CHEMOTAXIS IN THE AMOEBA

HARTMANNELLA RHYSODES

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ABSTRACT

A number of techniques have been considered for the measurement of chemotaxis of the soil amoeba Hartmannella rhysodes to food bacteria. It was found that only the Boyden Chamber technique would give any reasonable degree of quantitation. The mechanism responsible for the chemotactic response of amoebae to food material was investigated using a modified version of this technique. This resulted in the finding that soil amoeba may actively participate in the initiation of the chemotactic response by the release of substances, almost certainly enzymes, which may react with components of the bacteria to release small molecular weight chemotactic factor(s). Amoebae which were starved for up to four days were unable to cause the release of these factors, but responded to them by chemotaxis once they had been released into solution by other means. The physical and chemical properties of the active substance(s) were investigated, the results indicating that a very stable molecule or group of molecules of approximately 400 molecular weight, possessing a strong negative charge, was responsible for the induction of the chemotactic movement. However, a similar substance was also found to be produced by growing or lysed bacteria. The possible significance of these findings with respect to the present knowledge of recognition mechanisms in other protozoan and metazoan cells is discussed.

This thesis contains no material previously submitted by me for a degree in any University, and to the best of my knowledge and belief it contains no material previously published or written by another person except when reference is made in the text.

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INTRODUCTION

The ability to distinguish foreign matter from self components must have been one of the fundamental properties influencing the survival of the cell during evolution. The survival of species at the single cell level would necessitate the ability of a cell to distinguish cells of other species from those of its own, when seeking food. Boyden (1963) has suggested that the development of animals could not have proceeded beyond the metazoa if there had not existed some reliable means of preventing colonization of the tissues of multicellular animals by the micro-organisms ever present in the environment. It is also clear that the ability of cells to identify self from non-self is fundamental to the organization of tissues during embryogenesis.

The present thesis is concerned with the manner in which a highly specialized cell recognizes the presence of food particles in its environment. Basically the phenomenon can be divided into two parts. The first involves the movement of the phagocytic cells towards the foreign material, a process known as chemotaxis, and the second involves the uptake of the particle, i.e. phagocytosis. Studies with vertebrate phagocytic cells have indicated that both these parts depend on highly specific reactions. Despite the fact that people have demonstrated the movement of phagocytic cells of invertebrates towards a foreign body imbedded in the tissues and

subsequent phagocytosis, nothing is known concerning the mechanism of chemotaxis or phagocytosis in these animals. One of the problems of working with the invertebrates is that the phagocytic cells of these animals are as yet difficult to maintain in tissue culture, unlike similar cells from the vertebrates. However, many free living protozoa such as amoebae which may be maintained with ease in the laboratory display these phenomena. They move in a positive manner towards food particles and many species are highly selective in their feeding, preferring to feed on a certain organism rather than another if presented with both.

We have chosen to work with soil amoebae and study the first part of the recognition phenomenon, i.e. chemotaxis, in the hope that a better knowledge of this reaction with a particular type of phagocytic cell may give us some clue as to what may be happening in more complex invertebrate metazoan systems. However, one must bear in mind when using a model system such as this that natural selection may well have operated such that the mechanism of recognition may vary between phyla, though it is likely that there may be certain basic similarities.

The first part of the thesis deals with attempts to find a suitable quantitative technique to measure chemotaxis in soil amoebae, and the remainder with an analysis of the mechanisms involved in

chemotaxis, including an investigation into the nature of the active substances.

CHAPTER 1

THE PHENOMENON OF CHEMOTAXIS

The ability of a cell to sense chemically the presence of particulate or soluble matter some distance from it and to move directionally towards that matter has been termed chemotaxis. It is the resultant of the reaction between the cell and a chemical substance diffusing from the source, the cell moving along a concentration gradient of this substance. The phenomenon was first described by Pfeffer (1884) who attempted to discover the mechanism whereby bracken sperm were attracted to their ova. He found that these motile cells swam towards a source of sodium malate in the same directional manner as they did towards their ova. The attractant was fairly specific, in that only malic acid and a few of its salts produced directional migration. Brockaw (1958) has since extended this work and presented evidence that these malate ions are reversibly adsorbed to "combining sites" on the anterior ends of the sperm. He suggested that concentration gradients of this chemical cause the head to point in the direction of the source, the sperm thus swimming up a gradient.

Since the original discovery of this phenomenon by Pfeffer, numerous other examples have been found in many biological systems. These include the reproductive cells of plants and animals (reviewed by Rosen, 1962; Ziegler, 1962; and Rothschild, 1956), many

protozoans (reviewed by Mast, 1941; Wichterman, 1953), including the mycetazoa (Bonner, 1967) and the phagocytic cell systems of the metazoa (reviewed by McCutcheon, 1946; Harris, 1954, 1960). In this review, chemotaxis in some protozoan and metazoan amoeboid phagocytic cells will be considered.

1.1 Chemotaxis in metazoan amoeboid phagocytic cells

The cell system in which this phenomenon has been studied most widely is the group of cells responsible in the metazoans for the phagocytosis of foreign matter. The accumulation of large numbers of phagocytic cells at sites of inflammation led researchers to believe that chemotaxis was a fundamental aspect of the response. As early as 1843, Addison reported migration of leucocytes from blood vessels at sites of inflammation. Metchnikoff (1887, 1893) was prominent amongst the numerous investigators to observe this phenomenon in a variety of metazoa, and believed this cellular response to be the principal feature of metazoan defence against potential pathogenic organisms. However, the first to use the term "chemotaxis" with respect to leucocytes was Leber (1888, 1891). He saw a parallel between the process whereby female plant gametes or sodium malate attracted sperm, as described by Pfeffer (1884) and the way leucocytes could be attracted by chemical stimuli. This he demonstrated by observing the directional migration of leucocytes in a guinea pig eye

following injection of chemical substances into the cornea.

Numerous subsequent investigations of this phenomenon by a variety of techniques have until recently resulted in a mass of contradictory data. Almost every class of known biological substance was claimed to be chemotactic to leucocytes. These included proteins (Chambers and Grand, 1936), polysaccharides (Meier and Schär, 1954), simple sugars (Chambers and Grand, 1936), polypeptides (Menkin, 1956), tissue breakdown products (Massart and Bordet, 1890; Silverman, 1938), bacteria and other microorganisms (Leber, 1888; Metchnikoff, 1887; McCutcheon and Dixon, 1936; Harris, 1954). These varied findings may have resulted from the wide variety of techniques used to measure chemotaxis. Apart from the method used by Leber (1888), these include :

(i) Histological methods which demonstrate an accumulation of leucocytes at the site of injection of the test substance.

(ii) Tissue culture methods, where chemotactic substances were claimed to elongate the normally circular pattern of cells migrating from tissue explants (Kiaer, 1925).

(iii) In vivo observations, involving microcinematographic observations of the migration of cells towards sites of injection of test substances in either the tails of anaesthetised tadpoles (Clark and Clark, 1930) or in rabbit ear chambers (Allison, Smith and Wood, 1955; Cliff, 1966).

(iv) Use of capillary tubes, where substances were placed in these tubes and inserted either into the peritoneal cavity or beneath the skin of an animal or into a plasma clot. Chemotaxis was regarded as positive if the numbers of leucocytes present in the tube after a certain time were greater than in control tubes of saline. This method was used by Menkin (1936), Grand and Chambers (1936) and numerous other workers.

(v) Slide-coverslip methods, which involved placing a drop of blood between a slide and a coverslip, pressing them together and observing by microcinematography the movement of leucocytes towards particulate matter, or soluble substances adsorbed on to kaolin, which had previously been placed in the drop (Comandon, 1919; McCutcheon, 1946; Harris, 1953).

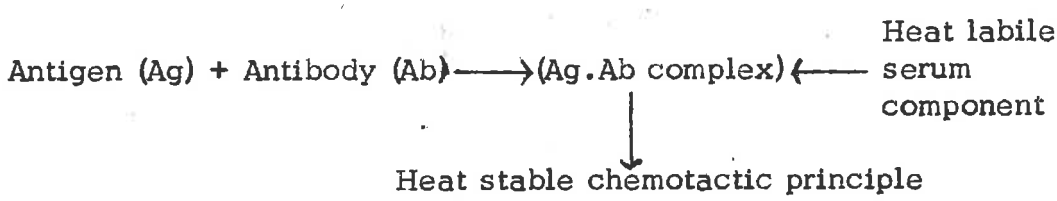
It is evident that many of these techniques do not give results which measure up to the requirements of the initial definition. Many factors other than chemotaxis may cause accumulations of cells at a site of injection or in a capillary tube containing the test substance. Pfoehl (1898) showed that convection currents due to differences in osmotic pressure would induce the migration of leucocytes into capillary tubes. While the experiments of Clark and Clark (1920) seem creditable, they did not differentiate between the accumulation of leucocytes at the injection sites due to the toxic effects of the test

substance and the accumulation due to chemical attraction. From their vivid descriptions of the events it seems that the former was the case with some of the materials tested, e.g. croton oil. The rabbit ear chamber also would seem to be a good in vivo system to test chemotaxis, and is used widely at present in investigations of inflammatory processes. However, while Clark and Clark (1935) reported many examples of chemotaxis using this method, later workers (Cliff, 1966; Allison et al., 1955) have been unable to detect chemotaxis with any of the commonly acknowledged chemotactic substances. Of the other methods mentioned, the slide-coverslip technique appears to demonstrate directional migration with minimal complication.

It was not until 1962 that a technique was introduced which enabled one to measure chemotaxis in a quantitative manner. Boyden (1962a) devised a Perspex chamber containing two compartments, separated by a membrane of such pore size that rabbit polymorphonuclear leucocytes could move through the membrane only by active migration. A cell suspension was placed in the upper compartment at the same time that the test medium was introduced into the lower one. After a period of incubation the membrane was removed, washed, fixed, stained and cleared. A microscopic count could then be made of cells that had migrated through to the lower side of the then transparent membrane.

Using this system, Boyden (1963) tested a large number of substances for their ability to attract rabbit polymorphonuclear phagocytes. A wide variety of substances was found to be chemotactic, but chemotaxis was only induced when the test substance was mixed with fresh serum. In general, he found that the further removed the source of the test material was phylogenetically from the rabbit, the more active it was in inducing chemotaxis. Even insoluble substances such as collodian were able to attract cells in the presence of fresh serum. Proteins from species closely related to the rabbit were hardly chemotactic in normal rabbit serum, but if these proteins were added to serum obtained from rabbits specifically immunized with these proteins, a very pronounced response was observed, being maximum around the equivalence point of antigen/antibody combination. Boyden concluded that the chemotactic response of polymorphonuclear leucocytes was mediated through serum antibody. However, if the antigen/antibody complexes were removed from serum by centrifugation, washed, and then tested in the chamber, they were found not to be chemotactic. Moreover, if the immune serum was heated at 56° for 30 minutes before adding the proteins, no chemotaxis occurred, despite the formation of antigen/antibody complexes. Further experiments indicated that if the immune serum was heated after the antigen/antibody reaction had taken place, then the mixture was still

chemotactic. This serum caused chemotaxis even when the antigen/antibody complexes were removed. Boyden concluded from these experiments that the antigen/antibody complexes fixed a heat labile component present in serum, which resulted in the release of a heat stable chemotactic principle. The reaction may be summarized as follows :



Boyden suggested that the heat labile component of serum was complement.*

It is clear from these findings why such a large number of substances was claimed by previous workers to be chemotactic. Many of the systems involved testing the substances in the presence of serum, this being a constituent of the tissue culture medium. Normal serum is known to contain small amounts of antibody (natural antibodies) to a variety of proteins and polysaccharides.

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* Complement is a complex mixture of proteins present in fresh serum of most vertebrates, consisting of 9 major functional components. These are fixed in an orderly fashion by antigen/antibody complexes.

During the last five years, study of this process has been carried on principally by two groups of workers, Ward and his colleagues in the U.S.A. and Keller and Sorkin in Switzerland. Ward has investigated the nature of the various chemotactic factors which may be released from complement, the mechanism whereby they induce directional migration, and factors released from bacteria, which are chemotactic to polymorphonuclear leucocytes and macrophages of mammals. His interest in the subject originated from findings with Cochrane (Ward and Cochrane, 1965) that the local binding of complement to antigen/antibody complexes associated with the walls of blood vessels caused an influx of polymorphonuclear leucocytes to the site. If by suitable treatments the animals were first depleted of complement, then the influx of polymorphonuclear cells failed to occur following the injection of antigen into the skin of a sensitized animal. Using the Boyden chamber, Ward, Cochrane and Müller-Eberhard (1965) found that, apart from antigen/antibody complexes, other substances known to fix complement, such as zymosan (an insoluble carbohydrate from yeast) and heat aggregated human γ globulin, also caused the liberation of chemotactic materials when mixed with fresh serum from different animal species. They also found that heat inactivation (56° for 30 minutes) of serum, as well as the presence of ethylene-diamine-tetracetic acid (E.D.T.A.)

prevented the formation of the factor. Sera from animals genetically deficient in one of the components of the complement system (C'6) were completely inactive. If, however, the missing complement component (C'6) was added to such sera, then activity was restored upon treatment with antigen/antibody complexes.

Serum containing the heat stable chemotactic principle formed as a result of fixation of complement by these complexes was subjected to column chromatography (such serum is frequently referred to as activated serum). The results of such experiments indicated that the active material was a protein complex consisting of C'5 and C'6 complement components. However, each component was inactive if tested individually. Further work led them to believe that C'7 was also associated with this chemotactic material (Ward, Cochrane and Müller-Eberhard, 1966). While many other substances may be produced in tissues following the reaction of complement with antigen/antibody complexes, none of the known mediators of hypersensitivity phenomena, e.g. serotonin, bradykinin, etc., were found by these people to be chemotactic. Subcutaneous injection of the trimolecular complex (C'5, 6, 7) into rabbits produced a marked infiltration of polymorphonuclear phagocytes into the injected areas.

It soon became clear, however, that there were substances chemotactic to polymorphonuclear leucocytes other than this

trimolecular complex. Taylor and Ward (1967) found that a certain ratio of plasminogen mixed with streptokinase resulted in the liberation of a low molecular weight fragment from the C'3 component of complement which was chemotactic to leucocytes. Ward (1967) later showed this "plasmin"-split fragment to be a heat labile, dialysable substance of molecular weight about 6000. This is very different from the large activated trimolecular complex, which is heat stable, and has a molecular weight of around 300,000. More recently, Ward (1968b) reported that two trypsin-split fragments, one from C'5, of molecular weight about 15,000, and the other from C'3 would also induce chemotaxis in vitro. Furthermore, Snyderman, Gewurz and Mergenhagen (1968) reported a 15,000-30,000 molecular weight substance produced when bacterial lipopolysaccharide was mixed with fresh serum which was chemotactic in vitro to rabbit polymorphonuclear leucocytes. This substance also appeared to be associated with the C'5 component of complement.

