiplication of bacteria in the test series was less than that in the control, since some bacteria were in the process of being phagocytosed. The actual numbers of bacteria phagocytosed were thus smaller than those implied in the calculation. This difference became increasingly important when being considered concurrently with a different rate of multiplication between edible and inedible bacteria.

Nevertheless, when this was taken into consideration, the results still suggested a relatively greater reduction of edible bacteria as compared with that of inedible bacteria.

In the light of these results that amoebae showed preferential ingestion for edible bacteria when they have equal opportunity to encounter both edible and inedible organisms, it seemed that this refinement of selection was very extraordinary and it required an explanation. This will be given at some length in Chapter VII.

5. Intracellular killing of bacteria by soil amoebae.

Survival of edible and inedible organisms in H. astronyxis was studied using two different techniques, i.e. phagocytosis experiments and single cell studies (Chapter II, pp.79 and 81).

1) Phagocytosis experiment.

Ps. fluorescens, S. enteritidis and E. coli 812 were used. The organisms were passaged through semi-solid medium (Lederberg, 1956) once or twice so that they became actively motile. This step was very important since, when non-motile organisms were used, the percentage phagocytosis was very small and consequently the rate of intracellular killing could not be measured. Broth culture of S. enteritidis was incubated overnight at 37° whereas those of Ps. fluorescens and E. coli 812 were incubated at 30°. In fact, E. coli 812 could be grown at 37°, but it was found that the organisms were actively motile only when they were grown

at 30°.

0.1 ml. of washed bacterial suspension in PPG containing approximately 10^6 organisms per ml. was added to 1.0 ml. PPG suspension of washed H. astronyxis in a series of siliconised tubes. The amoeba concentration was adjusted to be 4×10^5 cells per ml. The control series was also prepared by inoculating 0.1 ml. of bacterial suspension into 1.0 ml. of PPG alone. The percentage phagocytosis and killing of bacteria was determined using the method described in Chapter II, p.79.

Results.

The results in text - figs.16, 17 and 18, and in Tables XIV, show that the percentage phagocytosis and killing of Ps. fluorescens, S. enteritidis and E. coli 812 was comparable. These results thus indicate that under conditions employed in the tests, H. astronyxis effectively kills any bacteria entering the cells regardless of the difference in their acceptability as food organisms.

2) Killing of a single bacterium by a single amoeba.

In most phagocytosis experiments, assessment of the numbers of bacteria ingested by phagocytes was based on the differences in the numbers of bacterial cells recovered from the supernatant in the control and in the test series. It is clear that the observed numbers of bacteria ingested consist of those which are actually inside the cells and those which are adherent to the cell surfaces. It is conceivable
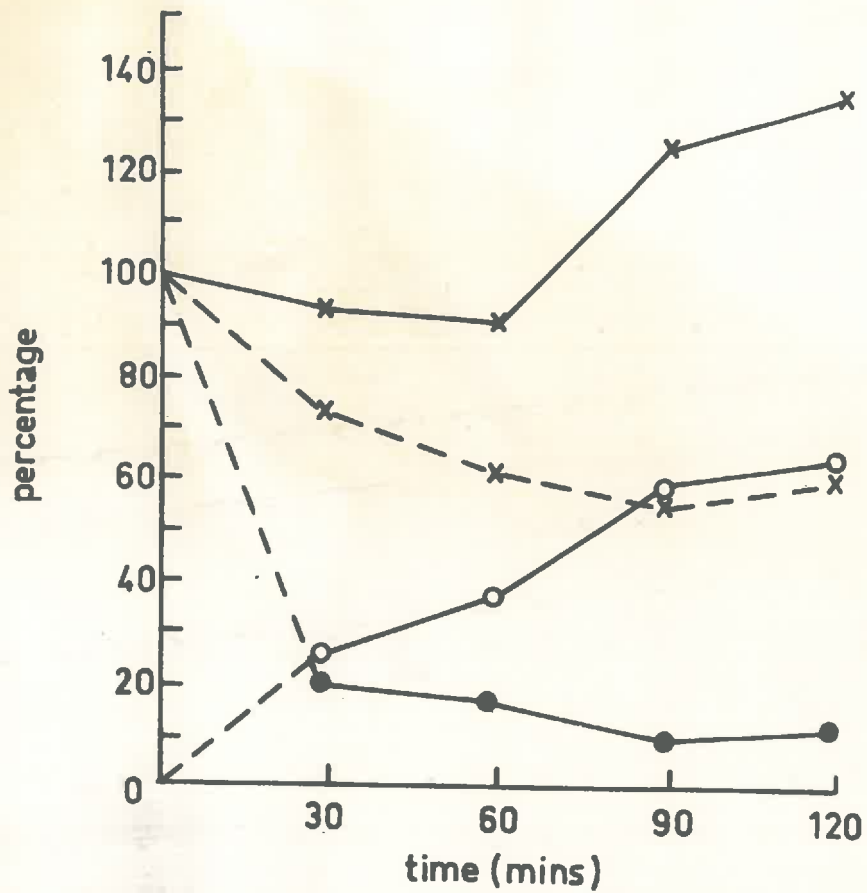
Text-fig. 16. Phagocytosis and killing of Pseudo-monas fluorescens by Hartmanella astronyxis.

x————x   Total recovery (control).

x– – – –x   Total recovery (test).

o————o   Phagocytosis.

●————●   Intracellular survival.
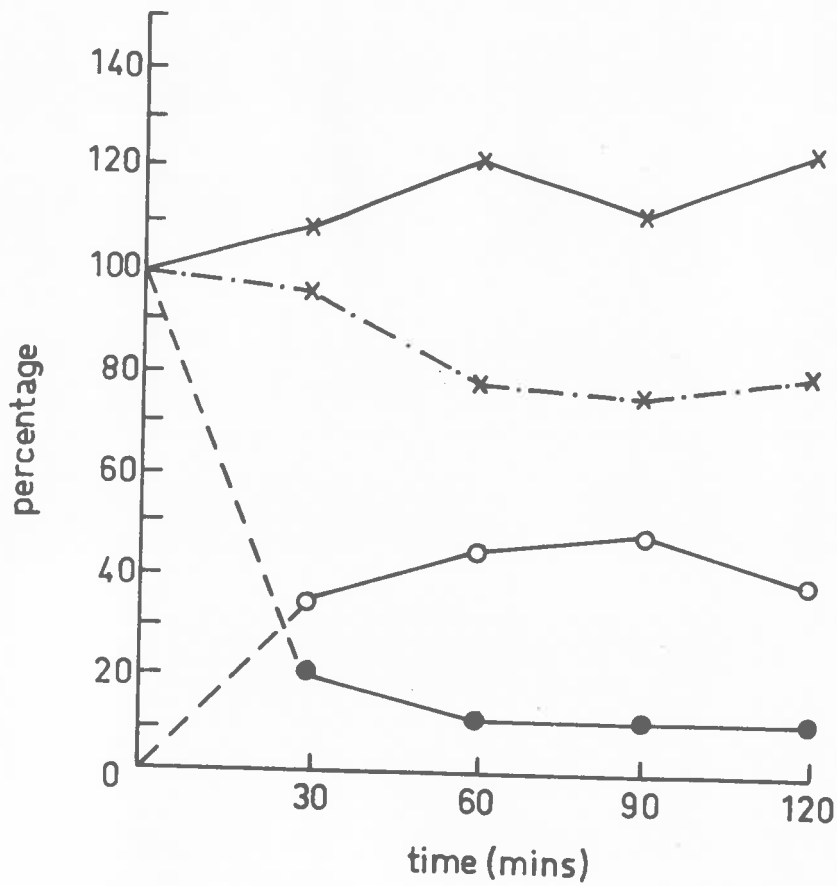
Text-fig. 17. Phagocytosis and killing of Salmon-
ella enteritidis by Hartmanella astronyxis.

x————x	Total recovery (control).
x—·—·—·—·x	Total recovery (test).
o————o	Phagocytosis.
●————●	Intracellular survival.
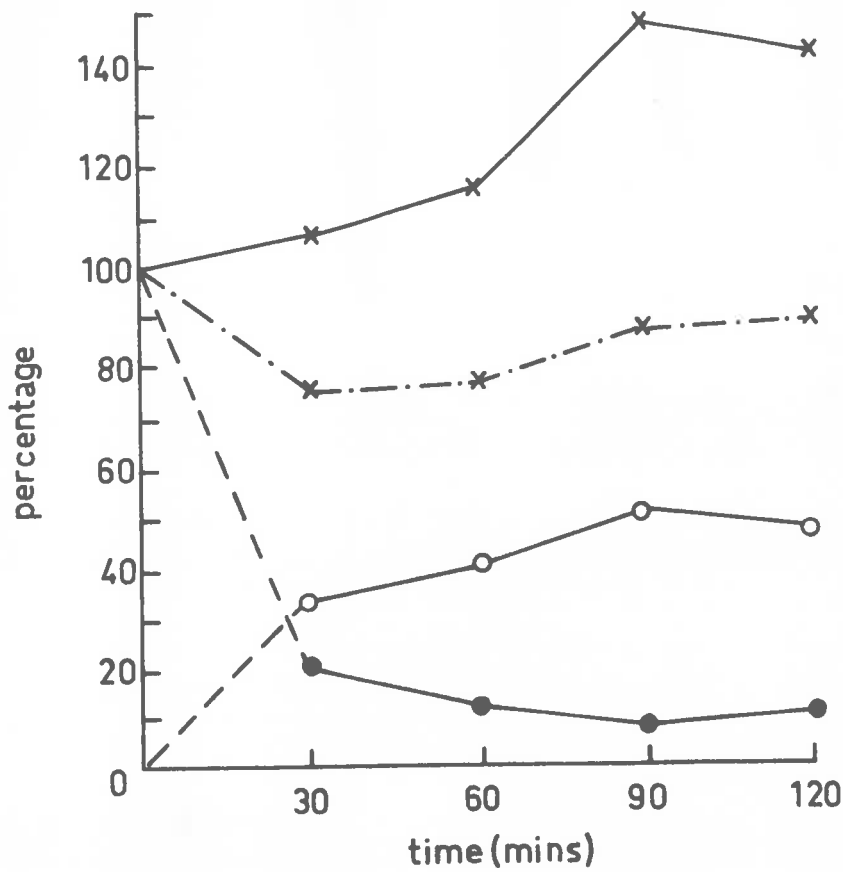
Text-fig. 18.   Phagocytosis and killing of Escheri-
chia coli 812 by Hartmanella astronyxis.

x————x   Total recovery (control).
x—·—·—·—·x   Total recovery (test).
o————o   Phagocytosis.
●————●   Intracellular survival.