Whilst the work of Boyden and Ward and his colleagues suggested that the fixation of complement was important for the production of chemotactic factors, Keller and Sorkin (1965a and 1965b) have shown that the process is not quite so straightforward. They find that under conditions where complement is reported not to be fixed by antigen/antibody complexes, i.e. at pH 4 (Barandun, Kistler, Jeunet and

Isliker, 1962), chemotactic activity may still be generated in serum. On the other hand, even though heat aggregated human γ globulin fixes pig, sheep and human haemolytic complement, they find no chemotactic activity in the supernatant. These investigators agree with Ward, Cochrane and Müller-Eberhard, (1965) that antigen/antibody complexes are necessary for the production of a chemotactic factor(s) in a wide range of fresh sera, but are at variance with these workers in reporting that heat aggregated human γ globulin and bovine γ globulin, despite their ability to fix complement, do not cause active substances to be formed in fresh sera other than rabbit. A further point made by Keller and Sorkin (1967c) is that even when antigen/antibody complexes are incubated in fresh serum, it is possible that several factors are generated. They mixed such complexes with samples of serum which had been preheated at varying temperatures up to 100°. It was found that antigen/antibody complexes mixed with sera heated above 56° still resulted in a small though definable response. The factor formed in this way was also heat stable. Furthermore, column chromatography of activated serum, using buffer at pH8, resulted in the elution of chemotactic material of approximately 30,000 molecular weight (Keller and Sorkin 1967a). Under these alkaline conditions, the C'5, 6, 7 complex is known to dissociate (Nilson and Müller-Eberhard, 1965).

To complicate matters even further, Stecker and Sorkin (1969) found that sera taken from rabbits genetically deficient in C'6 was

just as good as sera taken from normal animals in its ability to liberate chemotactic principles with antigen/antibody complexes.

Other investigations by these workers (Keller and Sorkin, 1967a, 1965b) resulted in the finding that many strains of bacteria or bacterial products, such as the lipopolysaccharide endotoxin from gram negative bacteria, and the purified protein derivative (PPD) from mycobacteria, would give rise to chemotactic substance when incubated in the presence of normal serum, this being due to the presence of natural antibodies in the serum to the various products. However, there were certain anomalies which were difficult to explain.

For example, endotoxin from a strain of Proteus was found to produce a chemotactic factor when incubated with normal rabbit serum, while that derived from Salmonella enteritidis would not. One might expect this to be due to the presence of natural antibodies in the serum to the O somatic antigen of the former organism, but not the latter. However, there was found to be no correlation between the capacity of either fresh normal rabbit serum or bovine serum to induce chemotaxis with various endotoxins and their ability to agglutinate endotoxin coated sheep erythrocytes. This may indicate that a different type of antibody is required for the production of chemotactic factor from that required for haemagglutination.

Moreover, these same workers found that the culture filtrates of some bacteria grown in media not containing serum were able to induce the migration of polymorphonuclear cells through pores in a membrane filter, the cells also being suspended in a serum-free system. Similar results were obtained later by Ward and his colleagues (Ward, Lepow and Newman, 1968). Activity appeared in the culture filtrate only after a considerable time of growth, which led both groups of workers to believe that the chemotactic factor was a product of metabolism. The factor was found to be a low molecular weight substance (less than 3,600) and relatively heat stable (50% loss in activity at 56° for 30 minutes). Factors obtained from the culture filtrates of various bacteria appeared to be of similar size and stability. Apart from these bacterial culture filtrates, Keller and Sorkin (1965b) have reported other substances to be strongly chemotactic to both granulocytes and agranulocytes in the absence of serum. These include two culture media, Witte's peptone and Bacto-Casitone, and casein.

Until quite recently, most of the experimental work has been carried out on the polymorphonuclear leucocytes. Keller and Sorkin (1967b) have recently modified the Boyden Chamber technique to study the chemotactic movement of macrophages. These workers found that the only substance chemotactic to macrophages was casein. Serum activated by immune complexes or products of bacterial metabolism

induced no response. Again, these experimental results are in marked contrast to those reported by Ward (1968a). He found that macrophages would respond just like polymorphonuclear phagocytes in the presence of immune complex activated serum, the plasmin split C'3 factor, and bacterial culture filtrates, though at a much lower rate. However, he found that the factor produced in fresh serum by immune complexes to which the macrophages respond was not the C'5, 6, 7 component, although a heat labile component of serum was necessary for its generation.

1.1a Mechanism of action of chemotactic factors on leucocytes

Ward and his colleagues have attempted to find the chemical receptors on leucocytes with which the chemotactic substances react. Becker and Austen (1964) had earlier demonstrated that phosphonate esters inhibited the antigen/antibody induced release of histamine from tissue slices and cell suspensions, a reaction which also involved components of complement. Ward and Becker (1967) found that phosphonate esters would also inhibit the chemotactic response of polymorphonuclear leucocytes to the activated serum complex (C'5, 6, 7). Becker, Fukute, Boone, Canham and Boger (1963) had demonstrated that these compounds inactivated specific groups of enzymes, the serine esterases, in a characteristically irreversible manner. In the inhibition of chemotaxis, Ward and Becker (1967)

found that there were two distinct types of inhibition. Polymorphonuclear leucocytes could be prevented from migrating in the presence of the chemotactic factor if they were first pretreated with a variety of phosphonate esters and washed free of these substances before being placed in the test chamber. Another type of inhibition occurred with certain phosphonate esters which was dependent on the substance being present in the system throughout the experiment. Prior pretreatment of cells with these esters did not prevent migration (Ward and Becker, 1967). These results led them to conclude that the inhibition of chemotaxis caused by pretreating the cells with certain phosphonate esters was due to the inactivation of some active cell-bound esterases. The other type of inhibition was due also to inactivation of a cell bound esterase, but this became active only after the leucocyte had contacted the chemotactic factor, i.e. an activatable esterase.

1.1b Summary

While a considerable degree of progress has been made in the study of chemotaxis in metazoan phagocytic cells since the introduction of the Boyden technique, it is quite clear that considerable confusion exists as to the nature of the chemotactic principle(s) and their mode of action. Taking the sum of the evidence we can say, however, that as far as the polymorphonuclear leucocyte is concerned,

combination of antibody with the foreign material plus the fixation of a heat labile component of serum, probably complement, initiates the first step involved in recognition, i.e. chemotaxis. Attempts will be made in Chapter 7 to explain some of the confusion resulting from these experiments, in light of our own findings, in studying chemotaxis in the amoeba Hartmannella rhysodes.

1.2 Chemotaxis in protozoan systems

It seems reasonable to assume that the ability of a protozoan cell to recognize the presence of food at a distance from it would endow it with a considerable survival advantage. Examples will be taken from three groups of amoeboid cells to illustrate this phenomenon.

1.2a Chemotaxis in large free living amoebae

Rhumbler (1898) appears to have been the first to notice the effect of chemical stimuli on the orientation of movement in free living amoebae. Schaeffer (1916, 1917) was the first to make a concentrated study of the ability of Amoeba proteus to recognize various materials. He observed that they could sense soluble substances such as tyrosine held in a capillary tube, or insoluble substances such as carmine, carbon, glass, silicic acid, at a distance and send out pseudopods to touch them. He concluded that asdiffusion of material from insoluble substances was not possible, the attraction must have a physical basis.

The only other study of chemotaxis in the large free living amoebae was by a group of workers at Kings College, London, who were principally interested in the mechanism of amoeboid movement. In the large amoeba, Amoeba proteus, a directional response is usually expressed by the formation of a pseudopod in the direction of the stimulus. Such formations were found by Jeon and Bell (1962) and Bell and Jeon (1962) to be produced in amoebae by extracts of Tetrahymena pyriformis or Hydra viridans held in capillary tubes, or to pieces of living or heat killed hydra. With these large amoebae, no response was observed to convection currents from such tubes, as occurred in other amoeboid cells. Time lapse cinematography was used to ensure that directional migration to the hydra pieces occurred.

In 1965, Jeon and Bell attempted to isolate the substance which induced the chemotactic movement in Amoeba proteus. Substances from large pieces of living hydra were found capable of attracting amoebae from distances of 900 μ. They assumed that the chemotactic agent must be liberated by the hydra tissue, and prepared papain digests of this material. However, papain itself was found to be very chemotactic and from it they extracted a large molecular weight, water soluble fraction by repeated trichloracetic acid precipitation. This chemotactic substance was found to be a heat stable, positively charged macromolecule, consisting mainly of protein. The molecule

did not retain any of the original enzyme activity. Fluorescent labelling of the substance showed that when the amoebae were suspended in a solution of it, the label remained attached to the mucous coat of the amoeba membrane. This very inadequate experiment led them to believe that this substance induced locomotion directionally by acting on the amoeba surface. Other substances of interest which did not induce pseudopod formation were amino acids, sucrose, versene, glutathione, β and γ globulins, and antibody against components of the amoeba membrane, as described by Wolpert and O'Neill (1962).

In 1962 Bingley and Thompson produced some interesting data on membrane potentials in Amoeba proteus. Using microelectrodes, they found that in normal resting amoeba the membrane potential of the cell was around -30mV. As pseudopodia formed, there was a rapid decrease in potential at the pseudopod surface, and when such formations retreated, their surface potential increased considerably. They concluded that directional movement could be initiated by depolarisation of the membrane at a particular point on the surface. Bingley, Bell and Jeon, (1962) found that hydra extract did reduce the membrane potential of amoeba by approximately 70%.

1.2b Chemotaxis in soil amoebae

The only study of chemotaxis that appears to have been made in

the small free living amoebae is that by Savanat (1965). Using a dark field photographic technique, he demonstrated that the soil amoeba, Hartmannella astronyxis would move towards a clump of bacteria. In his attempts to elucidate the mechanism of this attraction he found that if the bacteria were killed by heat, alcohol, or streptomycin, amoebaewere no longer able to recognize them. On the other hand, mild sonication of live bacteria did not affect the chemotactic recognition process. Following the experiments of Shaffer (1953), which will be discussed in detail in the next section, Savanat tested the possibility that a diffusible substance released by the living bacteria was responsible for inducing chemotaxis. He added small blocks of agar on which bacterial colonies had grown to cultures of amoebae, but was unable to observe any directional movement towards the agar blocks. However, it is very probable, as Savanat suggested, that there may have been insufficient concentrations of chemotactic substances present in the agar.

As will be described later (Chapter 3), Savanat (1965) demonstrated that certain bacteria were more readily cleared from agar plates by soil amoebae than were others. He also tested whether the attractiveness of bacteria corresponded to their edibility. Whereas inedible organisms generally were not chemotactic, Savanat found that not all edible bacteria induced a positive directional motion in amoebae.

However, the results were not very satisfactory, as occasionally amoebae were seen to move directionally towards clumps of inedible bacteria, though they were found not to remain at the site. A further experiment consisted in adapting the amoebae to grow on normally inedible, non-chemotactic bacteria, and then testing for recognition of the food organism. However, amoebae were still unable to respond chemotactically to the bacteria, even though they had been rendered edible to these amoebae.

In other experiments, Savanat (1965) found that if he placed clumps of inedible and edible organisms on a coverslip culture, the amoebae migrated towards the edible bacteria as though the inedible bacteria did not exist. Savanat was unable to conclude from his experiments the factors involved in chemotaxis.

1.2c Chemotaxis in the slime moulds

Among the first studies of the phenomenon of chemotaxis in these organisms were those of Stahl (1884) and Metchnikoff (1893). They observed that some plasmodia of the Mycetazoa (the true slime moulds) were attracted to extracts of dead leaves, and certain other substances. The organisms in which chemotaxis has been most intesely studied have been the Acrasiae, the cellular slime moulds. These organisms have a unique life cycle, commencing with the liberation from spores of uninucleate, free living myxamoebae, which feed by phagocytosing

bacteria. Once the food supply is exhausted, the randomly moving amoebae suddenly begin to aggregate to central collection points to form a multicellular 'slug' or 'pseudoplasmodium'. This multicellular organism moves in an organized manner and eventually forms a stalk, at the tip of which encysted cells form a fruiting body.

Chemotaxis has been found to play an important role in two of the developmental stages of this organism. It enables the amoebae to find food and it enables cells to find one another during the social or aggregative phase. The unique process of chemotaxis between aggregating cells was the first of the two processes to be discovered. Though Olive (1902) is reported as the first to suggest that chemotaxis was involved in this process, Runyon (1942) appears to have presented the first evidence that chemotaxis between aggregating cells did occur. He showed that cell aggregates could orientate freely moving amoebae, even though both groups of cells were separated by semi permeable membranes. Bonner (1947) added further evidence that slime mould amoebae were orientated to a gradient of a chemical by allowing a gentle stream of water to flow over a group of aggregating cells. Normally, a radial pattern of amoebae is seen swarming towards a central mass of cells. The water caused the centre to elongate considerably, as though a chemical substance was being removed from it with the flow of fluid. At that time, several theories were

in existence to explain the observed aggregative phenomena. Among the factors proposed were agglutination, the spreading of a molecular film to induce the patterned migration, the presence of a predetermined structural matrix, and the existence of galvanic or magnetic forces, inducing a radial attractive stimulus towards the centre. Bonner (1947) was able to systematically show that such factors were not involved. He called the chemical substance he believed responsible for these observed processes 'acrasin'.

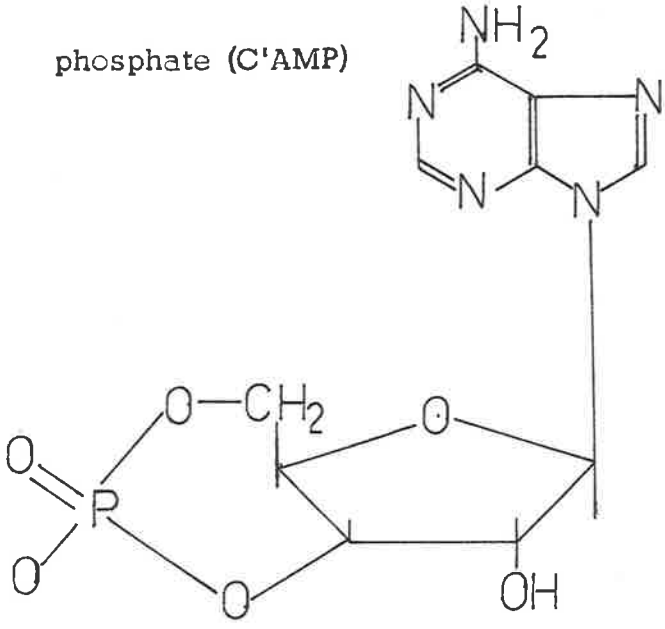
In 1953, Shaffer (1953) isolated 'acrasin' by allowing aggregates of amoebae to form on a cellophane sheet placed on agar. He found that the agar immediately opposite an aggregation centre, if removed and placed amongst a population of the same species of cells, would initiate aggregation of these cells. Shaffer (1957) also demonstrated that 'acrasins' formed by different genera of slime moulds were generally specific only for that genus. Testing three different species of Dictyosteliae, D. discoideum, D. mucoroides and D. purpureum, he found that any two of the above species will form a mixed aggregate. However, none of the above species will form an aggregate with species from the genus Polysphondylium. Claims were made of substances which were able to initiate chemotaxis in the myxamoebae. Wright (1964) found several hormones which were active at very low concentrations. Heftmann, Wright and Liddel

(1959, 1960) isolated a sterol (Δ^{22} - stigmasten - 3 - β - ol) with weak acrasin activity from Dictyostelium discoideum. Hostak and Raper (1960) reported positive results with alkaloids. However, in many instances, such claims were subject to criticism due to the techniques used in demonstrating directional migration.

Chemotaxis also plays a significant role in the feeding process of the myxamoebae, as mentioned earlier. Arndt (1937) appears to have been the first to suggest this, his suggestion resulting from the study of time lapse cinematography films of the behaviour of cells under various conditions. Samuel (1961) also found that the paths of individual amoebae were strongly oriented towards bacterial colonies, suggesting that some positive attractant may be liberated from these colonies. Konijn (1961) further found that the culture filtrates of food bacteria (E. coli) contained substances which induced chemotactic migration of vegetative myxamoebae. His assay system consisted in placing a small drop of a suspension of myxamoebae (at the stage of development when they were most sensitive to chemotactic stimuli) on to the surface of an agar medium which had been made hydrophobic by repeated washing of the agar with distilled water and placing a small drop of the chemotactic test substance close by. The movement of the cells out of the circular area in which they had been placed towards the test substance was his evidence for chemotaxis.

The factor produced by bacteria which is chemotactic to myxamoebae in the vegetative feeding stage caused much speculation by investigators in the field. Bonner, Kelso and Gillmore (1966), on showing that this substance could also attract aggregating cells, suggested that this substance may indeed be the natural 'acrasin'.

Konijn, Van de Meene, Bonner and Barkley (1967) proceeded to isolate this chemotactic substance from the culture filtrates of bacteria. They found the active component to be a low molecular weight (200-400), negatively charged, but stable substance, which absorbed strongly at 259 mμ. Purification was achieved by gel filtration using Sephadex G-10, followed by ion exchange chromatography (using DEAE cellulose), and absorption to charcoal (Konijn, 1968, personal communication). The active substance was found to be 3'5' Cyclic Adenosine monophosphate (C'AMP)



Konijn, Barkley, Chang and Bonner (1968) later reported that at concentrations as low at 10^{-4} mg./ml. this substance could induce directional migration in Dictyostelium discoideum amoebae, as well as those of other species of the Dictyosteliae at both feeding and aggregative stages, though cells of the Polysphondylium genus would respond only at the feeding stage of growth. The fact that this substance is able to induce aggregation in those myxamoebae appears to support the contention of these workers that C'AMP is the acrasin for Dictyosteliae.

This nucleotide is certainly a very interesting one. It was discovered originally by Sutherland and his co-workers (Sutherland and Rall, 1958) and has been found to act as a secondary mediator of numerous hormonal reactions in mammalian tissues, including those involving the catecholamines, glucagon, adreno-cortico-trophic hormone (A.C.T.H.), thyroid stimulating hormone (T.S.H.), insulin, tri-iodothyronine, histamine and serotonin (Butcher, Robison, Hardman and Sutherland, 1968). It is postulated that the hormones act on certain specific receptors on the cell surfaces, resulting in the activation of a membrane-bound enzyme, adenyl cyclase, which splits adenosine triphosphate (ATP) to give C'AMP. This substance then acts intracellularly to effect changes in cell activity. The discovery of the acrasin activity of this chemical substance will

no doubt lead to some very interesting studies on cell aggregation, and speculation on its role has already begun (Konijn et al. 1968).

Chang (1968) has isolated a specific enzyme from Dictyostelium discoideum which will hydrolyze C'AMP to 5' - adenosine monophosphate. This enzyme is produced by the amoebae extracellularly in quite high concentrations. The balance between enzyme and substrate at various phases of development of the myxamoebae is as yet unknown.

1.2d Summary

It will be very obvious that investigations into chemotaxis in the protozoan amoeboid phagocytic cells have, except in the example of the cellular slime moulds, yielded very little coherent information. The studies of the phenomenon in the myxamoebae may present an in interesting lead. One wonders if there is any parallel between this process and that acting in the metazoan phagocytes. While C'AMP does not attract leucocytes (Konijn, 1968, personal communication) or indeed any other amoeboid cell tested by Konijn, it would not be impossible for a basic mechanism involving nucleotides to be common to both systems.

CHAPTER 2.

MATERIALS AND METHODS, PART 1.

2.1 Preparation of glassware

All glassware used in the experiments with amoebae was boiled in Calgon (Albright and Wilson, Aust. Pty Ltd), and rinsed thoroughly in distilled water. Glass coverslips used in monolayer experiments were cleaned by being placed in 1% detergent (Pyroneg, Diversey Australia Pty Ltd), and subjected to ultrasound from an M.S.E. Ultrasonic Disintegrator (Model 11-62) for 15 minutes. The coverslips were washed free of detergent and subjected to the same treatment in distilled water. Following this, they were rinsed in deionized water and dried in an oven at 60°.

2.2 Maintenance of cultures of soil amoebae

The species of soil amoebae used most extensively in this study was :

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

These amoebae were either grown axenically in a liquid medium or together with bacteria on a solid medium. The liquid culture medium was a proteose peptone glucose solution (P.P.G.), (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 g.
Glucose	18 g.

Glass distilled water to 1 litre. Final pH 6.8

The media was autoclaved at 15 lb. per sq. in. at 121° for 10 minutes.

Amoebae were grown at 20° in 20 ml. of this medium in 4 oz. medicinal bottles placed horizontally. The cells were subcultured every 4 weeks, with care being taken to prevent bacterial contamination of the culture. By this time, approximately 10^7 amoebae were found to have grown in each bottle from approximately 5×10^5 amoebae in the original subculture.

The solid medium employed was a 0.1% yeast extract agar (Y.E.A), prepared as follows (Ray, 1951):

Yeast extract (Oxoid)	1 g.
Agar	15 g.

Glass distilled water to 1 litre.

Suitable food bacteria, e.g. Pseudomonas fluorescens, were streaked on to this medium in a Petri dish and the plate incubated for 24 hours.

A loopful of amoebae, containing approximately 300 cells, was inoculated at the centre of the streaks and the plate incubated at 20°. After clearing the bacteria from the plate, the amoebaeencysted, and could be kept for long periods of time in this state. Upon sub-culturing the cysts on to fresh bacterial streaks as above, they vegetated very quickly and proceeded to clear the bacteria from the plate.

Other soil amoebae used in minor studies were :

(a) Hartmannella astronyxis (Ray, Cambridge collection of protozoa and algae No. 1534/1). These amoebae were grown axenically in P.P.G. or on Y.E.A. plates streaked with Pseudomonas fluorescens. However, to obtain luxuriant growth in liquid media the P.P.G. needed to be supplemented with killed bacterial cells. Pseudomonas fluorescens, killed by heating at 56° for 1½ hours was added to give a concentration of approximately 5 x 10^7 bacteria/ml. (Drozanski and Drozanska, 1961).

(b) Acanthamoeba sp. (Neff; Cambridge collection of protozoa and algae No. 1501/1.) This amoeba was grown in the manner described for H. rhysodes.

(c) Schizopyrenus russelli

(d) Didascalus thorntoni

(e) Naegleria gruberi

(f) Hartmannella glebae

(g) Hartmannella 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. They were maintained on Y.E.A. streaked with Pseudomonas fluorescens.

2.3 Strains of bacteria used in experimental studies

2.3a Pseudomonas fluorescens. This strain was obtained from Mr. J. R. Harris, C.S.I.R.O. Division of Soil Microbiology, South Australia. It was very motile and would only grow at temperatures of 30° or lower. Growth was inhibited at 31°. It is a very common soil organism and was accepted as food by all species of amoebae tested.

2.3b Salmonellae species. Ten strains of Salmonellae were used in these studies. These are presented in the following Table 1, together with their corresponding antigenic determinants (Kauffmann, 1961). These organisms were kindly supplied by Dr. Nancy Atkinson, Department of Oral Biology, Dental School, University of Adelaide.

TABLE 1

Strains of Salmonellae used in chemotaxis experiments

Chemotype	Name of organism	Antigenic structure (Kauffmann, 1961)
B	S. budapest	1, 4, 12
	S. typhimurium (M206)*	1, 4, 5, 12
	S. typhimurium (C5)*	1, 4, 5, 12
	S. typhimurium	4, 5, 12
C3	S. virginia	8
	S. kentucky	8, 20
E1	S. uganda	3, 10
	S. london	3, 10
H	S. onderstepoort	1, 6, 14, 25
	S. carrau	6, 14, 25

* Furness and Rowley, 1956.

2.3c Bacterium H. (Savanat, 1965). This organism is a strain of Aerobacter, maintained by Mr. J. R. Harris, C.S.I.R.O. Division of Soil Microbiology, Adelaide. It is a Gram negative non-motile, cocco-bacillus, which will not grow at 37° but will grow at 30°. Its biochemical characteristics are described by Savanat (1965).

2.3d Micrococcus lysodeikticus.

2.3e Staphylococcus albus.

These two organisms (2.3d and 2.3e) were maintained in the Department of Microbiology, University of Adelaide.

2.3f Streptococcus pneumoniae. This organism was a rough strain of pneumococcus and was kindly supplied by Mr. R. Bateman, Department of Microbiology, University of Adelaide. Ampoules containing freeze-dried cultures were opened when required and the contents placed in a medium consisting basically of brain-heart infusion broth to which was added 1% glucose, 0.13% sodium bicarbonate and 1% foetal calf serum. Cultures were grown at 37° on a reciprocating shaker for 48 hours.

2.4 Maintenance of bacterial cultures

Unless otherwise stated, these organisms were maintained at 4° on nutrient agar slopes in 1 oz. screw-capped bottles. Each slope was opened no more than 10 times. Pseudomonas fluorescens was subcultured every two weeks, as cultures underwent autolysis and lost their viability after 1 month.

2.5 Cultivation and preparation of bacteria for experimental purposes

Except in special circumstances as mentioned earlier, bacteria were grown in nutrient broth at 37° on a reciprocating shaker. For experiments on chemotaxis, bacteria were washed twice with phosphate buffered saline (μ : 0.06) by centrifugation at 3,000 g. for 15 minutes in a Servall refrigerated centrifuge.

Phosphate buffered saline (P.B.S.) was prepared as follows :-

4M	NaH_2PO_4	1.85 ml.
5M	NaCl	8.25 ml.
0.5M	Na_2HPO_4	8.5 ml.

Glass distilled water to 1 litre.

Final pH 6.5

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

In a few instances bacteria were grown in a minimal medium as below (Davis and Mingioli, 1950) :-

Glucose	2 g.
K_2HPO_4	7 g.
KH_2PO_4	3 g.
Sodium citrate, $2H_2O$	0.5 g.
Mg $SO_4.7H_2O$	0.1 g.
$(NH_4)_2SO_4$	1.0 g.

Glass distilled water to 1 litre.

The glucose was made up separately from the rest of the medium and autoclaved at 15 lb. per sq. in.at 121° for 10 minutes.

2.6 Counting bacteria

This was achieved either by direct counting with the Petroff-Hausser counting chamber, or by estimation of the optical density of a culture using a Unicam S.P.600 Spectrophotometer.

The latter method was the one most commonly used. Concentrations were read from a standard curve prepared by measuring the optical density at 650 mμ of various concentrations of organisms in broth and plotting these readings against the numbers of bacteria counted using the Petroff-Hausser counting chamber for the same

concentration (Fig. 1).

2.7 Preparation of bacterial cell walls

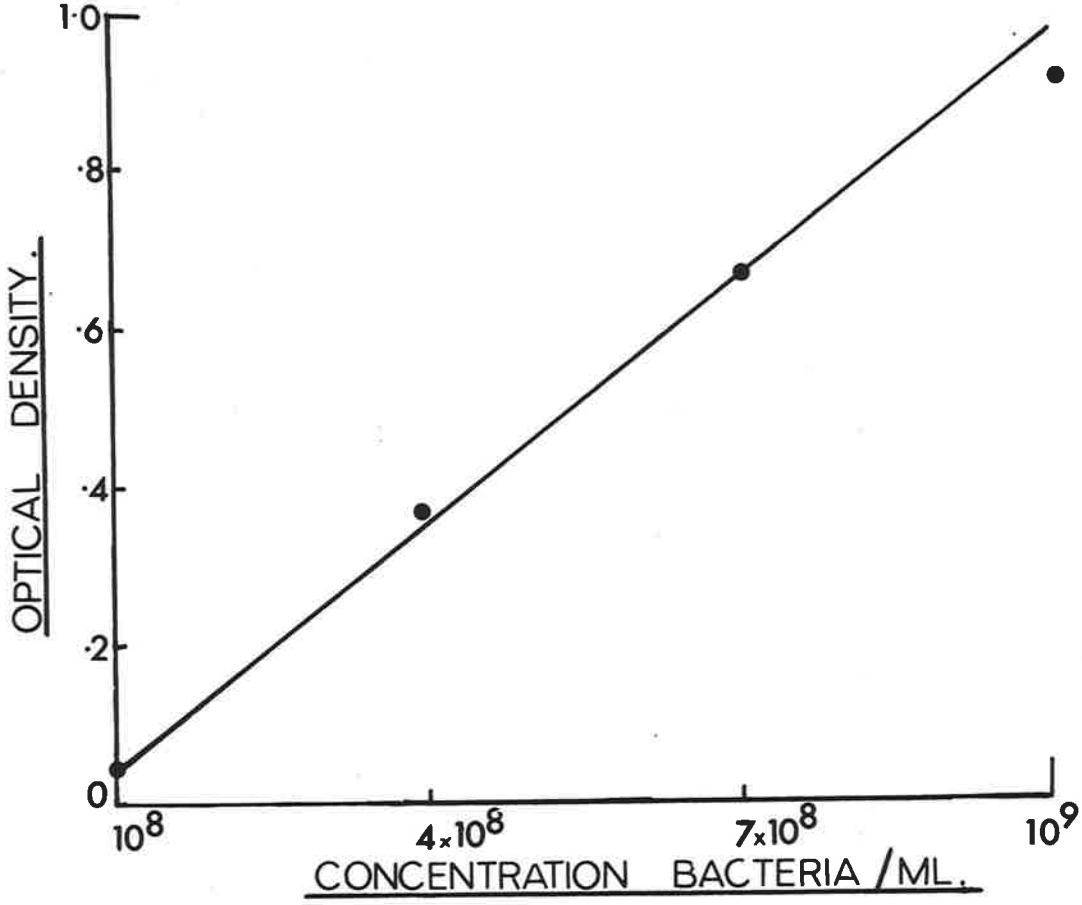
Roux bottles, each containing 125 ml. of nutrient agar, were inoculated with 1 ml. of an overnight culture of Pseudomonas fluorescens. After growth at 30° for 48 hours the cells were harvested and the resulting bacterial suspension concentrated by centrifugation, using a Servall refrigerated centrifuge and resuspended at a concentration of approximately 10mg. dry weight/ml.

The bacterial suspensions were chilled and placed in a Mullard Ultrasonic Disintegrator with a frequency output of 25 Kc/second . The suspensions were treated with ultrasound for 5 minutes and then centrifuged at 3,000 g. for 15 minutes to remove undisrupted cells. The supernatant was then centrifuged at 27,000 g. for 20 minutes and the deposit resuspended in molar saline and washed 3 times with the same. The cell walls were finally suspended in 0.05M phosphate buffer at pH 7.6. Trypsin was added to give a concentration of 0.5 mg./ml. and the suspension incubated at 37° for 3 hours. A few drops of toluene were added to inhibit the growth of any contaminants.

Following trypsin treatment, the cell walls were washed 3 times in distilled water at 27,000 g. for 20 minutes and finally resuspended in P.B.S. A final centrifugation at 3,000 g. for 15 minutes was made

FIG. 1.