TABLE XXV.

Phagocytosis and killing of bacteria by H. astronyxis.

Time (Mins.)	No. of bacteria in the control supernate (A)	No. of bacteria in the test supernate (B)	No. of bacteria associated with amoebae (C)	Total number of bacteria in the test system (T)	Percentage phagocytosis $\frac{A-B}{A} \times 100$	Percentage intracellular survival $\frac{C}{A-B} \times 100$
			1. Ps. fluorescens.		Inoculum $= 1.34 \times 10^6$	
30	1.24×10^6	9.25×10^5	6.21×10^4	9.87×10^5	25.4	19.7
60	1.22×10^6	7.65×10^5	6.68×10^4	8.32×10^5	37.3	14.7
90	1.68×10^6	6.90×10^5	9.32×10^4	7.83×10^5	58.9	9.4
120	1.81×10^6	6.70×10^5	1.55×10^5	8.25×10^5	62.9	13.6
			2. S. enteritidis		Inoculum $= 2.10 \times 10^6$	
30	2.26×10^6	1.48×10^6	1.51×10^5	2.01×10^6	34.5	19.4
60	2.54×10^6	1.43×10^6	1.29×10^5	1.63×10^6	43.7	11.6
90	2.34×10^6	1.24×10^6	1.21×10^5	1.56×10^6	47.0	11.0
120	2.52×10^6	1.57×10^6	1.04×10^5	1.66×10^6	37.7	10.9
			3. E. coli 812		Inoculum $= 2.60 \times 10^6$	
30	2.76×10^6	1.85×10^6	1.91×10^5	1.95×10^6	32.9	21.0
60	3.01×10^6	1.79×10^6	1.54×10^5	1.98×10^6	40.5	12.6
90	3.88×10^6	1.90×10^6	1.63×10^5	2.28×10^6	51.0	8.2
120	3.71×10^6	1.97×10^6	1.82×10^5	2.33×10^6	46.9	10.5

that the less numbers of bacteria adherent to the cells
and the more rapid engulfment of bacteria after adhesion
would contribute to the validity of the assessment of the
degree of phagocytosis. Unfortunately, in the case of
soil amoebae, it was found in preliminary tests that a con-
siderable time elapsed from the beginning of adhesion until
the completion of the engulfment. Taking this into con-
sideration, it is possible that the survival rate of bac-
teria in soil amoebae as determined by phagocytosis experi-
ments would be greater than the actual value. It is
thought that this difficulty could be overcome by using a
single cell study since, by this technique, it is possible
to measure accurately the period of intracellular residence.

Ps. fluorescens and E. coli 812 used in these exper-
iments were grown in broth overnight at 30°. A single
motile bacterium was isolated and added by means of a micro-
pipette to a single H. astronyxis in a drop of PIG. By
means of phase contrast microscopy, it is possible to follow
the process of adhesion and engulfment accurately (Chapter
II, p.81). Recovery of bacteria from the cell was done at
2 and 10 minutes after engulfment. The time taken from
the beginning of adhesion until the completion of engulf-
ment was also recorded. Colony forming ability of each
bacterium was determined by correlating the numbers of bac-
teria counted under phase contrast and the colony counts
when these were plated on nutrient agar.

<u>Results</u>.

The results in Table XXVI show that at 2 minutes after ingestion the majority of the bacteria were not killed, whereas at 10 minutes after ingestion almost 90 per cent. of them were killed. Furthermore, there was no significant difference between the rates of killing of <u>Ps. fluorescens</u> and <u>E. coli 812</u>, thus confirming the results in phagocytosis experiments.

6. <u>Measurement of growth rates of H. astronyxis feeding on colonies of different bacterial species</u>.

In the preceding section (p.147) it has been pointed out that measurement of clearing ability of bacterial streaks by soil amoebae does not necessarily indicate selective feeding, since amoebae may have different rates of multiplication. In order to study this possibility, the following experiments were performed.

Two <u>H. astronyxis</u> cells grown axenically in 3 per cent. yeast extract solution were inoculated on a single colony of bacteria prepared on 0.1 per cent. yeast extract agar on a glass coverslip according to the technique described in Chapter II, p.82. The numbers of amoebae in each bacterial colony were determined daily and the results were given in text - fig.19. It can be seen that in the presence of different species of bacteria, the amoebae had different rates of multiplication and these could be correlated with edibility of micro-organisms (see this chapter,

TABLE XXVI.

Killing of a single bacterium by a single amoeba.

Time (Min.)	Ps. fluorescens					E. coli 812				
	No. of amoebae tested	Total No. of bact. ingested	Time required for phagocytosis after adhesion	No. of bacteria recovered	Percent. intracell. survival	No. of amoebae tested	Total No. of bact. ingested	Time required for phagocytosis after adhesion	No. of bacteria recovered	Percent. intracell. survival
2	18	18	5.5 ± 4.8*	16	88.9	16	16	3.9 ± 3.1	12	75.0
10	20	20	4.6 ± 2.8	2	10	23	23	-	1	4.3

* Standard deviation.

Colony forming ability of Ps. fluorescens = 94.0% of E. coli 812 = 94.1%. (This was measured by correlating the number of bacteria counted under phase contrast and the number of colonies formed when these were plated on nutrient agar).
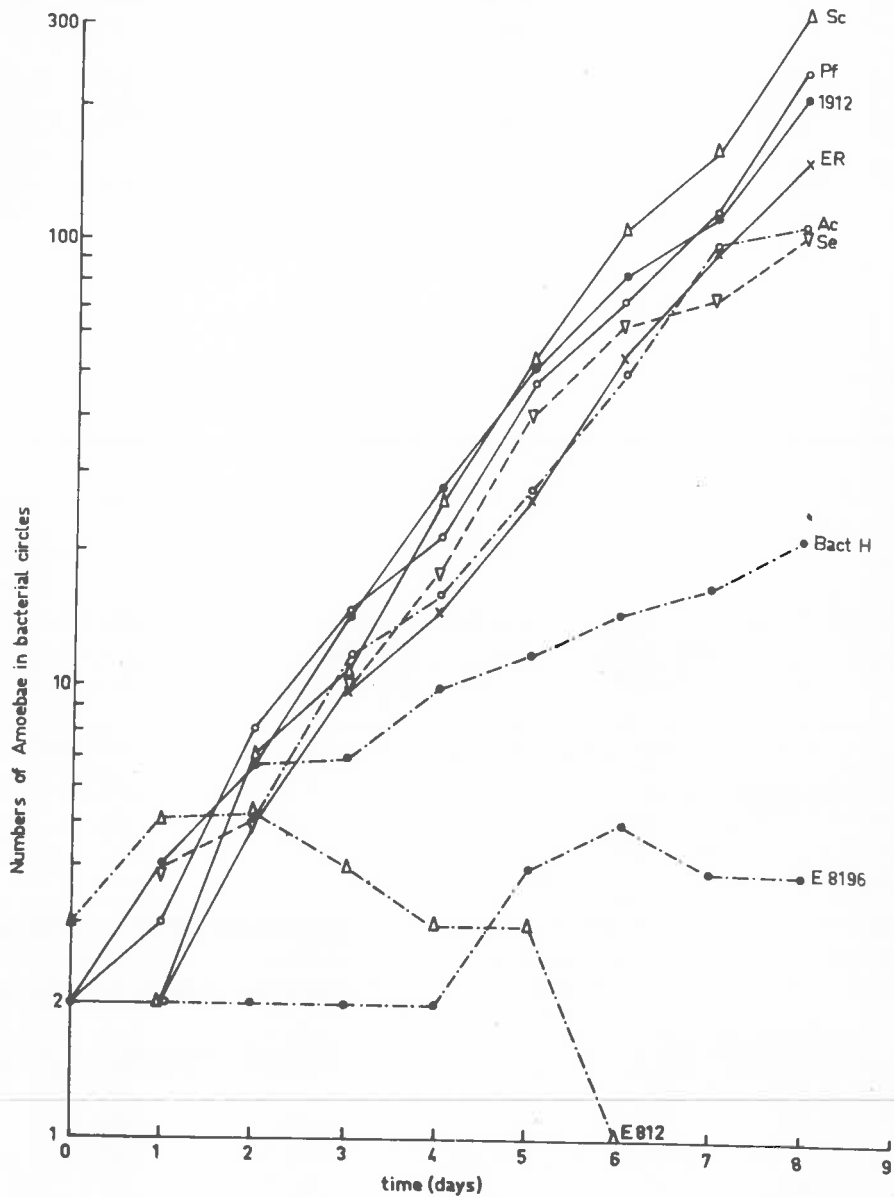
Text-fig. 19. Growth of Hartmanella astronyxis
feeding on colonies of different bacterial species.
Ac = Aerobacter cloacae, Bact H = Bacterium H, E812
= Escherichia coli strain 812, E8196 = E. coli NCTC
8196, ER = E. coli Rechner, Pf = Pseudomonas fluor-
escens, Sc = Salmonella cholerae-suis, Se = S. enter-
itidis, 1912 = Bacterium 1912.

p.134 for comparison).