Standard curve for the concentration of bacteria at 650 mμ on a Unicam S.P.600 spectrophotometer.


1·0
·8
·6
·4
·2
0
OPTICAL DENSITY.
10^8
4×10^8
7×10^8
10^9
CONCENTRATION BACTERIA / ML.

to remove any whole bacteria and the supernatant diluted to give a concentration of cell walls having a dry weight of 10 mg./ml.

Since the cell wall is approximately one quarter of the total dry weight of the bacterium, this quantity was equivalent to the weight of cell wall derived from 5×10^{10} bacteria/ml., the dry weight of which was 40 mg./ml. The cell wall suspension was divided into 2 ml. aliquots and stored at -20°.

2.8 Biological methods used during the course of the study

2.8a Phagocytosis of bacteria by soil amoebae

The technique used was a modification of one described by Rowley and Whitby (1959), who studied the phagocytosis of bacteria by mouse peritoneal macrophages.

Amoebae were washed twice in minimal medium (Davis and Mingioli, 1950) by centrifugation at 120 g. for 10 minutes and finally resuspended in this medium. The concentration of cells was estimated using a haemocytometer (the method for counting white cells being used) and was adjusted to give a concentration of 2×10^{6} cells/ml. Samples of 0.5 ml. of this suspension were placed in siliconised glass tubes 75 mm. long by 12 mm. diameter. Overnight cultures of bacteria were washed also in minimal medium by centrifugation at 3,000 g. for 15 minutes, and counts made using the Petroff-Hausser counting chamber.

The concentrations of bacterial suspensions were adjusted to 10^7 cells/ml. Samples of 0.5 ml. of these suspensions were added to the tubes containing amoebae giving an amoeba-bacterium ratio of 1:5, and to an equal number of tubes containing 0.5 ml. of minimal medium alone as controls. The tubes were then placed on a drum revolving at the rate of 1 revolution every 2 minutes, and the experiment carried out at room temperature. At zero time, and at intervals of time over a period of 5 hours, 0.1 ml. samples were taken from the control tubes and appropriately diluted in physiological saline. An aliquot of 0.1 ml. of this dilution was spread on nutrient agar and the plates incubated overnight. At similar time intervals a certain number of the experimental samples were emptied into small centrifuge tubes and spun at 120 g. for 5 minutes at 4°. Appropriate dilutions of the supernatant were made and plated out. The cell deposit was rewashed twice in saline at 4° and finally resuspended in 10 ml. of this medium. Following disruption by sonication for 10 seconds using an M.S.E. ultrasonic disintegrator, an aliquot of the disrupted cell suspension was suitably diluted and 0.1 ml. plated on to nutrient agar for a bacterial count. The percentage phagocytosis that occurred at any given time interval was given by the formula : $\frac{A - B}{A} \times 100$, where A = the supernatant count in the control tube and B = the supernatant count in the experimental tube. 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, and A-B the number of bacteria phagocytosed.

2.9 Chemical methods used in the purification of chemotactic substances.

2.9a Concentration of fluid samples

During investigations into the nature of the chemotactic substance it was found necessary to obtain large volumes (up to 7 litres) of certain materials and to concentrate these. Volumes above 3 litres were concentrated initially using a cyclone evaporator at a pressure of 18 mm. mercury and a temperature of 38°, to smaller volumes which could be concentrated further on a rotary evaporator. The bath temperature of the rotary evaporator was 70°, and the pressure of 18 mm. mercury was maintained by a Venturi pump. A continuous flow adaptor was fitted to the rotary evaporator.

During purification procedures, smaller volumes (e.g. 100 ml.) were concentrated by freeze drying on a New Brunswick Model B66 Freeze Dryer (New Brunswick Scientific Co. Inc., New Brunswick, U.S.A.).

2.9b Gel filtration chromatography

During the course of this study, columns were prepared of both

Sephadex G-25 (Pharmacia, Uppsala, Sweden) and Biogel P-2 (Calbiochem, Los Angeles, U.S.A.). Sephadex G-25, a polydextran, excludes substances of molecular weight approximately 5,000 and above. Biogel P-2 is a polyacrylamide gel with an exclusion limit of 1,600 molecular weight. For preparative work, the columns measured 60 cm. x 2.6 cm. diameter, while for analytical work they were of the same height but only 1 cm. in diameter. The gel powders were hydrated overnight by soaking in P.B.S.(μ : 0.06). After a series of washings in this buffer, the supernatant liquid being removed by decantation, the gels were packed into the vertical glass columns as follows. Columns were sealed at the lower end by a rubber stopper containing a flow tube which was separated from the gel by a piece of fine gauze. An excess liquid suspension of the gel was run into the column, and when the gel had settled out the excess fluid was removed and more gel suspension added until the upper level of packed material was within a short distance of the top end of the column. Prior to use the columns were equilibrated with phosphate buffered saline (pH 6.5, μ : 0.06). The final height of the gel in the analytical columns was 50 cm., while in the preparative it was 52 cm. The columns were attached to a fraction collector (Paton Industries, Beaumont, South Australia).

A sample for fractionation was placed carefully on to the surface of the gel so as to maintain an even layer. It was allowed to run into the gel, and the sides of the column above the surface of the gel were washed with buffer. Following the removal of the washings, buffer was again added to a height of about 4 cm. above the surface of the gel and the top of the column sealed by a rubber stopper. Through this stopper was a flow tube connected to a reservoir of buffer placed above the column. Flow of buffer through the column was maintained by gravity, the rubber seals maintaining sufficient air pressure in the column to control the amount of buffer entering. The flow rate was adjusted to approximately 1 ml./minute/cm^2. Care was taken during all these procedures to prevent the buffer level falling below that of the gel, causing the latter material to dry out. Samples of chemotactic material, eluted from the column, were examined in 1 cm. Silica Quartz cells in a Hitachi Perkin-Elmer Double Beam Spectrophotometer, to which was attached a Hitachi recorder. Ultraviolet absorption spectra of the eluted fractions were determined over a wavelength of 240-300 mμ. As analytical columns were used partially to determine the molecular weight of the chemotactic factor(s), calibration of these with substances of known molecular weight was carried out using the method of Andrews (1964). Andrews investigated the correlation between the elution volumes (Ve) and molecular weight, and found

that plots of Ve against log molecular weight were linear over the molecular weight ranges 3,000 - 35,000, and 5,000 - 60,000 for Sephadex G-75 and G-100 respectively. It was found that the plots of the elution volumes of various sugars from Sephadex G-25 against the logs of their molecular weights also followed this linear pattern. This method was also similar to that recommended by the suppliers of P2-Biogel for use of their product (Biorad Manual, 1968). The following substances were used to calibrate the column :-

(i) Bovine serum albumin (Mol. wt. 69,000) at a concentration of 1 mg./ml.

(ii) Dinitrophenol (Mol. wt. 182) at a concentration of 2 mg./ml.

(iii) Bacitracin (Mol. wt. 1470) at a concentration of 7 mg./ml.

(iv) Glycyl-l-tyrosine (Mol. wt. 384) at a concentration of 2 mg./ml.

(v) Glucose (Mol. wt. 180) at a concentration of 50%.

(vi) Sucrose (Mol. wt. 342) at a concentration of 10%.

(vii) Raffinose (Mol. wt. 506) at a concentration of 5%.

The elution pattern of the above substances from the column, apart from the sugars, was followed by spectrophotometric analysis of fractions of constant volume. Bovine serum albumin was read at a wavelength of 280 mμ, dinitrophenol at 347 mμ, bacitracin at 225 mμ, and glycyl-l-tyrosine at 275 mμ. The concentrations of sugars in the various fractions were determined using the anthrone reaction

(Kabat and Mayer, 1961).

2.9c Ion exchange chromatography

Sephadex A-25 DEAE (di-ethyl-amino-ethyl) cellulose (Pharmacia, Uppsala, Sweden) was used for ion exchange chromatography. The cellulose powder was suspended in distilled water, allowed to settle, and the excess fluid removed by decanting. The gel was then suspended in 0.1M hydrochloric acid, washed 3 times in distilled water, treated with 0.01M sodium hydroxide, washed 3 times with distilled water, and treated with 1.0M acetic acid. Following these procedures, the cellulose was washed 4 times with 400 ml. volumes of 0.025M ammonium acetate solution at pH 6.5. The ion exchange material was then packed into a glass column 50 cm. long and 1 cm. internal diameter. Acid resistant stoppers and tubing were used to connect the column to a reservoir. Prior to use the column was again washed with 500 ml. 0.025M ammonium acetate solution, the flow rate being 5 ml./minute. Samples of material for purification were placed on the column and washed through with 150 ml. of the 0.025M ammonium acetate (pH 6.5). Fractions of 10 ml. were collected in glass tubes, using a fraction collector (Paton Industries, Beaumont, South Australia) controlled on a constant volume basis. The material adsorbed to the column was further eluted with a step wise gradient of buffers of increasing ionic concentration and decreasing

pH, as follows :

(i)	150 ml.	0.1M acetic acid buffered to pH 5.
(ii)	150 ml.	0.5M acetic acid buffered to pH 4.
(iii)	150 ml.	1M acetic acid, pH 2.8.
(iv)	150 ml.	2M acetic acid, pH 2.3.

Volatile buffers were used for elution as the biological tests for chemotaxis required the presence of minimum concentrations of salts and other chemicals. The buffers were evaporated by rotary evaporation and the samples dissolved in water in preparation for chemotactic tests.

2.9d Paper chromatography

Samples of the chemotactic factor were freeze dried and a small volume of the solvent mixture added to the dry material. Samples (0.05 ml.) of the test substance were placed with a micropipette on to thin strips of Whatman No. 3 filter paper, about 2 cm. from one end. A warm stream of air was blown on to the filter paper during this time to dry excess moisture and keep the sample in a confined spot. The upper end of the strip of filter paper was attached to a glass rod such that about 1 cm. of the sample end dipped into the solvent in a large glass container which was well covered to prevent evaporation of the solvent. When the solvent had flowed to within 1 cm. of the top end of the paper strip the strip was removed from the tank and dried at room

temperature. It was cut into 10 segments and a water eluate from each segment tested for chemotactic activity.

CHAPTER 3

MATERIALS AND METHODS. PART 2.

AN ANALYSIS OF POSSIBLE METHODS TO QUANTITATE CHEMOTAXIS IN SOIL AMOEBAE

An essential prerequisite for the analysis of chemotaxis must be a reliable quantitative technique. It is evident from previous studies that the lack of suitable techniques has considerably hindered meaningful investigations into this phenomenon.

In the present section, various methods used by other workers will be analysed for their suitability to measure chemotaxis in a quantitative manner.

3.1 Method 1

Food selectivity

The first method tested made use of findings by several workers that soil amoebae were able to clear selectively some organisms growing on agar plates in preference to others (Severtzova, 1928; Singh, 1941, 1942, 1945; Savanat, 1965). Singh (1942, 1945) demonstrated that if two streaks of different organisms were placed closely parallel on an agar plate and soil amoebae inoculated at one end of the streaks, then in most instances the amoebae proceeded to clear one faster than the other, and finally when that streak was

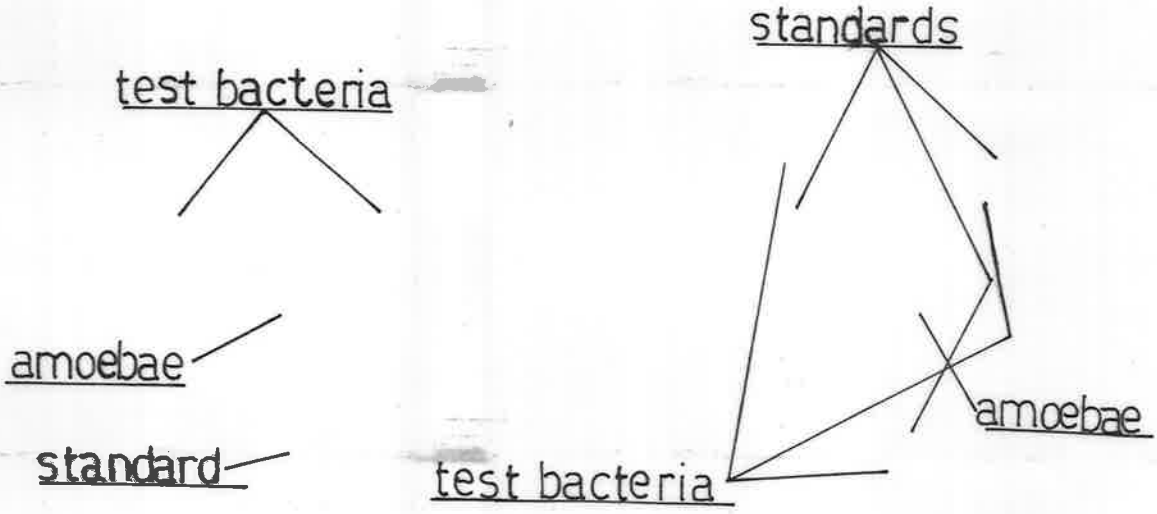
cleared, migrated across to feed on the other. He believed that this result demonstrated that the one was more acceptable as food, or edible, than the other. Savanat (1965) further demonstrated (using this method) that bacteria which were poorly accepted as food by amoebae were also not chemotactic to those amoebae when tested in coverslip cultures, as described in detail later (Method 2). Therefore, it seemed not unreasonable to suppose that among the more basic factors responsible for this food selection, chemotaxis would be of some importance. The reason why one strain of bacteria was cleared more rapidly than another could be because it attracted more amoebae to it. One also hoped at the onset of the experiments that one might find some correlation between the chemical structure of the surface of the bacteria and their attractiveness for amoebae. This is why Salmonella strains were often chosen as test organisms.

The first technique used was similar to that described by Severtzova (1928). Overnight cultures of the different bacteria were streaked on to agar plates in the form of a radiating star, as in Figure 2(1a). These were allowed to grow at 30° for 48 hours, and then a loopful of a culture of amoebae, containing approximately 5×10^{3} cells, was inoculated into the centre of the plates and spread so as to just touch the beginning of each streak. The plates were incubated at 20° for 8-12 days, during which time the amoebae cleared the bacterial

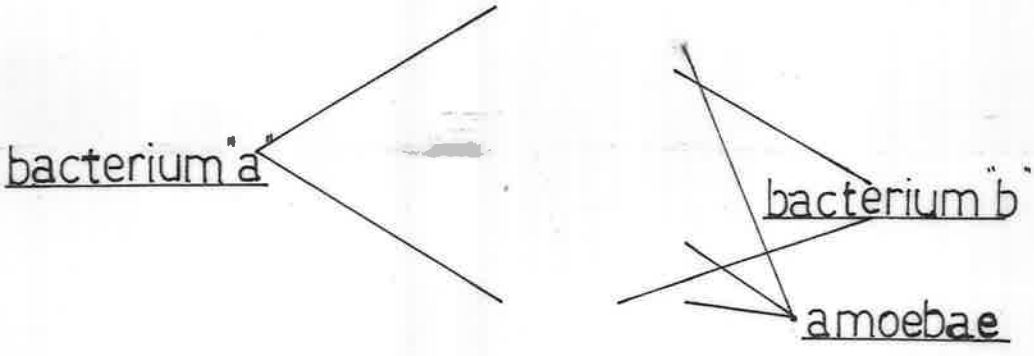
FIG. 2.