7. Studies on the mechanisms of bacterial agglutination on the surface of soil amoebae.

Agglutination of flagellated bacteria on the surface of soil amoebae has been well documented (Mouton, 1902; Ray, 1951); nevertheless the mechanisms underlying this phenomenon are not yet understood. Ray (1951) suggested that two possibilities could be accounted for this reaction, namely, secretion by amoebae of slime or adhesive substances and physico-chemical factors such as electrostatic charges on the surface of amoebae.

The purpose of the work described herein is to re-investigate this problem in relation to these two possibilities. It is thought that if agglutination is brought about by physico-chemical reactions at the surface of amoebae, any changes in the physical properties of the medium in which the cells are suspended would probably affect this process. It was decided, therefore, to study the influence of physical factors such as hydrogen ion concentration, ionic strength, and chelating agents on surface adhesion of bacteria. The work was also extended to include the influence of the nucleus, a biological factor, on this process. An attempt was also made to inhibit competitively any receptor site 'specific' for flagellated bacteria on the surface of amoebae by pre-treating the protozoa with homologous flagellar preparation.

1. Factors affecting the process of bacterial agglutination.

Unless otherwise stated, amoebae used in the tests were H. astronyxis grown in PPG in the presence of heat killed Ps. fluorescens. The cells were washed while adherent to the culture vessel with two changes of physiological saline and finally added to the test medium in a depression slide. After a brief exposure (5 minutes), the amoebae were transferred unwashed, together with the suspending medium, on to a coverslip in an oil chamber in which the medium spread out to form a drop of approximately 2 mm. in diameter. At intervals varying from 30 to 120 minutes, motile S. enteritidis suspended in broth was added by means of a micro-pipette to amoebae in the drop and the process of agglutination observed under phase contrast.

a) Effect of hydrogen ion concentration on bacterial adhesion.

The medium used was PPG which was previously adjusted with either HCl or NaOH to give a pH range of 3 to 9 with a difference of 1 pH unit. It was found that at pH 4 to 9, amoebae appeared normal morphologically and bacterial agglutination was clearly demonstrated. At pH 3, however, the result was inconclusive, since the amoebae were round up and did not adhere to glass; and the bacteria became non-motile and tended to agglutinate spontaneously.

b) **Effect of the ionic strength on bacterial adhesion.**

The test solutions were phosphate buffered sodium chloride solutions pH 6.5 having the ionic strength of 0.02, 0.06, 0.1, 0.25, 0.5 and 1 μ.   These solutions were made up as follows:

Ionic strength (μ)	0.02	0.06	0.1	0.25	0.5	1.0
5M NaCl (ml.)	0.50	8.25	16.0	46.0	96.0	196.0
0.5M Na_2HPO_4 (ml.)	8.5	8.5	8.5	8.5	8.5	8.5
4M NaH_2PO_4 (ml.)	1.85	1.85	1.85	1.85	1.85	1.85

Glass distilled water added to make 1 litre.

It was found that bacterial adhesion on the surface of amoebae occurred in all the solutions tested.   On exposure to the medium having ionic strength up to 0.1 μ, the amoebae looked morphologically normal and adhered firmly to the glass coverslip.   In contrast, amoebae exposed to the medium having ionic strength of 0.25 μ or higher were sick as judged by their appearance and their ability to adhere to glass, i.e. the cells were round up, the pseudopods became filiforms and had irregular movement.

c) **Effect of EDTA on bacterial adhesion.**

The media used were 1 per cent. proteose-peptone solutions to which EDTA was added to give a final concentration of 0.01, 0.05, 0.1 and 0.2 molar.   It was found that **S. enteritidis** wells were adherent to the surface of amoebae at all the concentration tested.   As regards morphological

appearance of amoebae, it was found that the cells were normal on exposure to EDTA at the concentrations of 0.01 and 0.05 molar whereas at higher concentrations they were round up and did not attach to glass.

It is concluded that EDTA has no influence on bacterial adhesion: in other words, divalent cations were not required for this process.

d) <u>Effect of the nucleus on bacterial adhesion</u>.

Amoebae used in this study were <u>H. rhysodes</u> instead of <u>H. astronyxis</u> since, in the preliminary observations, it was found that <u>H. rhysodes</u> adhered to glass much firmer than the latter and therefore rendered the ease in cell sectioning. Moreover, these protozoa remained active in a micro-drop of PPG for a very long period of time and accordingly there was no need to change the medium during the time of observations.

A single amoeba was first isolated in a micro-drop of PPG on a coverslip in an oil chamber. It was then merotomized into two halves by a micropipette controlled by a micromanipulator. Both halves were maintained in the drop of the culture medium at 20° for a period of up to 7 days. During this period, some nucleated amoebae divided but this number was not determined numerically. Five pairs of amoebae were tested daily for their ability to agglutinate <u>S. enteritidis</u>. It was found that the bacteria became adherent to both halves equally well (figs.30a and b), even

Figs. 30. Photographs showing adhesion of Sal-
monella enteritidis on the surface of the nuclear
half and the cytoplasmic half of Hartmanella rhys-
odes four days after cutting. (Phase contrast).

a. The nuclear half.

b. The cytoplasmic half.

Fig. 31. Photograph showing adhesion of Sal-
monella enteritidis on the surface of the cell
ghost of Hartmanella astronyxis.
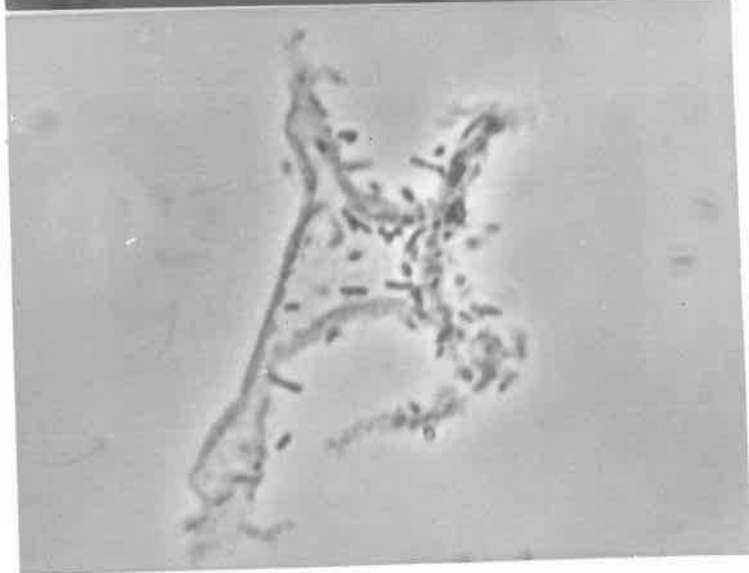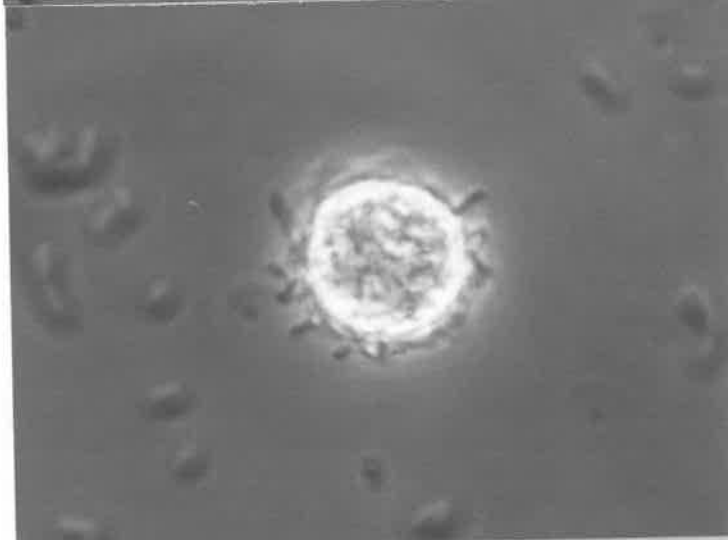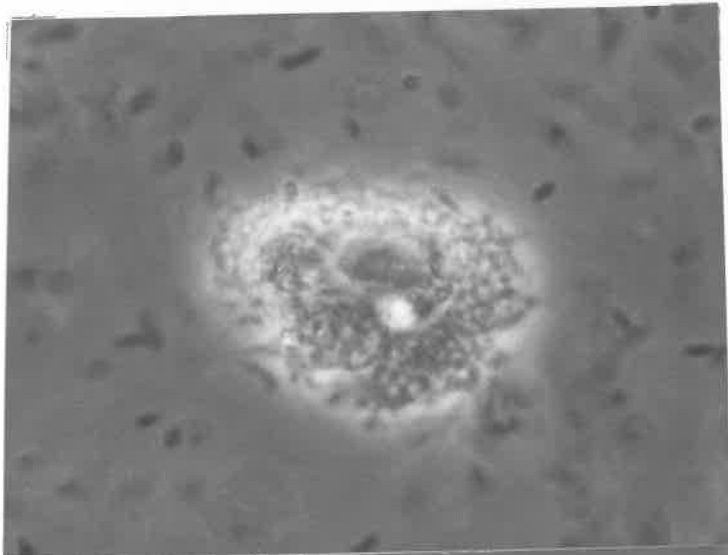
at 7 days after cutting.   The only difference between
these two halves was in their ability to re-orientate the
organisms which had been adherent on the cell surface to
become localised at the tail region, and this could be
demonstrated only in the nuclear half.   This finding could
be explained in relation to what is known in the whole am-
oebae that the membrane is rolled backwards as the cells
move forward (Goldacre, 1961).   It is conceivable there-
fore that in nucleate cells, any particles adherent to the
surface would eventually be carried passively to the tail
region.   In the cytoplasmic halves, however, the cells
could neither attach to glass nor move;   and it was poss-
ible that the membrane did not roll backwards and conse-
quently no re-orientation of adhering bacteria took place.

    2.   <u>Failure to inhibit competitively agglutination of
flagellated bacteria by flagellar preparation from homol-
ogous organism</u>.

      In 1951, Ray reported that all flagellated bacteria
tested were agglutinated on the surface of a species of
limax amoebae, and this finding was supported by our observ-
ation which showed that all motile bacteria tested, viz.
<u>S. enteritidis</u>, <u>S. choleraesuis</u>, <u>S. adelaide</u>, <u>S. typhimur-
ium</u> M_{206}, <u>Ps. fluorescens</u>, <u>Ps. chloroaphis</u> and <u>A. cloacae</u>
were adherent to the surface of <u>H. astronyxis</u>.   It is not
known, however, as to whether surface agglutination is a
non-specific reaction for all flagellated bacteria or

whether amoebae have many receptor sites and each one is specific for a particular organism. If the latter is the case, one would expect that bacterial agglutination would be inhibited competitively by treating amoebae with flagella preparation of homologous organisms. The following experiment was then performed to test this possibility.

Purified flagellar preparation of S. typhimurium M206 used in this test was kindly provided by Mr. A. Blaskett, Department of Microbiology, the University of Adelaide. This flagellar preparation was diluted in PPG so that the final concentration was 1 mg. per ml. Approximately 100 H. astronyxis were exposed to 1.0 ml. of this flagellar suspension for 5 minutes and the cells were transferred unwashed together with the suspending medium on to a coverslip in an oil chamber. At 30 to 90 minutes after exposure, the amoebae were tested for their ability to agglutinate motile S. typhimurium M206. It was found that adhesion of bacteria on the surface of amoebae still occurred. This unsuccessful attempt to inhibit bacterial agglutination does not disprove entirely the idea of the existence of specific receptor sites, since it is possible that the amount of flagellar preparation as used in this test might be inadequate to block completely all the available sites specific for S. typhimurium M206. It is also possible that the membrane of amoebae was being synthesised continuously and production of new receptor sites would

never be exhausted. On the other hand, this result could be interpreted to mean that agglutination of bacteria on the amoeban surface is a non-specific reaction brought about by other factors; in other words, there are no specific receptor sites whatsoever.

3. Demonstration of bacterial agglutination on a cell ghost of soil amoeba.

During the course of the study of the effect of the nucleus on bacterial agglutination, it occurred accidentally that as a micropipette was pressed on H. astronyxis cell adhering to a glass coverslip in a drop of PPG, the cell was suddenly disrupted in such a way that the cell ghost was formed. When S. enteritidis was added to the ghost, bacterial adhesion on its surface was seen (fig.31). This observation should rule out the possibility that agglutination was due to the surface charge, since this should be profoundly affected when the cell was ruptured and the electrostatic charge on the cell ghost should be equivalent to that of the medium.

4. Failure to demonstrate agglutination of formalinised flagellated bacteria by saline extracts of soil amoebae.

Since the evidence obtained from the preceding sections suggested that agglutination of flagellated bacteria on the surface of soil amoebae was likely to be due to a secretion from these protozoa, a more direct support for this contention was needed. In the preliminary test, it

was found that the extract of H. rhysodes failed to ag-
glutinate formalinised broth culture of Ps. fluorescens
or S. enteritidis. It was thought that if the extract of
amoebae could be adsorbed on to the surface of bacteria,
one would expect to be able to demonstrate agglutination
when antiserum prepared against amoebae was added to the
organisms pretreated with the extract of amoebae.

H. rhysodes cells used in this experiment were
washed three times in chilled physiological saline by cent-
rifugation at 1000 r.p.m. for 3 minutes and finally resus-
pended in the same solution. The cell concentration was
adjusted to be 10^6 amoebae per ml. (This was equivalent
to 0.85 mg. dry weight per ml.). After sonication for 10
seconds using a MSE ultrasonic disintegrator, the super-
natant was separated from the disrupted cells by centrifug-
ation at 4000 r.p.m. for 15 minutes, after which it was re-
moved by means of a Pasteur pipette into a screw-capped
bottle and used immediately.

Overnight broth culture of Ps. fluorescens contain-
ing 5×10^8 bacteria per ml. was treated with 0.1 per cent.
formalin and the cell suspension was incubated further at
30^0 overnight and finally stored at 4^0 until use.

Equal volumes (1.0 ml.) of the amoeba extract (un-
diluted, and diluted 1:10 and 1:50 in physiological saline)
and the bacterial suspension were mixed and incubated at 20^0
for 18 hours. In some tubes the supernatant from the am-

oeba culture or physiological saline alone were used in place of the amoeba extract. Following incubation, the bacteria were washed once in physiological saline and finally resuspended in 0.5 ml. of the same solution. A volume (0.5 ml.) of _H. rhysodes_ antiserum diluted 1:10 in physiological saline was then added to each tube, and the mixtures were incubated at 20° for a further 4 hours.

On examination (macroscopically and microscopically), it was found that no agglutination of bacteria could be demonstrated.

Discussion.

The results from these studies did not prove one way or the other as to how flagellated bacteria become adherent to the surface of soil amoebae. It is unlikely, however, that this was brought about by physico-chemical changes, e.g. the surface charge effect, since the process of adhesion was not affected under the conditions in which physico-chemical properties of the cell surface should be profoundly changed, e.g. after the alteration in pH, ionic strength or addition of EDTA in the medium. The observation that flagellated bacteria were adherent to the surface of the cell ghost suggested strongly that secretory substances from amoebae might play an important role. Failure to prove this by the method similar to Indirect Coomb's test does not necessarily rule out this possibility, since it is possible that these substances may be destroyed during the disruption

of the cells by sonication or conversely the substances may remain associated with the cell residue. If bacterial adhesion on amoeba surface is due to the secretory substances, the finding that adhesion occurred on the surface of enucleate amoebae would suggest that these substances may be secreted by the cytoplasm independent of the nuclear control.

CHAPTER VII.

DISCUSSION.

In 1893, Metchnikoff drew attention to the similarity between the functions of vertebrate phagocytes and free living protozoa such as amoebae in phagocytosis and killing of bacteria.   He commented that phagocytosis in amoebae was the principal means whereby the cells obtained nutrients whereas this process in higher animals had evolved into an important means of combating infection.   It is conceivable that any study of phagocytosis in cells of different phylogeny may contribute to a better understanding of the development of host defence.   If all phagocytic cells have evolved from a common ancestor, it is possible that they might share common bactericidal mechanisms and any factor which acts to promote phagocytosis and killing of bacteria in one situation should have a similar action in the other.   In this discussion, attempts will be made to compare various aspects of phagocytosis in amoebae with the existing knowledge known to occur in mammalian phagocytes.

Killing of salmonellae by mammalian phagocytes and by amoebae.

In vitro studies of Rowley and Whitby (1959) showed that the average half-life of salmonellae opsonised with normal human serum in mouse peritoneal macrophages was approximately 15 minutes.   In the present studies, it was shown that the average half-life of opsonised or unopsonised S. enteritidis or S. typhimurium in A. proteus was approxim-

ately 1 hour (Chapter IV, p.106).   The difference in the
rate of intracellular survival of these organisms in these
two types of phagocytic cells could be attributed to the
difference in the temperature in which the tests were per-
formed, i.e. 37^0 for mammalian phagocytes and 20^0 for am-
oebae.   In the experiment performed at 30^0, it was shown
that the intracellular survival of S. enteritidis in A.
proteus was considerably reduced, i.e. their intracellular
half-life was approximately 40 minutes (Chapter IV, p.108).
It thus seems likely that killing of this organism by A.
proteus follows the Laws of Thermodynamics, i.e. a twofold
increase in the rate of bacterial killing for every 10^0 rise
in temperature.   Should the test be performed at 37^0, the
ingested bacteria would probably be killed at the rate sim-
ilar to that in mammalian macrophages.

The mechanisms whereby the ingested bacteria are
killed in amoebae or in other phagocytic cells are not known.
Two possibilities are generally considered, i.e. killing by
specific bactericidal substances and killing by an accum-
ulation of non-specific factors which act by changing the
environment in the vacuoles so that the bacteria fail to
survive.   At least two bactericidal substances, phagocytin
and lysozyme have been shown to be localised in the granules
in mammalian polymorphonuclear leucocytes (Hirsch, 1956;
Cohn and Hirsch, 1960).   After phagocytosis, these granules

lyse and liberate their contents into the vacuole or its vicinity (Hirsch and Cohn, 1960; Hirsch, 1962). Evidence from electron microscopic studies showed that membranes of these granules fused with those of vacuoles and their contents were discharged into the vacuoles (Lockwood and Allison, 1963; 1964; Zucker-Franklin and Hirsch, 1964). In view of the fact that the vacuolar environment is acidic (Sprick, 1956) and hence creates ideal condition for optimum action of both phagocytin and lysozyme (Hirsch, 1960), it is highly likely that these substances are important in killing of bacteria inside polymorphonuclear leucocytes.