Methods of plating bacteria for clearance studies.

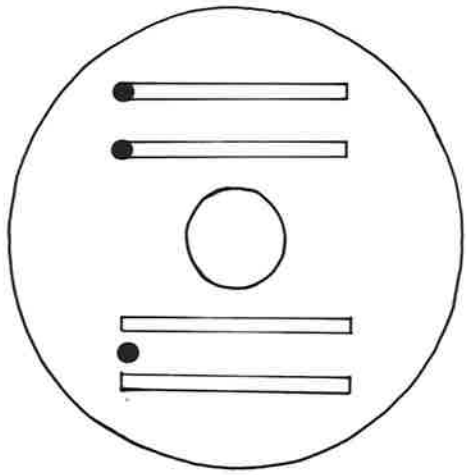
Methods 1a, 1b, 1c.

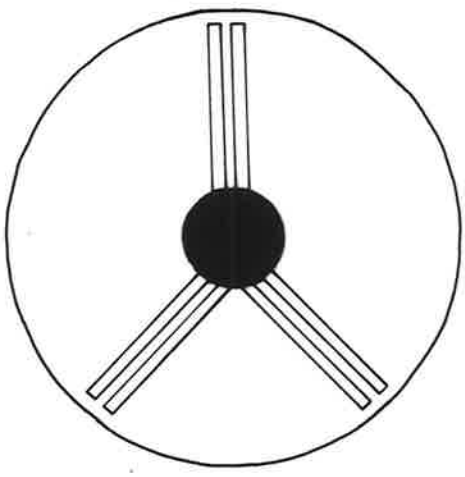

test bacteria
amoebae
standard
standards
amoebae
test bacteria

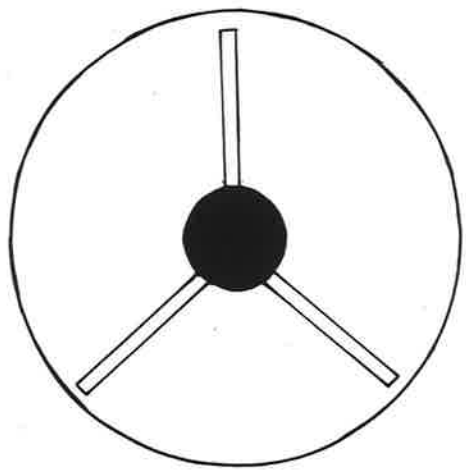
1a 1b


bacterium "a"
bacterium "b"
amoebae

1c







streaks to varying degrees. The acceptability of each organism as food for a particular amoeba was considered to be proportional to the length of streak cleared. To enable a comparison to be made between results from different plates, a standard bacterium was included on each one. This organism was Pseudomonas fluorescens, a strain of bacteria shown by Savanat (1965) to be acceptable as food by all the species of soil amoebae he tested, as well as being very chemotactic. The clearance of streaks of bacteria was followed daily and without exception, the strain of Pseudomonas was cleared more rapidly than the others. The length of the streak of the standard organism was 22 mm, and by comparing the rate of clearance of this standard strain between plates, one could ensure that the plates were under similar experimental conditions and thus satisfactorily compare the rate of clearance of a test strain on one plate with that on another. A selection of bacteria consisting of representatives of chemotypes B, C, E and H from the genus Salmonella, a bacterium shown by Savanat (1965) to be completely inacceptable as food by all the species of amoebae he tested (Bacterium H), and the standard strain Pseudomonas fluorescens were plated on to Y.E.A. in the manner described earlier. The results of this experiment are given in Table 2. A picture of a sample plate showing the clearance of 3 of these organisms may be seen in Fig. 3(1a). When the experiments

TABLE 2

Clearance of various strains of bacteria by Hartmannella rhysodes

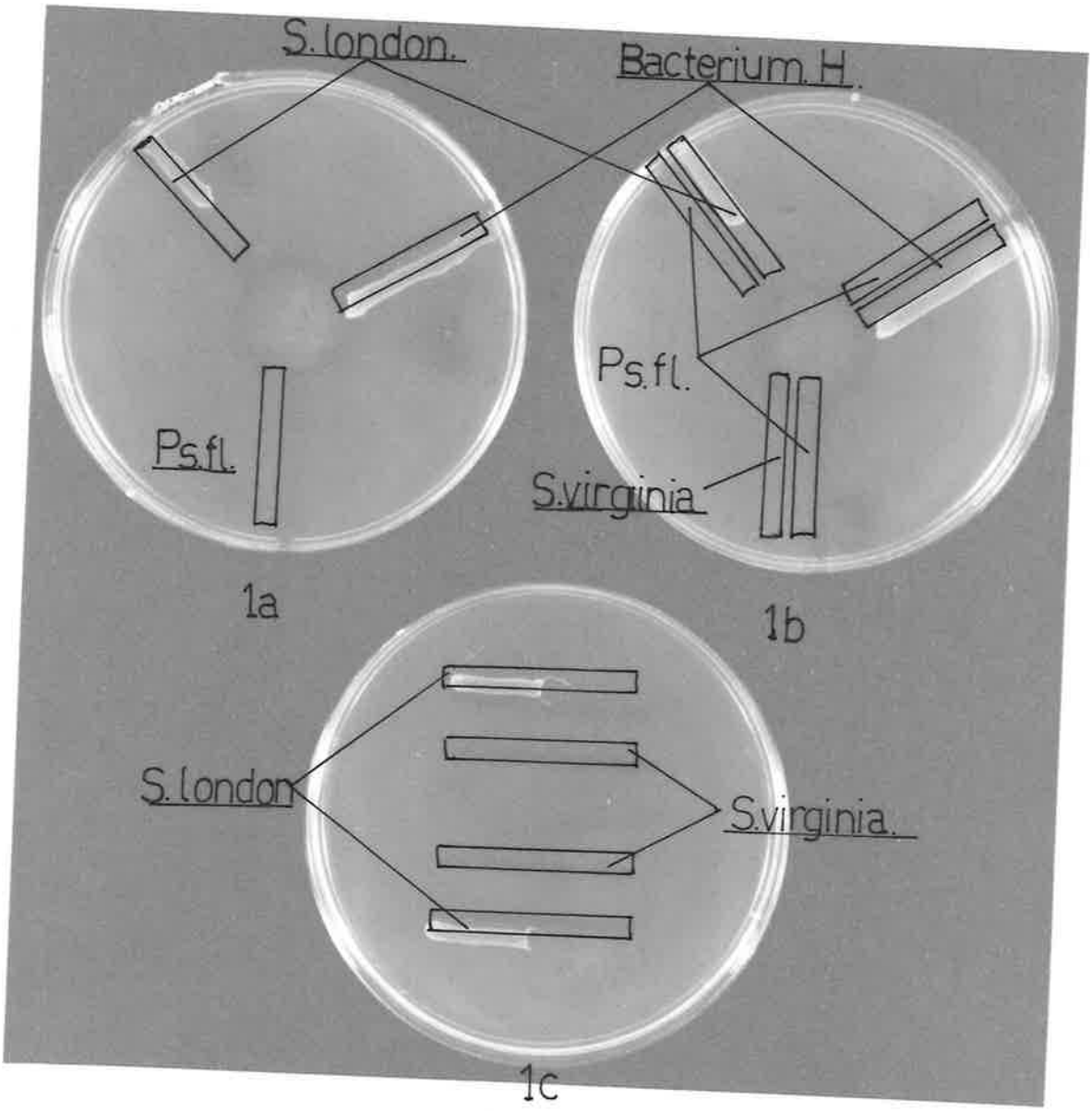
Bacterial strain	Chemotype*	Antigenic structure*	Length of streak cleared (mm)
Pseudomonas fluorescens			22
Bacterium H			0
S. typhimurium (M206)	B	1, 4, 5, 12	14
S. typhimurium (C5)	B	1, 4, 5, 12	19
S. typhimurium	B	4, 5, 12	16
S. budapest	B	1, 4, 12	16
S. virginia	C3	8	22
S. kentucky	C3	8, 20	14
S. uganda	E1	3, 10	14
S. london	E1	3, 10	11
S. onderstepoort	H	1, 6, 14, 25	13
S. carrau	H	6, 14, 25	13

* See Kauffmann, F. 1961 "Die Bakteriologie der Salmonella Species," Munksgaard, Kopenhagen.

FIG. 3.

Clearance of various strains of bacteria from yeast extract agar by Hartmannella rhysodes.

Methods 1a, 1b, 1c.


S. london.
Ps.fl.
1a
Bacterium. H.
Ps.fl.
S.virginia
1b
S. london
S.virginia.
1c

were repeated it was found that for any particular strain, the rate of clearance was remarkably constant. The results given in Table 2 are the averages from three separate experiments. The plates were always read at the time the standard strain had just been cleared. It is obvious from the data that there is no apparent relationship between the chemotype and the rate of clearance of the strains of Salmonella. Even organisms known to have similar antigenic determinants (viz. S. typhimurium strains C5 and M206) were cleared at widely differing rates. The organism Bacterium H was not cleared at all by this amoeba.

The experiment was repeated with the same bacteria, testing their acceptability as food to a number of species of soil amoebae (Table 3). A wide variation in the rates of clearance of the strains of Salmonella by each species of amoeba was evident. Again, no correlation between surface antigenic characteristics of each strain of Salmonella and its acceptability as food was observed. However, as found by Savanat (1965), Pseudomonas fluorescens was cleared quickly by all the species of amoebae tested, and Bacterium H not at all.

The second technique was that originally used by Singh (1942, 1945) and was a modified version of the first method. Two bacterial streaks were placed side by side at intervals around an agar plate in

TABLE 3

Clearance of various strains of bacteria by 7 other species of soil amoebae

Chemo type *	Strain of bacteria	Species of amoeba: Acanthamoeba	H.Astonyxis	Naegleria gruberi	Didascalus thorntoni	Schizopyrenus russelli	H. glebae	H. rhysodes (strain 15)
		Length of streak cleared (mm)						
	Pseudomonas fluorescens	22	22	22	22	22	22	22
	Bacterium H.	0	0	0	0	0	0	0
B	S. typhimurium (M206)	13	20	9	13	18	19	12
B	S. typhimurium (C5)	17	8	19	17	17	22	10
B	S. typhimurium	18	12	20	14	22	19	20
B	S. budapest	10	9	14	14	17	17	16
C3	S. virginia	22	15	22	22	15	19	14
C3	S. kentucky	15	10	17	14	19	17	17
E1	S. uganda	13	8	9	22	12	5	6
E1	S. london	13	4	12	15	13	7	10
H	S. onderstepoort	11	8	3	19	14	15	15
H	S. carrau	14	9	0	22	16	15	13

* See Kauffmann, F. 1961. "Die Bakteriologie der Salmonella Species," Munksgaard, Kopenhagen.

the form of a radiating star (Fig. 2(1b)), one of the organisms from each pair being common to every pair in all the experimental plates. This provided a standard against which the migration of the amoebae along each test streak of bacteria could be measured. Singh (1942) claimed that this method demonstrated food selection more adequately than the above technique in that the amoebae at the end of each pair of streaks, had the choice of which streak to use as food and would migrate towards the one most acceptable. However, it was found that the results obtained by this method did not differ from those obtained using the earlier method (1a), indicating that both methods were demonstrating selectivity to the same extent. A picture of a sample plate in which this method was used may be seen in Fig. 3(1b). In order to establish that chemotaxis played a role in food selectivity it was decided to examine more closely the clearance of three of the organisms, S. virginia, S. london and Bacterium H by the amoeba Hartmannella rhysodes.

3.1a The effect of increasing the distance of amoebae from the food source on food selection

It was observed by Samuels (1961) that myxamoebae were attracted towards colonies of bacteria on agar plates from relatively large distances. It may be expected that if chemotaxis plays a part in the selection of one strain of bacterium as food in preference to

another by an amoeba, then if the amoebae are separated by an equal distance from streaks of both organisms, selection as expressed by rate of clearance may be enhanced in favour of the more acceptable organism. To test this possibility, streaks of S. virginia and S. london were placed 1 cm. apart and parallel on Y.E.A, as in Fig. 2(1c). Previous experiments (see Table 2) had indicated that S. virginia was cleared more rapidly on Y.E.A. than S.london. A loopful of amoebae was inoculated either immediately between the ends of the two streaks, or at the end of each streak. However, the differences between the lengths of both streaks cleared in both instances remained constant, as may be seen in Fig. 3(1c). These data argued against chemotaxis being a significant factor in influencing the selection of food by amoebae under these conditions.

3.1b Clearance of streaks of mixed cultures of bacteria by amoebae

The results from the above experiments suggested two possibilities for the faster rate of clearance of one strain of bacteria compared with another. One possibility was that strains of bacteria that are cleared very slowly are, for some reason, poorly digested by amoebae and thus the amoebae fail to multiply. The second possibility was that these strains produce substances which may be toxic to these phagocytic cells. The following experiment was designed to test

these possibilities. Equal volumes of cultures of S. virginia and S. london containing similar concentrations of bacteria were mixed and the mixed cultures streaked on toY.E.A. as in the first method used for demonstrating selection, and grown for 48 hours at 30°. Amoebae were inoculated at the ends of the mixed streaks and the rate of clearance followed. It was found that the rate of clearance of the mixed streaks was identical to that of the least acceptable organism if this were tested alone (Table 4). These results were even more striking if S. virginia was mixed with Bacterium H, a strain that is not cleared at all. With such mixed streaks no clearance was observed. The results argued very strongly against chemotaxis playing a role in the selection of food organisms, and indeed suggested that these techniques are not even measuring selection, but rather the toxic effect of bi-products of bacterial metabolism on the amoebae. This conclusion was substantiated by the following experiments.

3.1c Clearance of bacteria grown on various media

It was evident from the previous result that a product released by certain bacteria exerted an inhibitory effect on amoebae in their ability to feed on bacteria from Y.E.A. plates.It was decided to test if variations in cultural media altered the acceptability of these bacteria as food. Streaks of the three organisms were grown for 48 hours at 30° on 1.5% agar containing the following nutrient

TABLE 4

Clearance of streaks of mixed cultures of bacteria by H. rhysodes

Bacterial strain	Clearance of organisms from 3 plates (mm)		
	Plate 1	Plate 2	Plate 3
S. virginia	22	21	23
S. london	10	12	11
S. virginia + S. london	11	9	9
S. virginia	20	22	22
Bacterium H.	0	0	0
S. virginia + Bacterium H.	0	0	0

materials :

(i) 10% Minimal Medium (M.M.), including 1% glucose (Davis and Mingioli, 1950).

(ii) 10% P.P.G., as prepared in Chapter 2.2.

(iii) Yeast extract, 0.1% (Y.E.A.).

Amoebae (H. rhysodes) were inoculated into the centre of the streaks as in the first method used to demonstrate selection. The results (Table 5) showed that Bacterium H, which was not cleared at all from Y.E.A, was cleared almost to the same extent as was S. virginia when grown on M.M. or P.P.G. agar. However, S. london became almost completely inacceptable as food when grown on M.M. agar. It was difficult to believe that a change in culture media could so extensively alter the ability of amoebae to phagocytose and kill bacteria grown on these media. Whatever the explanation of the results, they supported the belief that these methods do not demonstrate active selection of food satisfactorily.

3.1d Phagocytosis of bacteria by amoebae

A further demonstration of the presence of substances produced by some bacteria which affect the ability of amoebae to feed on them resulted from the following experiment. Cultures of each of the strains of bacteria S. virginia, S. london and Bacterium H were grown in minimal medium (Davis and Mingioli, 1950) for 7 days at

TABLE 5

Clearance of bacteria from various media by H. rhysodes

Strain of bacteria	Y.E.Agar			M.M.Agar (10%)			P.P.G.Agar (10%)		
	Clearance of bacteria from 3 plates (mm)								
	1	2	3	1	2	3	1	2	3
S. virginia	22	22	17	17	14	12	21	23	19
S. london	11	13	11	2	2	2	19	15	16
Bacterium H.	0	0	0	13	16	16	23	21	23

25^{o}. Each culture was then divided into 2 aliquots. One aliquot was washed thoroughly in fresh minimal medium by centrifugation at 10,000 g. for 15 minutes. The bacteria were finally suspended in fresh minimal medium and the concentration of bacteria adjusted to 10^{7}/ml. The original old culture medium from these 7 day cultures was kept. The other unwashed aliquot was diluted with this old culture medium so that the concentration of bacteria was also at 10^{7}/ml. The bacteria were counted using a Petroff-Hausser counting chamber.

A suspension of amoebae at a concentration of 2×10^{6}/ml. prepared as described in Chapter 2.8 was also divided into 2 aliquots. One was subjected to centrifugation at 120 g. for 10 minutes and the supernatant discarded. The cells were resuspended in a similar volume of 7 day old bacterial culture medium. Roller tubes containing bacteria and amoebae in the ratio 5 : 1, or bacteria alone, were set up as described in Chapter 2.8 to give the following pattern of experiments for each strain of bacterium :

(i) Bacterial controls	(12 tubes)	]	suspended in fresh
(ii) Bacteria + amoebae	(10 tubes)	]	minimal medium.
(iii) Bacterial controls	(12 tubes)	]	suspended in 7 day old
(iv) Bacteria + amoebae	(10 tubes)	]	culture minimal medium.

Viable counts of bacteria present in two tubes each of (i) and (iii) were made at zero time. At 1 hour and hourly intervals up to 5 hours,

two tubes were taken from each of the four categories and viable counts made as described in Chapter 2.8, together with counts of the number of viable bacteria associated with the amoebae in categories (ii) and (iv).

These results (Table 6A,B,C, Fig. 4A,B,C) showed that when bacteria were washed and resuspended in fresh medium, no apparent difference in the rates of phagocytosis of any of the three strains of bacteria could be detected over a 5 hour period. However, the phagocytosis of S. london in a 7 day growth medium was considerably inhibited. Also, it appeared that the intracellular killing of this organism was correspondingly reduced. Both phagocytosis and killing of Bacterium H were comparable with that of S. virginia. This result was similar to that obtained when the three bacterial strains were cleared from agar containing 10% minimal medium.

3.1e Discussion

While it may be possible that amoebae are attracted by chemotactic substances to colonies of certain bacteria grown on agar plates, it is certain that the process of clearance of streaks of bacteria from agar tested in this investigation does not depict a chemotactic response of amoebae to these bacteria, or even active selection of one food organism in preference to another. Rather does it seem most probable

TABLE 6

Phagocytosis of bacteria by H. rhysodes

(A) S. virginia

Time (hrs)	No. bact. in control supernatant (A)	No. bact. in test supernatant (B)	No. bact. associated with amoebae (C)	% phagocytosis $\frac{A-B}{A} \times 100$	% intracellular survival $\frac{C}{A-B} \times 100$
S. virginia in fresh minimal medium (inoculum; 4.71×10^6 bacteria/ml.)					
1	4.82×10^6	1.57×10^6	8.99×10^5	67.4	24.5
2	5.1×10^6	1.45×10^6	6.94×10^5	70.0	19.0
3	5.28×10^6	1.82×10^6	3.72×10^5	65.5	10.7
4	4.77×10^6	1.28×10^6	2.46×10^5	73.2	7.0
5	5.38×10^6	1.22×10^6	2.9×10^5	77.3	6.9
S. virginia in 7 day old culture minimal medium (inoculum; 3.17×10^6/ bacteria/ml)					
1	3.18×10^6	1.58×10^6	7.76×10^5	50.3	48.5
2	3.47×10^6	1.32×10^6	8.21×10^5	61.9	38.1
3	3.09×10^6	1.61×10^6	5.19×10^5	47.9	35.0
4	2.98×10^6	1.37×10^6	5.0×10^5	54.0	31.0
5	3.23×10^6	1.31×10^6	5.09×10^5	59.4	26.5

Concentration of amoebae ; 1.2×10^6/ml.

FIG. 4A

Phagocytosis of bacteria by Hartmannella rhysodes

A. S. virginia

△——△ Phagocytosis* in fresh minimal medium.

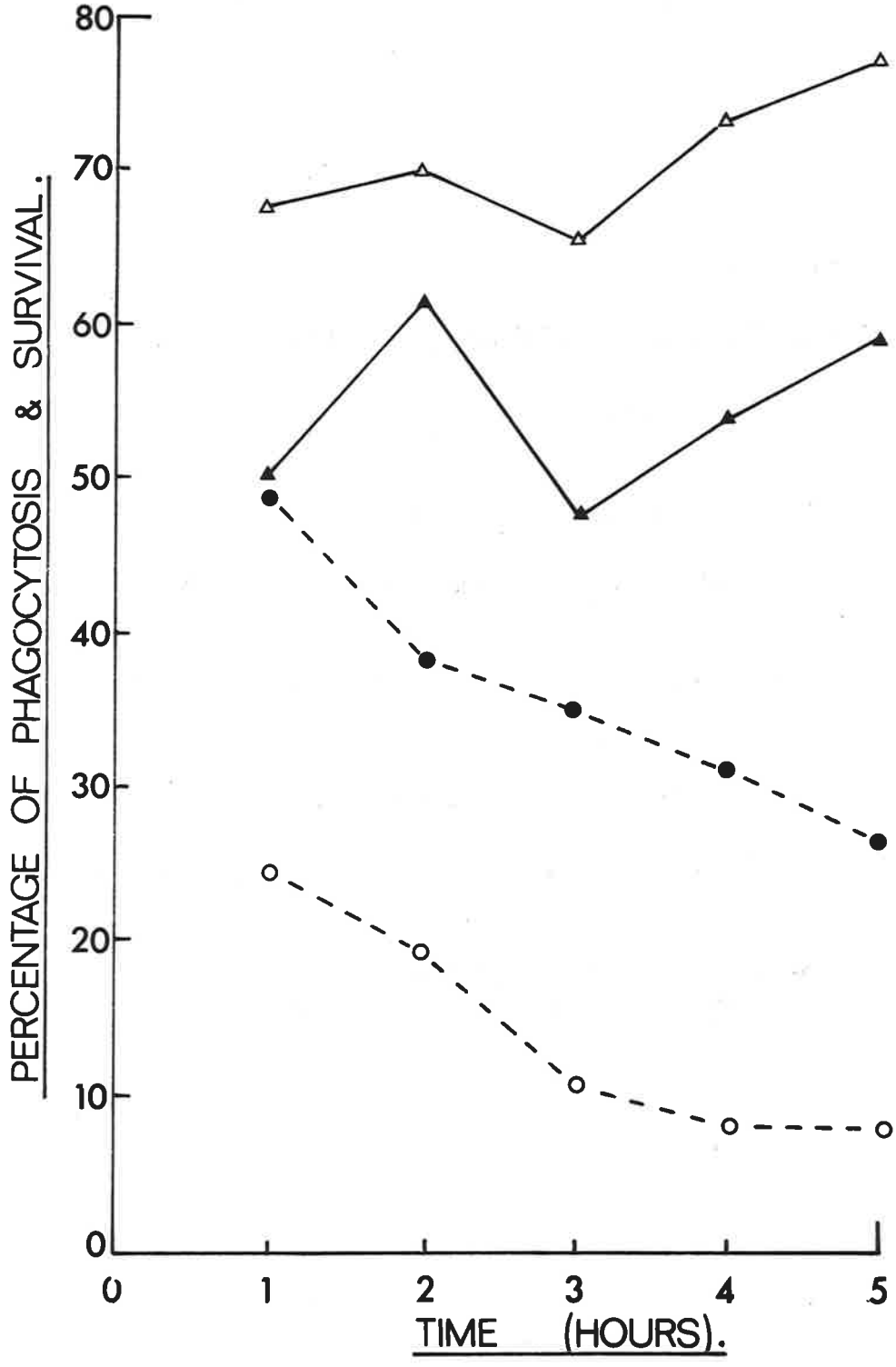
▲——▲ Phagocytosis* in 7 day old culture minimal medium.

* Phagocytosis as % of original inoculum of bacteria.

○--○ Survival* in fresh minimal medium.

●--● Survival* in 7 day old culture minimal medium.

* Survival as % of phagocytosed organisms remaining associated with amoebae (viable bacteria only).


80
70
60
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40
30
20
10
0
PERCENTAGE OF PHAGOCYTOSIS & SURVIVAL.
0
1
2
3
4
5
TIME (HOURS).

TABLE 6 (cntd)

Phagocytosis of bacteria by H. rhysodes

(B) S. london

Time (hrs)	No. bact. in control supernatant (A)	No. bact. in test supernatant (B)	No. bact. associated with amoebae (C)	% phagocytosis $(\frac{A-B}{A} \times 100)$	% intracellular survival $(\frac{C}{A-B} \times 100)$
S. london in fresh minimal medium (inoculum; 3.92×10^6 bacteria/ml.)					
1	3.11×10^6	1.61×10^6	6.99×10^5	48.2	46.6
2	3.93×10^6	1.49×10^6	6.94×10^5	62.0	28.4
3	4.28×10^6	1.58×10^6	5.28×10^5	63.0	19.5
4	3.74×10^6	1.44×10^6	3.13×10^5	61.5	13.6
5	3.61×10^6	1.37×10^6	1.87×10^5	62.0	8.3
S. london in 7 day old culture minimal medium (inoculum; 3.93×10^6/ bacteria/ml.)					
1	3.92×10^6	3.34×10^6	4.16×10^5	14.8	71.7
2	3.76×10^6	3.08×10^6	6.62×10^5	18.0	97.3
3	3.82×10^6	2.94×10^6	7.55×10^5	22.5	85.7
4	3.69×10^6	2.71×10^6	8.94×10^5	26.5	91.2
5	3.73×10^6	2.58×10^6	1.07×10^6	30.8	93.0

Concentration of amoebae ; 1.2×10^6/ml.

FIG. 4B

Phagocytosis of bacteria by Hartmannella rhysodes

B. S. london

△——△ Phagocytosis* in fresh minimal medium.

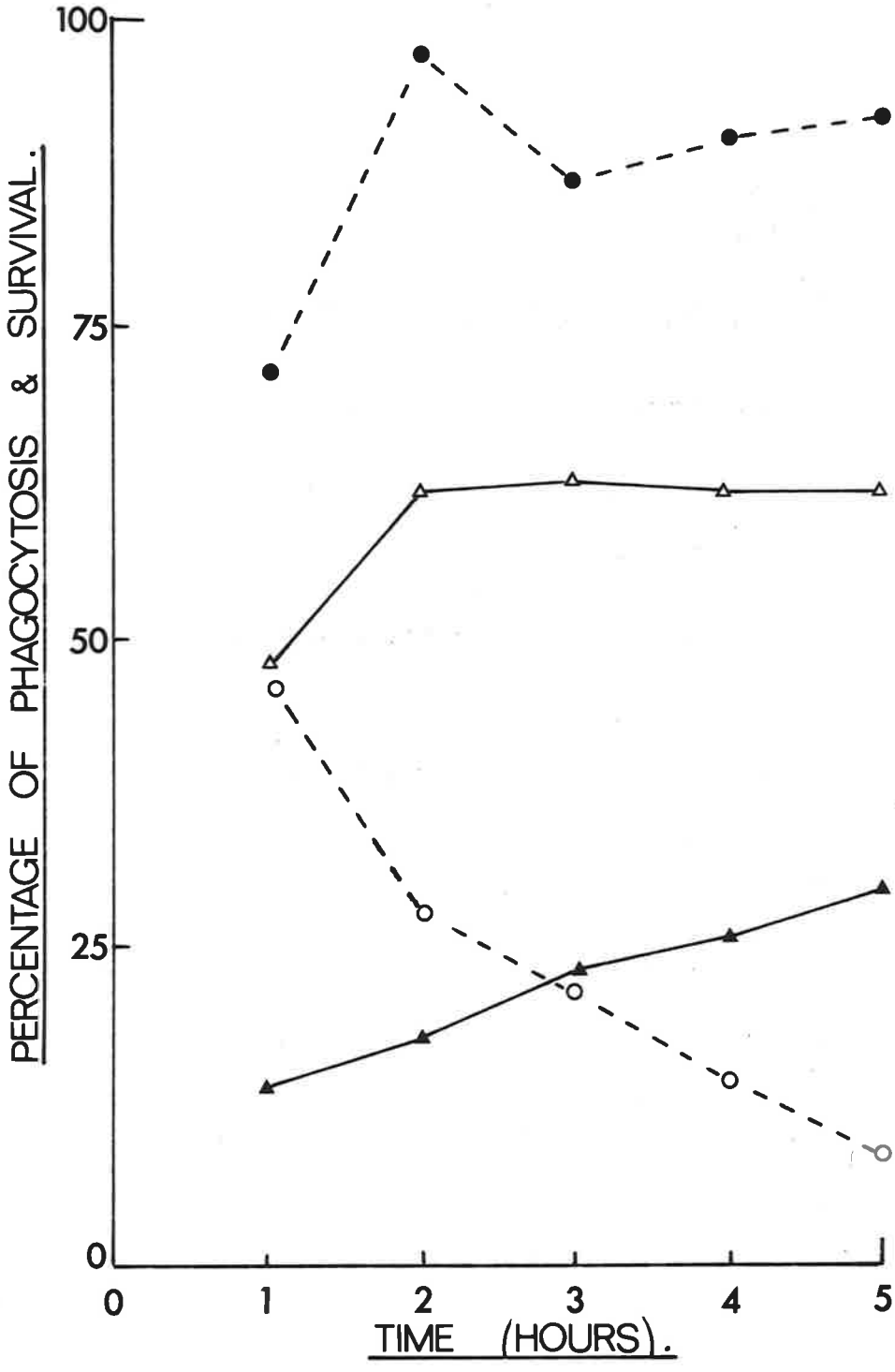
▲——▲ Phagocytosis* in 7 day old culture minimal medium.

* Phagocytosis as % of original inoculum of bacteria.

○--○ Survival* in fresh minimal medium.

●--● Survival* in 7 day old culture minimal medium.

* Survival as % of phagocytosed organisms remaining associated with amoebae (viable bacteria only).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75
50
25
0
PERCENTAGE OF PHAGOCYTOSIS & SURVIVAL.
0
1
2
3
4
5
TIME (HOURS).