Killing of bacteria by mononuclear cells seems to follow a similar chain of events to that in polymorphonuclear cells (Cohn and Weiner, 1963; North and Mackaness, 1963). Nevertheless, these cells do not have phagocytin (Hirsch, 1960) and the amount of lysozyme they contain is very small (Myrvik, Leake and Farris, 1961). Furthermore, the extract from them in contrast to polymorphonuclear leucocytes is not bactericidal for a number of bacteria tested (Rowley, 1959). In the light of these findings, it is possible to assume that the mechanisms of killing of bacteria in these cells may be different from those in polymorphonuclear leucocytes.

In amoebae, no specific bactericidal substances have been demonstrated. Nevertheless, these protozoa contain many hydrolytic enzymes and some of them, i.e. acid phos-

phatase, proteinase and succinic dehydrogenase, were shown
to be associated with cytoplasmic granules (Holter, 1954;
Holter and Lowy, 1959). Cytochemical staining of acid
phosphatase showed that this enzyme is concentrated in
pinocytotic and food vacuoles (Birns, 1960; Novikoff,
1960). The study of Müller et al. (1962) showed that
there was a progressive increase in the intensity of acid
phosphatase staining in the food vacuole of A. proteus
after ingestion of acid killed tetrahymenae. Electron-
microscope studies in Chapter III (p.96) revealed the pres-
ence in the cytoplasm of A. proteus of small vesicles con-
taining electron dense materials which were similar to
those described earlier by Roth (1960). Furthermore, it
was found that the contents of these vesicles were very
similar to that found in the vacuole containing injected
particles (bacteria or spores). Our recent electron micro-
scopic studies (Savanat and Casley-Smith, 1965, personal
observation) showed that these vesicles in effect contained
acid phosphatase. In the light of this evidence, it is
possible to assume that these vesicles released their con-
tents into the food vacuoles. It follows that killing of
bacteria by amoebae may have a sequence of events similar
to that in mammalian phagocytes.

Different rates of intracellular killing in amoebae of

salmonellae introduced into the cells by different routes.

It is clear from the present study that the rate of killing of naturally phagocytosed S. enteritidis in A. proteus was faster than that when the organisms were introduced into the cells by a micro-injection technique (Chapters III and IV). Electron-microscopic studies in Chapter III, p. 96, showed that at 2 hours after injection only small numbers of bacteria or bacterial spores were enclosed in the vacuoles, whereas at 6 or 24 hours after injection the great majority of them were confined in the vacuoles. This finding suggests that the delay in killing of injected S. enteritidis by A. proteus could be due to the time taken for the vacuoles to be formed around them. Possible reasons why a vacuolar environment should be more detrimental for bacteria than the cytoplasmic environment are given in the discussion at the end of Chapter IV.

The influence of serum antibodies on phagocytosis and intra-

cellular killing of bacteria by mammalian phagocytes and

by amoebae.

The enhancing effect of serum factors on phagocytosis of bacteria and other particulate matters by mammalian phagocytes has been well documented (Wright and Douglas, 1903; Mudd et al., 1934), but their influence on intracellular killing is still a matter of conjecture. Most

comparative phagocytic experiments suffer from the diffi-
culty of inducing a comparable degree of phagocytosis of
opsonised and unopsonised bacteria since, in the absence
of serum factors, little or no phagocytosis takes place
and consequently the rate of intracellular killing is un-
measurable.   This dilemma was partially circumvented by
an ingenious technique of Jenkin (1963) by means of which
unopsonised bacteria could be phagocytosed at the rate com-
parable to that of opsonised bacteria.   He showed that
S. typhimurium with adherent phage P_{22} could be phagocytos-
ed by mouse peritoneal macrophages in the presence of phage
antibodies at the rate comparable to that of opsonised or-
ganisms, but in contrast to opsonised bacteria they were
not killed.   Li, Mudd and Kapral (1963) presented evidence
to indicate that serum factors which acted in promoting
phagocytosis could be dissociated from those which acted in
promoting intracellular killing.   They showed that heat
inactivation of normal human serum resulted in a loss of
its ability to enhance killing of Staphylococcus aureus by
human polymorphonuclear leucocytes, even though its ability
to promote phagocytosis was retained.   However, under dif-
ferent experimental conditions, serum factors have been
shown to play an insignificant part in intracellular killing.
Miki and Mackaness (1964) showed that in vitro and in vivo
survival of Listeria monocytogenes was not influenced by the
presence of serum antibodies during the 5 hour period of

observation.   Thorpe and Marcus (1964) showed that immune serum had no effect on intracellular killing of Pasteurella tularensis by rabbit peritoneal macrophages harvested at 60 hours after saline stimulation.

In the present studies it was shown that pre-treatment of salmonellae with mammalian antibodies did not alter their survival rates in A. proteus when they were introduced into the cells either by micro-injection or by natural phagocytosis (Chapters III and IV).   Failure of opsonins to enhance killing in this situation could be because amoebae have not evolved in a way that such an effect would endow the cells with any survival advantage.   On the other hand, it could be that in the amoebae system, opsonin factors have already been provided (probably by the amoebae themselves) and hence provision of more in the form of mammalian antibody might have no effect.

Mammalian opsonins were shown, however, to promote phagocytosis of salmonellae in A. proteus, but the degree of enhancement was very much less than that to be expected if mammalian phagocytes were used.   Furthermore, this enhancement could be partly due to physical effects, since the bacteria formed clumps when the concentration of specific antibody used was equivalent to 1 minimal agglutinating dose per ml. or more.

The influence of complement on intracellular bactericidal mechanisms.

The studies in Chapter III (p.88) showed that an additional presence of fresh rabbit serum previously ab- sorbed with homologous organisms enhanced killing of opson- ised S. enteritidis when these were injected into the cyto- plasm of A. proteus.   It is highly likely that the enhanc- ing factors in the serum were in effect complement, since this serum failed to enhance killing if opsonisation was done at 4°.   (Under this condition, it is generally accept- ed that complement is only fixed very slowly).   The mech- anisms by which complement acts in promoting intracellular killing of smooth organisms in the presence of antibody are not known.   Wardlaw (1964) suggested that smooth organisms had on their surface the substrate for complement and this would probably be lipid or lipid-protein complexes.   Under normal circumstances, however, complement could not gain access to its substrate because of a protective covering of O-somatic antigen on the bacterial surface and because of a hindrance created by the highly negative charge on the bac- terial cell.   As soon as these effects were removed by re- action with O-antibody, complement was then able to combine with the lipoprotein on the bacterial cell or it might form a bridge connecting between the antibody and the cell wall lipoprotein.   This would trigger off a series of changes

which finally make the bacterial cells susceptible to the lysozyme action. Wardlaw (1962) showed that the bactericidal action of normal human serum was profoundly diminished when serum lysozyme was depleted even though the complement level was not appreciably altered. In keeping with this idea, it is possible to postulate the mechanism by which A. proteus kills more effectively smooth organisms treated with specific antibody in the presence of complement as follows: serum antibody acts first with the lipopolysaccharide on the bacterial surface and consequently makes available the substrate for complement to act resulting in physico-chemical changes on the bacterial surface and the organisms eventually become susceptible to enzymatic digestion by amoebae.

The mechanisms of membrane formation around the organisms injected into the cytoplasm of A. proteus.

In Chapter III, p.96, electron microscopic evidence is presented to demonstrate that membranes are synthesised de novo to enclose the particles injected into the cytoplasm of A. proteus. The process of membrane formation could be explained in relation to a group of cytoplasmic organelles called 'the reticulosome' (Pollak and Shorey, 1964; Pollak, 1965). They find that at one stage in the development of chicken liver, they can centrifuge out masses of these particles; later, when the endoplasmic reticulum has developed, the particles are much scarcer. The reticulosomes are morphologically very similar to the

ribosomes, including staining with lead and lying free in
the cytoplasm, but they are electrophoretically distinct
from ribosomes and are not destroyed by RNA-ase.   Pollak
believes that these particles are in effect prefabricated
pieces of membranes.   In the present study, it is shown
that the particles seen to surround the injected material
are also similar morphologically to the ribosomes, they
sometimes seem to have pale centres with an electron-dense
outer rim, and they are replaced by a unit membrane.   It
would thus appear probable that they are reticulosomes, that
they lie pre-formed in the cytoplasmic matrix, and that they
are converted into the vacuolar membranes.   The observa-
tions on the amoebae incubated at 4° indicate that, while
cellular energy may be needed to form the reticulosomes, it
is not required to make them aggregate around an injected
particle.   However, cellular energy is needed to transform
them from approximately spherical particles to become a part
of a sheet of a unit-membrane.   This behaviour of the retic-
ulosomes may be in accord with the observations that cell-
ular energy is necessary for the ingestion of large ($\sim 1\,\mu$)
particles and for the formation of phagocytic vacuoles (Kar-
novsky, 1962).

It could be argued that the small particles are in
fact ribosomes and that the membranes formed in relation to
them precisely because one of the functions of ribosomes
could be to form membranes, but this does not appear very

likely.   Apart from Pollak 's evidence that there are
reticulosomes, quite distinct from ribosomes, there is the
fact that the new membranes appear to replace the particles,
not just form in relation to them (fig.15, 16).   The dis-
appearance of the one coinciding with the appearance of
the other is the best evidence for the transformation of
the one into the other, rather than the manufacture of the
one by the other.