TABLE 6 (cntd)

Phagocytosis of bacteria by H. rhysodes

(C) Bacterium H

Time (hrs)	No. bact. in control supernatant (A)	No. bact. in test supernatant (B)	No. bact. associated with amoebae (C)	% phagocytosis $(\frac{A-B}{A} \times 100)$	% intracellular survival $(\frac{C}{A-B} \times 100)$
Bacterium H in fresh minimal medium (inoculum; 3.9×10^6 bacteria/ml.)					
1	4.21×10^6	1.42×10^6	9.16×10^5	64.3	32.8
2	4.37×10^6	1.50×10^6	6.98×10^5	65.6	24.3
3	4.29×10^6	1.43×10^6	5.19×10^5	66.6	18.1
4	4.76×10^6	1.40×10^6	3.92×10^5	70.5	11.6
5	4.39×10^6	1.32×10^6	3.91×10^5	69.9	12.7
Bacterium H in 7 day old culture minimal medium (inoculum; 4.89×10^6/ bacteria/ml.)					
1	5.1×10^6	1.81×10^6	6.84×10^5	64.5	20.7
2	5.3×10^6	1.74×10^6	8.62×10^5	67.1	24.2
3	5.19×10^6	1.79×10^6	7.38×10^5	65.5	21.7
4	5.43×10^6	1.60×10^6	9.10×10^5	70.5	23.7
5	5.21×10^6	1.72×10^6	7.94×10^5	66.9	22.7

Concentration of amoebae ; 1.2×10^6/ml.

FIG. 4C

Phagocytosis of bacteria by Hartmannella rhysodes

C. Bacterium H.

△——△ Phagocytosis* in fresh minimal medium.

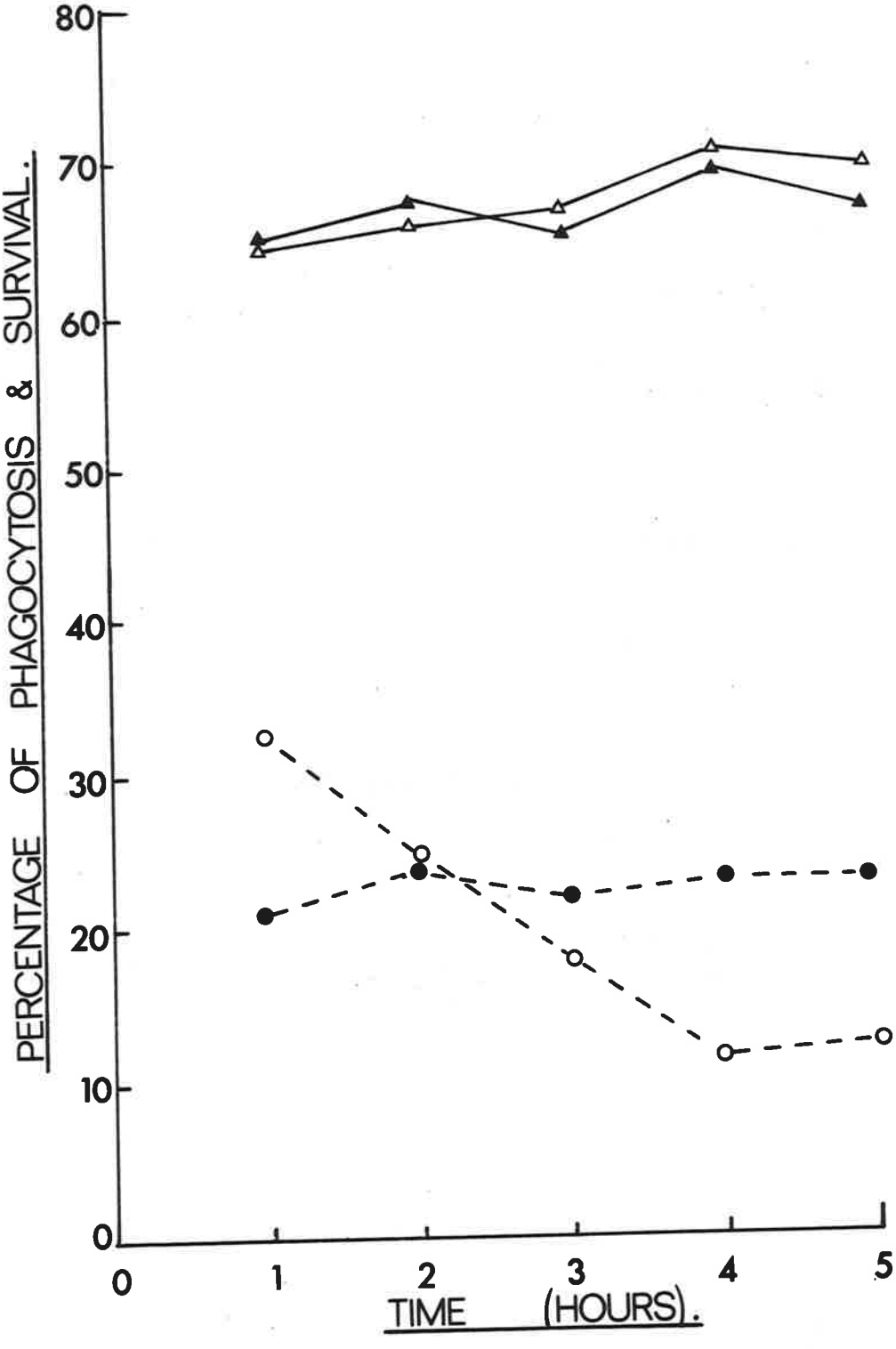
▲——▲ Phagocytosis* in 7 day old culture minimal medium.

* Phagocytosis as % of original inoculum of bacteria.

○---○ Survival* in fresh minimal medium.

●---● Survival* in 7 day old culture minimal medium.

* Survival as % of phagocytosed organisms remaining associated with amoebae (viable bacteria only).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PERCENTAGE OF PHAGOCYTOSIS & SURVIVAL.
0
1
2
3
4
5
TIME (HOURS).

that the difference in rates of clearance is a result of the release of metabolic products by some strains of bacteria which are toxic to certain amoebae, and inhibit, among other activities, phagocytosis of bacteria by these amoebae.

3.2 Method 2

The coverslip culture method, as used by Savanat (1965)

Amoebae were washed and suspended in P.B.S. and placed in a small oil chamber. This consisted of a coverslip which had been attached with molten wax to a rectangular perspex container so as to form a shallow chamber. It was filled with paraffin oil (see Fig. 5) which had been freed of fatty acids by heating at 70° for 10 minutes with activated charcoal and filtered. All cultures were placed on to the coverslip with a micropipette controlled by mouth suction. The cells adhered to the glass surface beneath the oil and formed mono-layers.

An overnight culture of bacteria was washed in P.B.S. and the final supernatant discarded. One drop of the bacterial deposit (0.02 ml.) containing approximately 10^{10} organisms, was mixed in a depression slide with the same volume of warm 2.0% neutral agar. After solidification, the agar was broken into small pieces. A piece approximately the same size as an amoeba (20μ diameter) was selected, sucked into a micropipette and washed three times by

FIG. 5

Diagram of oil chamber used in Method 2 (coverslip culture method)

A. Perspex frame from above.

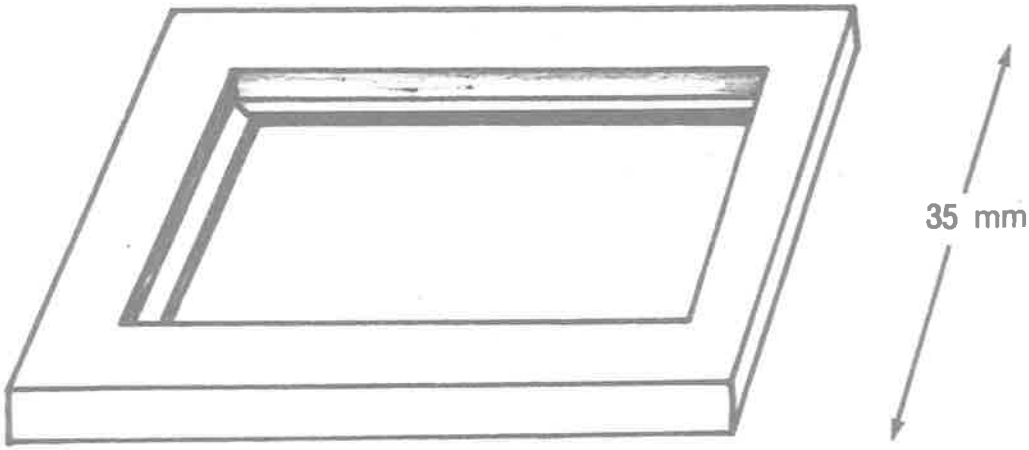
B. Side view.

(a) Coverslip

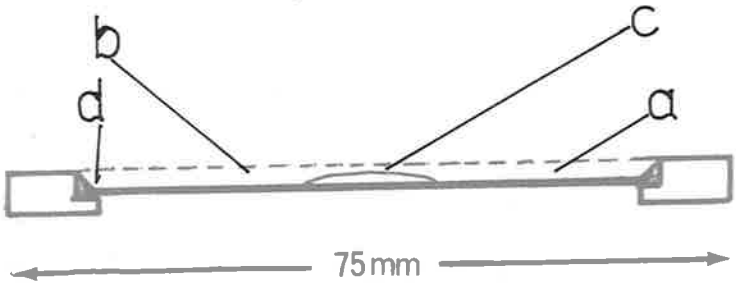
(b) Oil

(c) Culture of amoebae

(d) Wax



A



B

successive rinses in sterile P.B.S. It was then placed on to the coverslip among the amoebae.

Migration of the 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. A control piece of agar not containing bacteria, but mixed with 0.02 ml. P.B.S. of similar size to the bacterial clump, was added to a monolayer of the same amoebae on another coverslip, and again a dark ground tracing of their movements made.

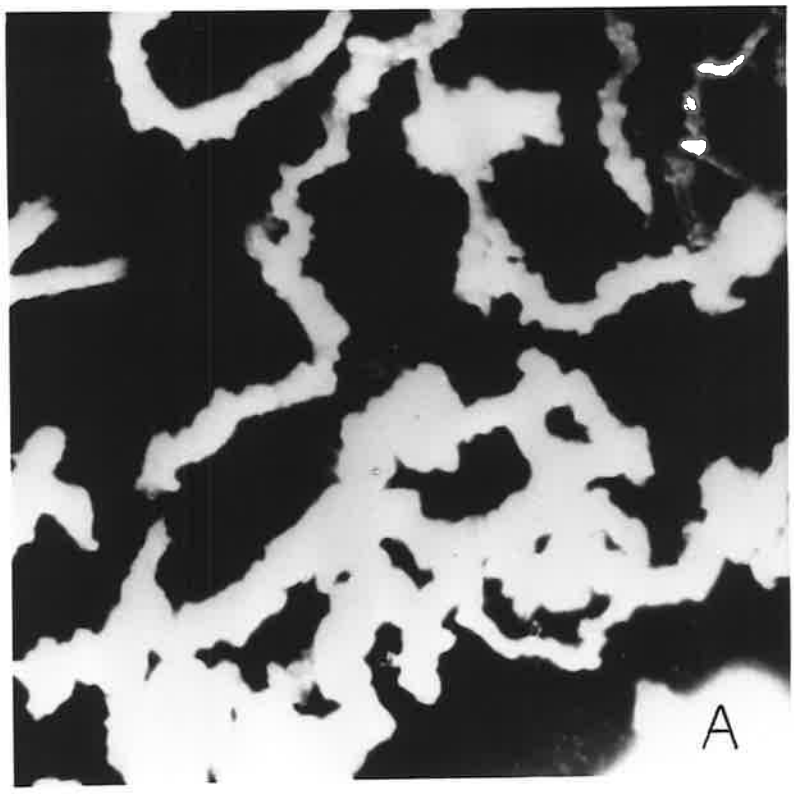
In initial experiments, clumps of Pseudomonas fluorescens were found to be very chemotactic to the soil amoeba Hartmannella rhysodes. Fig. 6b illustrates the directional migration of these cells towards the clump over a period of 30 minutes, while the random movement of amoebae adjacent to a large piece of agar alone is illustrated in Fig. 6a. Static pictures of this migration are also presented in Fig. 7.

It would seem that the only means whereby quantitation may be achieved is by measurement of the distance over which stimuli act. For this to be done, a method would also be required to ensure similar quantities of test material were added to the culture, and also that the numbers and condition of the amoebae in the culture remained the same.

FIG. 6.

(a) The random movement of amoebae adjacent to a large piece of agar (A) as depicted using a dark ground tracing technique. (Exposure time : 30 minutes, X 70).

(b) The directional movement of amoebae towards a clump of Pseudomonas fluorescens (B) using the same technique. (Exposure time : 30 minutes, X 70).


A

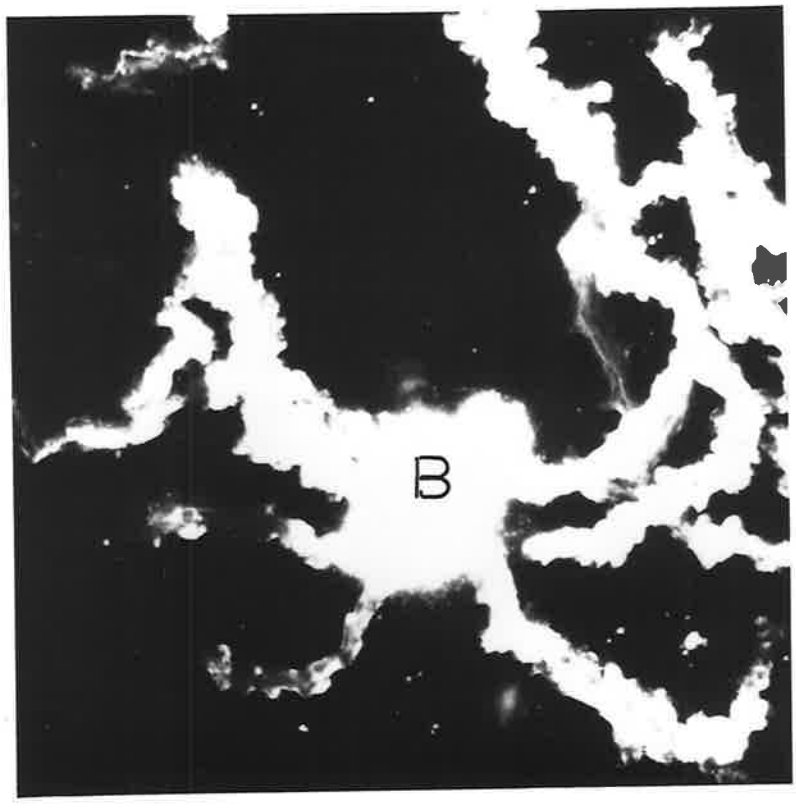

B

FIG. 7

Serial photographs showing the directional movement of H. rhysodes towards a clump of food bacteria (Pseudomonas fluorescens)