The reticulosome should have a great effect on our
concepts of membrane physiology.   These pre-formed units
can presumably enter and unite with pre-existing membranes
at any point.   Yet they can be present in the cytoplasm in
large numbers and form new membranes - endoplasmic reticul-
um or vacuoles - rather than simply joining the pre-existing
membranes.   Evidently there are various factors which det-
ermine when they join and when they do not.   The unravel-
ling of these factors should do much to elucidate the be-
haviour of membranes.

Micro-injection of particles into amoebae and their
subsequent localisation with the electron microscope has
also been performed by Feldherr (1962) using ferritin as a
marker.   He found the particles in the ground cytoplasm
and the ground nucleoplasm, between the two membranes of
mitochondria, in vacuoles and in food vacuoles.   In the
present study, particles were only found in the ground cyto-
plasm, in vacuoles and in food vacuoles.   They presumably

were all too large to enter the nuclear pores, and were obviously too large to be contained between the membranes of mitochondria.

The significance of the formation of membranes around injected organisms in relation to host defence.

Kopac (1958) predicted that by means of a micro-injection technique, it was possible to transplant micro-organisms such as zoochlorellae into the cytoplasm of am-oebae and these would probably evade intracellular killing and digestion by the protozoa and finally become established in the cytoplasm as xenic 'sub-cellular' inclusions. The results in Chapter III, however, indicate that such a phen-omenon is unlikely to occur, since the bacteria or bacterial spores injected into the cytoplasmic matrix were finally surrounded by vacuolar membranes, and the organisms were eventually killed. It is possible that de novo formation of the membrane in this instance may be a primitive means of protection in phagocytic cells against micro-organisms which are able to penetrate the cell membrane. It follows that recognition mechanisms at a sub-cellular level may depend on this process. It is also possible that success-ful parasitisation of any micro-organism in the cytoplasm of a given cell depends on its ability to inhibit the form-ation of the membrane around it.

Chemotaxis and food selection.

The studies on selection of bacterial food by soil amoebae reveal that this may operate at various levels. At the level of food seeking, it was shown that some edible bacteria were chemotactic whereas inedible bacteria failed to be so (Chapter VI, pp.137, 141). Chemotactic principles seemed to be associated only with living bacterial cells since chemotaxis was lost when the edible organisms were killed by heat, alcohol or streptomycin. The exact nature of the substance or substances chemotactic for amoebae is not known. In contrast, the general characters of these substances which attract mammalian phagocytes are fairly well defined. Boyden (1962) showed by means of a milipore chamber technique that the chemotactic principle for mammalian polymorphonuclear leucocytes was, in effect, a heat stable substance which was produced as the result of the activation of heat labile components of normal serum by the antigen antibody complex. The finding that inedible bacteria treated with serum antibodies became chemotactic for soil amoebae suggests that perhaps a similar mechanism may be operative. It is not impossible that amoebae secrete substances which interact with food substances or bacteria resulting in liberation of chemotactic agents. In the case of inedible bacteria, secretory substances from amoebae may be prevented from interacting with their substrates on the bacterial cell probably by steric hindrance caused by

some surface antigens. As soon as this inhibitory effect was removed by serum antibody, the secretory substances from amoebae are able to react with their substrates and the chemotactic principle is consequently released.

The mechanisms whereby amoebae respond to chemo-tactic stimuli are not clear. It has been shown that A. proteus could be induced to send out pseudopods which are subsequently developed into food cups by the extracts of Tetrahymena pyriformis or Hydra viridans (Jeon and Bell, 1962) and by heparin (Bell and Jeon, 1962). Furthermore, Bingley and co-workers (1962) showed that these inducers were able to depolarise the membranes of A. proteus. In the light of this evidence, Bell and Jeon (1963) suggested that the response of A. proteus to chemotactic stimuli was brought about by the action of chemotactic substances on the surface of amoebae and hence caused depolarisation of the membrane, and the resulting local depolarisation cur-rent might be the signal for attracting cytoplasmic move-ment into the stimulated spot.

It was frequently observed that as soon as the am-oebae arrived at the clump of edible bacteria, ingestion followed almost immediately. It is possible that the stim-ulation which acts in eliciting chemotaxis might also act in the induction of food cup, the formation of which might depend on the strength of the chemotactic substances. This idea at first seems to be contradictory to the observations

mentioned earlier in the introductory chapter (p.6) that
engulfment is not necessarily an inevitable consequence of
chemotaxis.   However, this contradiction could be explain-
ed in terms of the adverse effect of the chemotactic sub-
stances which might repel the cells when their concentra-
tion becomes too intense.

Chemotaxis, however, is unlikely to be the sole
basis of food selection in soil amoebae, since not all ed-
ible bacteria are chemotactic (Chapter VI, p.143).   Further-
more, it was shown that amoebae ingested preferentially ed-
ible bacteria even when they were present together with in-
edible ones in a colony of mixed cultures.   It is conceiv-
able that under this condition, chemotaxis may not come into
play.   Selective feeding in this instance indicates the
fineness of discrimination and this will be discussed in
the following section.

The mechanism of selective feeding of edible bacteria in a
    colony of mixed cultures.

It is reasonable to believe that in nature amoebae
must have a means to control the amount of bacteria ingested
otherwise they might suffer from over-ingestion and the
cells might even burst.   It can be deduced from the work
of Bell and co-workers (see the review by Bell, 1963) that
the formation of food cups is preceded by depolarisation of

the membrane and hence it is likely that mechanisms which regulate ingestion may depend on polarisation of the cell membrane. It is possible that after ingestion of sufficient food, the cell surface may become refractory to a further stimulation by substances which are able to depolarise the membrane. In this respect, different species of bacteria may cause different degrees of alteration on the membrane potentials of amoebae and it is likely that edible organisms may exert more profound changes than their counterparts which are inedible. When the food has been digested, the membrane potential might gradually change and at a certain level it becomes susceptible only to stimulation by edible bacteria but not by inedible organisms. By this means the cells would keep on ingesting edible organisms and ignoring the inedible ones. This idea, however, is purely speculative and, as far as the current reports go, no experiments have been done to study the relationship between membrane potentials and feeding habits in amoebae.

One of the interesting findings which comes out from the study on food selection of bacteria in a mixed colony is the demonstration of a comparatively faster rate of killing of edible bacteria (Chapter VI, p.146). This result contrasts with that obtained from phagocytosis experiments in which it was shown that killing of edible and inedible bacteria proceeded at a comparable rate. This contradiction, however, could be explained in relation to the numbers of

bacteria ingested, i.e. in the study of phagocytosis in
colonies of mixed cultures a large number of bacteria was
ingested per amoeba, whereas in the study of phagocytosis
in the liquid medium only one bacterium was associated with
an amoeba.   It is likely that the inedible bacteria are
less sensitive to the bactericidal action of amoebae than
the edible ones.   When small numbers of bacteria were phag-
ocytosed, the enzymes participating in killing and digestion
of bacteria would be efficient in exerting a bactericidal
effect for both edible and inedible bacteria.   On the con-
trary, when a large number of bacteria were ingested, the
amount of these enzymes might fall below a critical bacter-
icidal level for the inedible bacteria, nevertheless this
level might be high enough to inactivate the edible ones.
Bacterial agglutination as a means which facilitates feeding.

The means by which soil amoebae obtain their food
depends not only on their ability to move directionally to-
wards edible bacteria (chemotaxis), but also on their abil-
ity to capture motile organisms which happen to come into
contact with the amoeban surface and hence become adherent
to the cells.   The phenomenon of surface adhesion was first
observed by Mouton (1902) and later confirmed by Ray (1951).
Ray observed that there was a correlation between the pat-
tern of agglutination and the position of flagellae on the
bacterial cells and hence she concluded that this reaction
was in effect due to adhesion of flagellae on to the surface

of amoebae.   She suggested that this could be brought about by either a secretion from amoebae or by physico-chemical reactions, e.g. surface charge effects.   The results in the present studies (Chapter VI, p.154) do not prove this point one way or the other, but they suggest that this is likely to be due to a secretion rather than physico-chemical reactions at the cell surface (p.162).   If this is true, this substance may be regarded as 'natural' opsonin for soil amoebae, since it facilitates feeding in these protozoa. During the course of evolution in which the structures of the animal bodies become more and more complex, it is possible that the phagocytes can no longer synthesise opsonins by themselves and the ability to manufacture opsonins is handed on to antibody forming cells.   Nevertheless, these antibodies can be adsorbed on to the surface of phagocytes and thus enable them to phagocytose bacteria or other particulate matters in the absence of serum antibodies in the medium.   The existence of 'cytophilic' antibody has been clearly demonstrated by Boyden and Sorkin (1960, 1961), who showed that normal rabbit spleen cells treated in vitro with serum antibodies against human serum albumin were capable of adsorbing this antigen.   Jenkin, Rowley and Auzins (1964) reported that peritoneal macrophages harvested from mice at 14 days after being rendered 'carriers' of S. typhimurium were able to phagocytose and kill homologous organisms in vitro even when antibody was lacking in the medium.   Rowley,

Turner and Jenkin (1964) showed that these macrophages acted
by virtue of 'cell bound' antibody and this could be removed
by trypsin treatment. Similar results were reported by
Bennett, Old and Boyse (1964) in the studies of phagocytosis
of tumour cells by immune mouse peritoneal macrophages.
These authors showed that macrophages from immune mice were
able to phagocytose tumour cells in the absence of immune
antibody in the medium. This ability was abolished by
washing the peritoneal cells with balanced salt solution,
but was restored by the addition of isoantibody against the
tumour cells.

In the course of the study of intracellular killing
of bacteria by soil amoebae, a single cell technique has
been developed. It is evident that with this technique it
is possible to measure accurately the rate of intracellular
survival of bacteria. Should this technique be applied to
the mammalian phagocytic cell system, it might be possible
to solve some perplexing problems such as the rates of kill-
ing of virulent and avirulent bacteria, the effect of opsonin
on intracellular killing and even on the controversial prob-
lem of 'cellular' immunity.

REFERENCES.

Adams, M.H. (1959a): 'Bacteriophages'. Interscience Publishers, Inc., N.Y., p.456.

Adams, M.H. (1959b): idem, p.450.

Adams, M.H. (1959c): idem, p.463.

Adams, M.H. (1959d): idem, p.234.

Auzins, I. and Rowley, D. (1961): Aust. J. exp. Biol. med. Sci., 40, 283.

Band, R.N. (1959): J. Gen. Microbiol., 21, 80.

Bell, L.G.E. (1963): 'Some observations concerning cell movement and cell cleavage'. In 'Symposia of the international society for cell biology'. Vol.2, pp.215-228.

Bell, L.G.E. and Jeon, K.W. (1962): Nature, 195, 400.

Bell, L.G.E. and Jeon, K.W. (1963): Nature, 198, 675.

Bennett, B., Old, L.J. and Boyse, E.A. (1964): Transplantation, 2, 183.

Berry, L.J. and Spies, T.D. (1949): Medicine, 28, 239.

Bingley, M.S., Bell, L.G.E. and Jeon, K.W. (1962): Exp. Cell. Res., 28, 208.

Biozzi, G., Benacerraf, B. and Halpern, B.N. (1953): Brit. J. exp. Path., 34, 426.

Birns, M. (1960): Exp. Cell. Res., 20, 202.

Boyden, S.V. (1962): J. exp. Med., 115, 453.

Boyden, S.V. and Sorkin, E. (1960): Immunol., 3, 272.

Boyden, S.V. and Sorkin, E. (1961): Immung., 4, 244.

Bozler, E. (1924): Arch. Protistenk, 49, 163.

Bragg, A.N. (1936): Physiol. Zool., 9, 433.

Brachet, J. (1955): Biochim. Biophys. Acta., 18, 247.

Brachet, J. (1961): 'Nucleocytoplasmic interactions in
     unicellular organisms'. In 'The cell', vol.2, pp.771-
     841. Brachet, J. and Mirsky, A.E., ed. Academic Press,
     N.Y.

Brandt, P.W. (1958): Exp. Cell. Res., 15, 300.

Brandt, P.W. and Pappas, G.D. (1962): J. cell. Biol.,
     15, 55.

Briggs, J.D. (1958): J. exp. Zool. 138, 155.

Brumfitt, W. and Glynn, A.A. (1961): Brit. J. exp. Path.,
     42, 408.

Caufield, J.B. (1957): J. Biophys. Biochem. Cytol., 3, 827.

Chalkley, W.H. (1930): Science, 71, 442.

Chambers, R. and Chambers, E.L. (1961): 'Explorations into
     the nature of the living cell'. Harvard Univ. Press,
     Cambridge, Mass., p.134.

Chambers, R., Pollack, H. and Hiller, S. (1927): Proc.
     Soc. exp. Biol. Med., 24, 760.

Chang, S.L. (1960): Canad. J. Microbiol., 6, 397.

Chapman-Andresen, C. (1962): Compt. Rend. Trav. Lab.
     Carlsberg, 33, 73.

Chi, L., Vogel, J.E. and Shelokov, A. (1959): Science, 130, 1763.

Clark, A.M. (1943): Aust. J. exp. Biol. med. Sci., 21, 215.

Clark, E.R., Clark, E.L. and Rex, R.O. (1936): Am. J. Anat. 59, 123.

Cohen, A.I. (1959): Ann. N.Y. Acad. Sci., 78, 609.

Cohn, Z.A. (1964): J. exp. Med., 120, 869.

Cohn, Z.A. and Hirsch, J.G. (1960): J. exp. Med., 112, 983.

Cohn, Z.A. and Morse, S.I. (1959): J. exp. Med., 110, 419.

Cohn, Z.A. and Morse, S.I. (1960): J. exp. Med., 111, 667.

Cohn, Z.A. and Weiner, E. (1963): J. exp. Med., 118, 1009.

Comandon, J. and de Fonbrune, P. (1936): C. R. Soc. Biol. Paris, 123, 1170.

Comandon, J. and de Fonbrune, P. (1939a): C. R. Soc. Biol. Paris, 130, 740.

Comandon, J. and de Fonbrune, P. (1939b): C. R. Soc. Biol. Paris, 130, 744.

Comandon, J. and de Fonbrune, P. (1953): In 'Traite de zoologie' Tome I, Fasc. 2. Masson et Cie, Paris. p.25, Grasse, P.P. ed.

Cutler, D.W. and Crump, L.M. (1928): Brit. J. exp. Biol., 5, 155.

Daniels, E.W. (1962): J. Protozool., 9, 183.

Davis, B.D. and Mingioli, E.S. (1950): J. Bact., 60, 17.

De Fonbrune, P. (1949): 'Technique de micromanipulation'. Masson et Cie, Editeurs, Libraires de l'Académie de Médecine, Paris.

De Tara, N. and Rustad, R.C. (1959): Exp. Cell. Res., 17, 191.

Drożański, W. (1963): Acta Microbiol. Polon., 12, 281.

Drożański, W. and Drożańska, D. (1961): Acta microbiol. Polon., 10, 379.

Dubos, R.J. (1954): In 'Biochemical determinants of microbial diseases'. Harvard Univ. Press, Cambridge, Mass., p.42.

Dudziak, B. (1962): Acta Microbiol. Polon., 11, 223.

Ecker, E.E. and Lopez-Castro, G. (1947): J. Immunol., 55, 169.

Edwards, J.G. (1923): Brit. J. exp. Biol., 1, 571.

Edwards, J.G. (1925): Biol. Bull., 48, 236.

Essner, E. (1960): J. Biophys. Biochem. Cytol., 7, 329.

Feldherr, C.M. (1962): J. cell. Biol., 12, 159.

Fenn, W.O. (1922): J. Gen. Physiol., 4, 331.

Fenn, W.O. (1923): J. Gen. Physiol., 5, 311.

Finstad, J. and Good, R.A. (1964): J. exp. Med., 120, 1151.

Fishman, M. and Silverman, M.S. (1957): J. exp. Med., 105, 521.

Fleming, A. (1933): Proc. Roy. Soc. Med., 26, 71.

Frosch, P. (1897): Centralbl. f. Bakt., Abt. I, 21, 926.

Goldacre, R.J. (1961): Exp. Cell. Res. Sup., 8, 1.

Goldstein, L. (1958): Exp. Cell. Res., 15, 635.

Goldstein, L. and Plaut, W. (1955): Proc. Natl. Acad. Sci.
41, 874.

Goodman, J.R. and Moore, R.E. (1956): J. Bact., 71, 547.

Hanks, J.H. (1955): In 'Introduction to cell and tissue
culture'. Burgess Publishing Co., Minneapolis, Minn.,
p.2.

Harris, H. (1953): J. Path. Bact., 66, 135.

Harris, H. (1954): Physiol. Rev., 24, 529.

Harris, H. (1960): Bact. Rev., 24, 3.

Hayward, A.F. (1963): Compt. Rend. Trav. Lab. Carlsberg,
33, 535.

Heller, I.M. (1959): Ann. N.Y. Acad. Sci. 78, 602.

Heller, I.M. and Kopac, M.J. (1955): Exp. Cell. Res., 8, 62.

Hirsch, J.G. (1956): J. exp. Med., 103, 589.

Hirsch, J.G. (1960): Bact. Rev., 24, 133.

Hirsch, J.G. (1962): J. exp. Med., 116, 827.

Hirsch, J.G. and Cohn, Z.A. (1960): J. exp. Med., 112, 1005.

Hofer, B. (1889): Jenaische Zeitschrift., 24, 109. Quoted
by Metchnikoff, E. (1893) in 'Lectures on the compar-
ative pathology of inflammation', p.19.

Holter, H. (1954): Proc. Roy. Soc. Lond. ser. B., 142, 140.

Holter, H. and Lowy, B.A. (1959): Compt. Rend. Trav. Lab.
Carlsberg, 31, 105.

Hopkins, D.L. and Warner, K.L. (1946): J. Parasitol., 32, 175.

Howland, R.B. and Pollack, H. (1927): J. exp. Zool., 48, 441.

Inoue, K., Tanigawa, Y., Tabubo, M., Satani, M. and Amano, T. (1959): Biken's Journal., 2, 1.

Ivanic, M., (1933): Arch. Protistenk., 79, 200.

Ivanic, M., (1936): Cellule, 44, 367.

Jenkin, C.R. (1963): Brit. J. exp. Path., 44, 47.

Jenkin, C.R. and Benacerraf, B. (1960): J. exp. Med., 112, 403.

Jenkin, C.R. and Rowley, D. (1959): Nature, 184, 474.

Jenkin, C.R., Rowley, D. and Auzins, I. (1964): Aust. J. exp. Biol. med. Sci., 42, 215.

Jennings, H.S. (1931): In 'Behaviour of lower organisms'. Columbia Univ. Press.

Jeon, K.W. (1963): Cited by Bell, L.G.E. (1963).

Jeon, K.W. and Bell, L.G.E. (1962): Exp. Cell. Res., 27, 350.

Karrer, E.H. (1960): J. Biophys. Biochem. Cytol., 7, 357.

Karnovsky, M.L. (1962): Physiol. Rev. 42, 143.

Kopac, M.J. (1958): Ann. N.Y. Acad. Sci. 78, 696.

Lederberg, J. (1956): Genetics, 41, 845.

Leische, W. (1938): Arch. Protistenk., 91, 135.

Lewis, W. (1931): Bull. Johns Hopkins Hosp., 49, 19.

Li, I.W., Mudd, S. and Kapral, F.A. (1963): J. Immunol.,
90, 804.

Lilleengen, K. (1948): Acta Pathol. Microbiol. Scand. Supp.
77.

Looke, E. and Rowley, D. (1962): Aust. J. exp. Biol. Med.
Sci., 40, 315.

Lockwood, W.R. and Allison, F. (1963): Brit. J. exp. Path.,
44, 593.

Lockwood, W.R. and Allison, F. (1964): Brit. J. exp. Path.,
45, 294.

Lorch, I.J. and Danielli, J.F. (1953): Quart. J. Microsc.
Sci., 94, 461.

Losina-Losinsky, L.K. (1931): Arch. Protistenk., 74, 18.

Lucke, B., McCutcheon, M., Strumia, M. and Mudd, S. (1929):
J. exp. Med., 49, 797.

Lund, E.J. (1914): J. exp. Zool., 16, 1.

Maaløe, O. (1946): 'On the relation between alexin and
opsonin', Ejnar Munksgaard, Copenhagen.

McCutcheon, M. (1946): Physiol. Rev., 26, 319.

McCutcheon, M. (1955): Ann. N.Y. Acad. Sci., 59, 941.

Madsen, T. and Watabiki, T. (1919): Medd. k. Setakad.

Nobelinst, V. Quoted by Fenn, W.O. (1922).

Marchant, J. (1952): Quart. J. Microsc. Sci., 93, 395.

Mast, S.O. (1932): Physiol. Zool., 5, 1.

Mast, S.O. (1942): Biol. Bull., 83, 173.

Mast, S.O. (1947): Biol. Bull., 92, 31.

Mast, S.O. and Bowen, W.J. (1944): Biol. Bull., 87, 188.

Mast, S.O. and Doyle, W.L. (1934): Protoplasma, 20, 555.

Mast, S.O. and Fennel, R.A. (1938): Physiol. Zool., 11, 1.

Mast, S.O. and Hahnert, W.F. (1935): Physiol. Zool., 8, 255.

Mast, S.O. and Prosser, C.L. (1932): J. Cell. Compar.
    Physiol., 1, 333.

Mercer, E.H. (1959): Proc. Roy. Soc. Lond. ser. B., 150,
    216.

Metalnikov, S. (1912): Arch. Zool. expér. gén. 5° ser.,
    2, 373.

Metchnikoff, E. (1893): 'Lectures on the comparative path-
    ology of inflammation'. Translated by Starling, F.A.
    and Starling, E.H., Kegan Paul, Trench, Trubner & Co.
    Ltd., Lond.

Miki, K. and Mackaness, G.B. (1964): J. exp. Med., 120, 93.

Miya, F. and Marcus, S. (1961): J. Immunol., 86, 652.

Mouton, H. (1902): Ann. Inst. Past., 16, 457.

Mudd, S., Lucke, B., McCutcheon, M. and Strumia, M. (1929):
    J. exp. Med., 49, 779.

Mudd, S., McCutcheon, M. and Lucke, B. (1934): Physiol.
    Rev., 14, 210.

Müller, M., Tóth, J. and Téro, I. (1962): Acta. Biol. Acad.
    Sci. Hung., 13, 105.

Myrvik, Q.N. and Weiser, R.S. (1955): J. Immunol., 74, 9.

Myrvik, Q.N., Leake, E.S. and Fariss, B. (1961): J. Immunol.,

86, 133.

Nelson, R.A. and Lebrun, J. (1956): J. Hyg., 54, 8.

North, R.J. and Mackaness, G.B. (1963): Brit. J. exp.
Path., 44, 601.

Northrop, J.H. and De Kruif, P.H.C. (1922): J. Gen.
Physiol., 4, 655.

Novikoff, A.B. (1960): In 'Developing cell systems and
their control'. D. Rudnik ed. pp.167-203. Ronald
Press. N.Y.

Oehler, R. (1916): Arch. Protistenk., 37, 175.

Osler, A.G. (1961): Adv. Immunol., 1, 131.

Papermaster, B.W., Condie, R.M., Finstad, F. and Good, R.A.
(1964): J. exp. Med., 119, 105.

Parks, H.F. and Chiquoine, A.D. (1956): Anat. Rec., 124,
343.

Pavillard, E.R.J. (1963): Aust. J. exp. Biol. med. Sci.,
41, 265.

Pavolova, E.A. (1938): Med. Parasitol. Parasitic. Dis.
(Russ.), 7, 119.

Penard, E. (1905): Arch. Protistenk., 6, 175.

Pitts, R.F. and Mast, S.O. (1933): J. cell. comp. Physiol.,
3, 449.

Pollack, H. (1928): Biol. Bull., 55, 383.

Pollak, J.K. and Shorey, C.D. (1964): Biochem.J., 93, 36c.

Pollak, J.K. (1965): Personal communication.

Potter, E.V. and Stollerman, G.H. (1961): J. Immunol.,
87, 110.

Prescott, D.M. and James, T.W. (1955): Exp. Cell. Res., 8, 256.

Ray, D.L. (1951): J. exp. Zool., 118, 443.

Repaske, R. (1956): Biochim. Biophys. Acta, 22, 189.

Reynolds, B.D. (1924): Biol. Bull., 46, 106.

Reynolds, E.S. (1963): J. cell. Biol., 17, 208.

Reznikoff, P. and Pollack, H. (1928): Biol. Bull., 55, 377.

Rhumbler, L. (1910): Arch. Entw. Mechan. d. Organ., 30, 194.

Rice, N.E. (1935): Arch. Protistenk., 85, 350.

Rice, N.E. (1938): Arch. Protistenk., 90, 354.

Ridley, F. (1928): Proc. Roy. Soc. Med., 21, 1495.

Robertson, J.D. (1960): Progr. in Biophys. Chem., 10, 343.

Roth, L.E. (1960): J. Protozool., 7, 176.

Rother, K., Rother, U., Petersen, K.F., Gemsa, D. and Mitze, F. (1964): J. Immunol., 93, 319.

Roux, J., Serre, A. and Bassouls, C. (1964): C. R. Soc. Biol. Paris, 158, 613.

Rowley, D. (1958): Nature, 181, 1738.

Rowley, D. (1959): Quoted by Rowley, D. (1962).

Rowley, D. (1962): Adv. Immunol., 2, 241.

Rowley, D., Turner, K.J. and Jenkin, C.R. (1964): Aust. J. exp. Biol. med. Sci., 42, 237.

Rowley, D. and Whitby, J.L. (1959): Brit. J. exp. Path., 40, 507.

Rytel, M.W. and Stollerman, G.H. (1963): J. Immunol., 90, 607.

Schaeffer, A.A. (1910): J. exp. Zool., 8, 75.

Schaeffer, A.A. (1916a): Biol. Bull., 31, 303.

Schaeffer, A.A. (1916b): J. exp. Zool., 20, 529.

Schaeffer, A.A. (1917): J. anim. Behav., 7, 220.

Schumaker, V.N. (1958): Exp. Cell. Res., 15, 314.

Semenoff, W.E. (1938): Bull. Biol. Med. Exp. USSR., 5, 186.

Severtzova, L.B. (1928): Centralbl. f. Bakt. Abt. II, 73, 162.

Shaffer, B.M. (1956): J. exp. Biol., 33, 645.

Singh, B.N. (1941): Ann. Appl. Biol., 28, 52.

Singh, B.N. (1942): Ann. Appl. Biol., 29, 18.

Singh, B.N. (1945): Brit. J. exp. Path., 26, 316.

Sprick, M.G. (1956): Am. Rev. Tuberc., 74, 552.

Stahl (1884): Botanische Zeitung, No. 10 - 12. Quoted by
    Metchnikoff, E. (1893) in 'Lectures on the comparative
    pathology of inflammation', p.33.

Thorbecke, G.J., Old, L.J., Benacerraf, B. and Clarke, D.A.
    (1961): J. Histochem. Cytochem., 9, 392.

Thornton, F.E. (1932): Physiol. Zool., 5, 246.

Thorpe, B.D. and Marcus, S. (1964): J. Immunol., 92, 657.

Tsujitani, J., (1898): Centralbl. f. Bakt. Abt. I, 24, 666.

Van Rooyen, C.E. (1932): J. trop. Med. Hyg., 35, 259.

Vickerman, K., (1962): Exp. Cell. Res., 26, 497.

Wenrich, D.H. (1941): Biol. Bull., 81, 324.

Wiercinski, F. (1944): Biol. Bull., 86, 98.

Wardlaw, A.C. (1962): J. exp. Med., $\underline{115}$, 1231.

Wardlaw, A.C. (1964): In 'Bacterial endotoxins', pp. 81-88, Quinn & Boden Co., Inc., Rahway, N.J., Landy, M. and Braun, W., ed.

Wilson, A.T., Wiley, G.G. and Bruno, P. (1957): J. exp. Med., $\underline{106}$, 777.

Wright, A.E. and Douglas, S.R. (1903): Proc. Roy. Soc. Lond. ser. B. $\underline{72}$, 357.

Wu, W.G. and Marcus, S. (1964): J. Immunol., $\underline{92}$, 397.

Zucker-Franklin, D. and Hirsch, J.G. (1964): J. exp. Med. $\underline{120}$, 569.