A. At 2 minutes after placement of the food.

B. At 5 minutes.

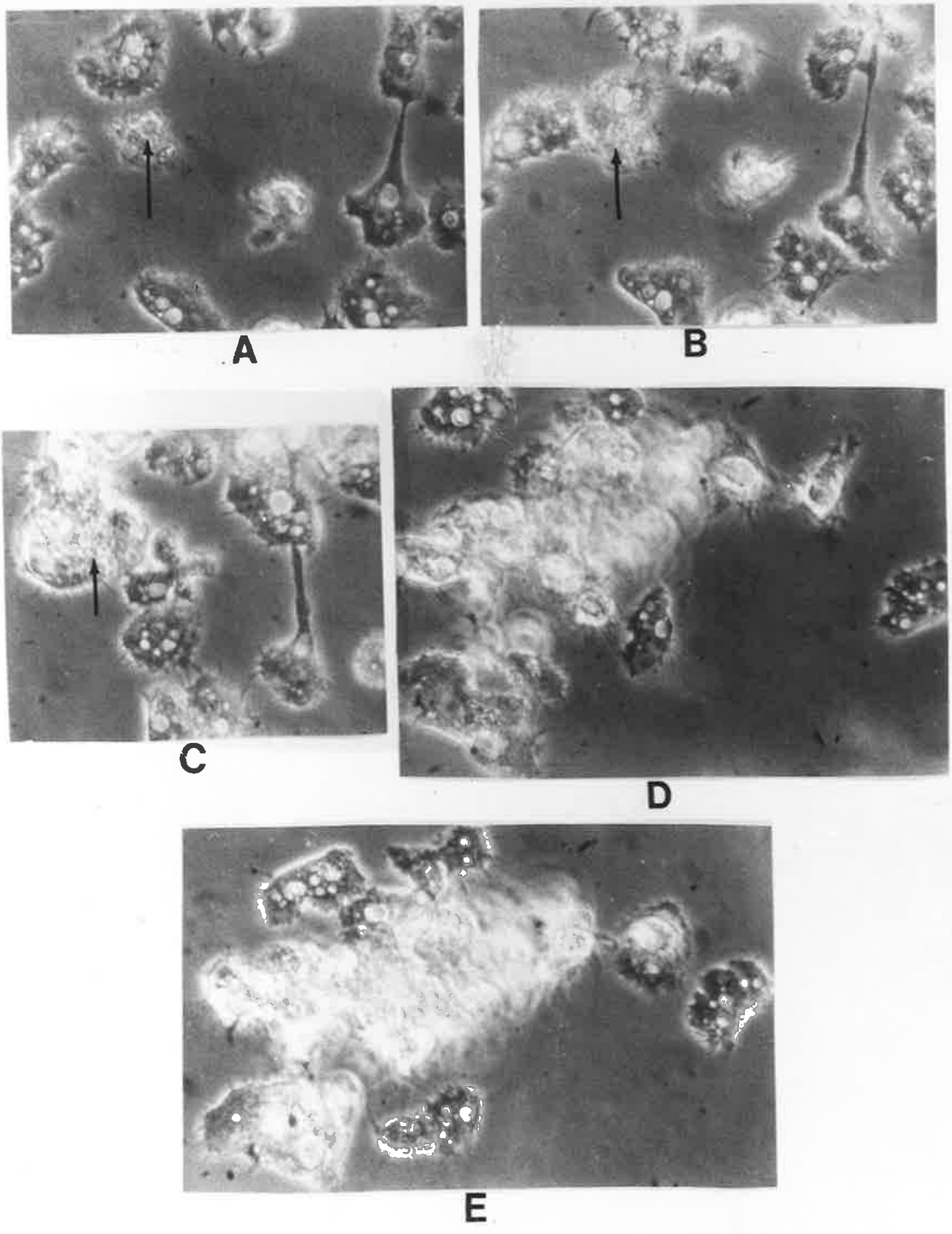
C. At 10 minutes.

D. At 30 minutes.

E. At 40 minutes.

(X 400, using phase contrast).

The arrow indicates the clump of bacteria.


A
B
C
D
E

It was extremely difficult to ensure that all these factors were kept constant.

Even factors such as the size of the agar block and the quantity of bacteria incorporated into the minute piece of agar used (about 20μ diameter), were impossible to keep constant. For these reasons, successive experiments with clumps of Pseudomonas fluorescens resulted in widely varying amounts of migration. It became fairly evident that even a reasonable degree of quantitation was not possible. However, this method appeared still to be very useful in demonstrating strong chemotactic responses, and it was thought that one may still be able to use it for analysing aspects of the chemotactic response. In view of the widespread evidence that products of food organisms are chemotactic for amoeboid cells, and the demonstration by Savanat (1965) that killed bacteria were not chemotactic, high concentrations of bacteria (10^{10}/ml) were suspended in P.B.S. for several hours. The mixture was then centrifuged at 12,000 g. for 20 minutes and the resulting supernatant tested for its ability to attract amoebae. However, considerable difficulty was met in attempts to introduce the material to localized areas in the test chamber. Among the methods used were :-

(a) Incorporation into agar pieces, as with bacteria.

(b) Use of a micropipette, which was then held in place in the culture by a micromanipulator.

(c) Formation of a small bulb (about 8μ diameter) near the tip of a micropipette, using a microforge (de Fonbrune, 1947). The test fluid was sucked into the bulb, and the pipette introduced into the culture. The pipette was then broken in front of the bulb so as to allow the bulb to remain in the culture. The fluid could diffuse through minute orifices (1μ diameter) on both sides.

(d) Beeswax was melted into a 0.01 pore size millipore filter (Millipore Membrane Co., U.S.A.) leaving one very small area unobstructed. A small reservoir of wax was built around this area and test fluid added to the reservoir, which was placed so as to be in contact with the tissue culture media just above the layer of amoebae. Test fluid was expected to diffuse through the filter to a reasonably localized area among the amoebae.

However, no definite evidence for chemotaxis could be seen except where micropipettes were used. Intense migration of cells directionally towards the tip of the pipette was evident in every case. This was found also to occur, however, when normal culture medium (P.B.S.) was present in the pipette. It appeared from further analysis

that the cells were extremely sensitive to convection currents at the tip of the pipette. All efforts to minimise these currents were of no avail. The method was abandoned when a more suitable technique was developed, using the principle of Boyden's chamber.

3.3 Method 3

Development of a modification of the Boyden Chamber technique for the measurement of chemotaxis

The third technique tested for its usefulness in demonstrating and measuring chemotaxis in soil amoebae was that originally used by Boyden (1962a) to measure the chemotactic response of rabbit polymorphonuclear leucocytes. The apparatus used was a perspex chamber (see diagram Fig. 8), consisting of two compartments (A) and (B) which may be separated by a membrane filter (C) of such pore size that cells may pass through it only by active migration (3μ pores for leucocytes). This membrane was held in place by a set of rings (D).

In Boyden's studies the normal experimental procedure consisted in pipetting a test substance into compartment B while a suspension of polymorphonuclear leucocytes was run simultaneously into compartment A, precautions being taken not to trap air bubbles beneath the membrane. After incubation at 37° for 3 hours the membranes were removed from the chambers, fixed, stained, and

FIG. 8

Sectional diagram of the original Boyden chamber

A. Compartment for the suspension of phagocytic cells.

B. Compartment for substance being tested for chemotactic properties.

C. Membrane.

D. Perspex rings.

FIG. 9

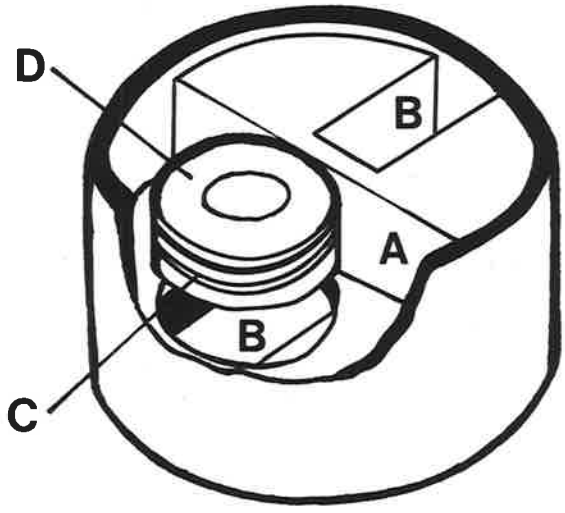
Diagram of the modified Boyden chamber

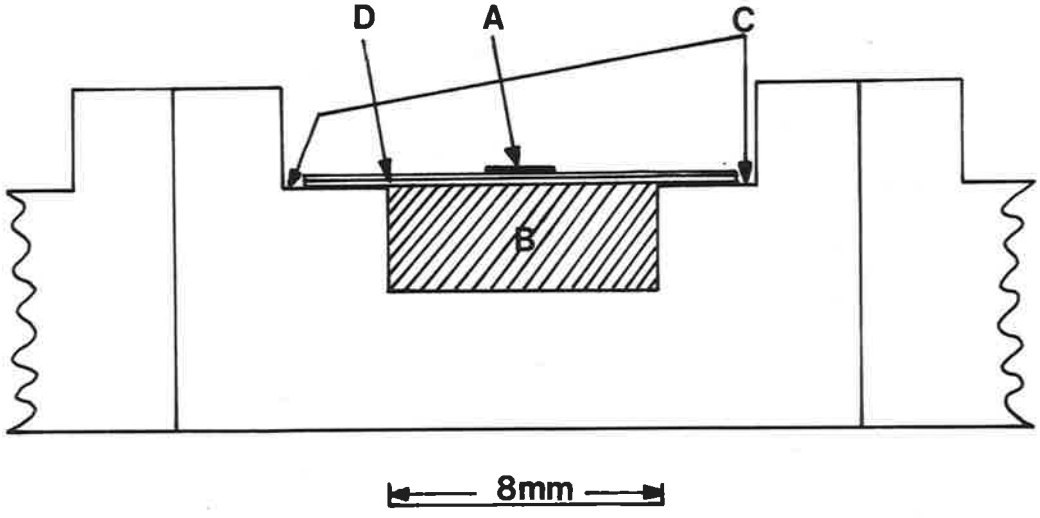
A. Amoeba suspension

B. Compartment for substance being tested for chemotactic properties.

C. Shoulders of chamber.

D. Membrane.


D
B
A
B
C


D
A
C
B
8mm

cleared in xylol. Microscopic examination of the membranes enabled a count to be made of the numbers of cells which had migrated through to the lower side.

In testing the ability of a species of soil amoebae, Hartmanella rhysodes, to migrate under somewhat similar experimental conditions, those grown in axenic culture were found most suitable. It was found impossible to free amoebae of bacteria when they were grown in the presence of the food source. A suspension of Pseudomonas fluorescens (10^{10}/ml.), the organism shown by Savanat (1965) to be chemotactic for this species of soil amoebae, was used as the test substance. For reasons that are not understood, amoebae would not migrate through the membrane in the presence of a chemotactic substance if fluid was present in compartment A, even after incubation for up to 5 hours at 20° (the optimal temperature for amoeboid movement), and using 5μ and 8μ pore sized membranes.

However, it was found that if amoebae were not covered by fluid but were layered on to a moist 8μ membrane, considerable migration occurred if chemotactic substances were present in compartment B. Also, during these experiments it was realized that a smaller type of chamber, requiring smaller volumes of test fluid, would be suitable for chemotactic assays. A diagram of this chamber may be seen in

Fig. 9. The volume of the test compartment was 0.175 ml. as compared with approximately 2 ml. with the larger original Boyden's chamber.

The basic procedure for these experiments was as follows. A sample of test substance suspended in phosphate buffered saline (P.B.S.) was placed in the test compartment (B), the fluid being level with the shoulders (C). Samples of Hartmannella rhysodes were washed twice in P.B.S. by centrifugation at 120 g. for 10 minutes and finally resuspended in the same medium to give a concentration of 2×10^7/ml. Membrane filters (Sartorius Membrane Filter Co., Germany) of pore size 8μ and 13 mm diameter were moistened in P.B.S. and placed on a filter paper to remove excess fluid. A small cylindrical loop was used to transfer a sample of amoebae on to the membrane. This resulted in a layer of cells about 2 mm in diameter on the membrane (A). The membrane was then layered across the shoulders (C) in contact with the test fluid. Precautions were taken to ensure that air bubbles were not trapped beneath the membrane. The membrane was kept in place by surface tension. The chambers were covered with a perspex sheet to minimize evaporation. Following a known period of time at 20°, each membrane was removed and prepared for microscopic examination by the following procedure :

(a) fixed in methanol for 5 seconds.

(b) washed in distilled water.

(c) stained in Ehrlich's haematoxylin for 5 minutes.

(d) washed in distilled water.

(e) treated with 1% acid alcohol for 1 minute.

(f) washed in distilled water.

(g) treated with a blueing agent ($MgSO_4$, 20 g; $NaHCO_3$, 2 g; distilled water to 1 litre) for 2 minutes.

(h) washed in distilled water.

(i) treated with 70% alcohol for 1 minute.

(j) treated with 95% alcohol for 1 minute.

(k) treated with 100% alcohol for 3 minutes.

(l) dried of excess alcohol and cleared in xylol.

The membrane was then placed on a coverslip so that the lower surface could be examined using a times 40 objective. The cells appeared stained blue against a fairly clear background, and could be seen at all levels through the membrane if migration had occurred. Ten random fields per membrane were counted, and the number of amoebae that had migrated to the lower side of the membrane was expressed as the average of the fields.

The degree of chemotaxis is expressed as a chemotactic index, which is the ratio of the average number of amoebae migrating

through the membrane in the experimental chamber, compared to the number migrating through the membrane in the control chamber. For example, if the average number of amoebae migrating through the membrane in the test chamber was 52, and the number in the control was 4, then the chemotactic index would be 13. The concentration of bacteria given in the various Figures indicates the total count of bacteria as measured using a Petroff-Hausser counter. Four chambers were used for each concentration of bacteria. For each experiment an equal number of control chambers were set up containing only P.B.S. in the test compartment.

An analysis of various aspects of the system was carried out to determine the kinetics of the process of migration.

3.3a Variation of chemotaxis index with time

An experiment was designed to see if the chemotactic index varied greatly with time. A number of chambers were set up using Pseudomonas fluorescens (10^{10}/ml.) as the test substance. At known time intervals membranes were removed from an experimental and a control chamber, stained, and the chemotactic index calculated. The results of the experiment are given in Table 7, Fig. 10, where it may be seen that after a period of 150 minutes the chemotactic index fell considerably, partly due to the increasing numbers of amoebae moving through the membranes in the control series. A close microscopic

TABLE 7

Variation of chemotactic index with time

Time (min.)	Control counts	Av./ membrane	Experimental counts	Av./ membrane	C.I.*
60	1, 0 1, 1, 1, 0, 1, 0, 0, 1	0.6	9, 6, 8, 7, 9, 8, 7, 5, 8, 9	7.6 ± 1.1	12.6
90	3, 1, 4, 3, 3, 1, 1, 2, 3, 4	2.5 ± 1.0	28,29,20,23,31,20,29,23, 26,21	25 ± 3.6	10.0
120	7, 5, 8, 5, 5, 6, 6, 7, 5, 7	6.1 ± 0.9	72,68,64,68,58,65,70,59, 63,72	65.9 ± 39	10.8
150	10,13,14,9,13,10,14,8,12, 14	11.7 ± 2.0	130,141,131,127,133,135, 141,122,137,127	132.4 ± 5.0	11.4
180	31,22,28,34,37,24,23,27, 30,34	29.0 ± 4.2	149,165,163,171,174,165, 160,168,159,151	162.5 ± 6.2	5.6
210	29,29,35,33,31,36,37,30, 27,37	32.4 ± 3.2	192,195,199,168,177,179, 194,187,193,195	187.9 ± 8.1	5.8
240	61,58,69,72,69,59,57,59, 69,74	64.7 ± 5.9	222,191,198,217,214,221, 203,209,203,192	207.0 ± 9.6	3.2

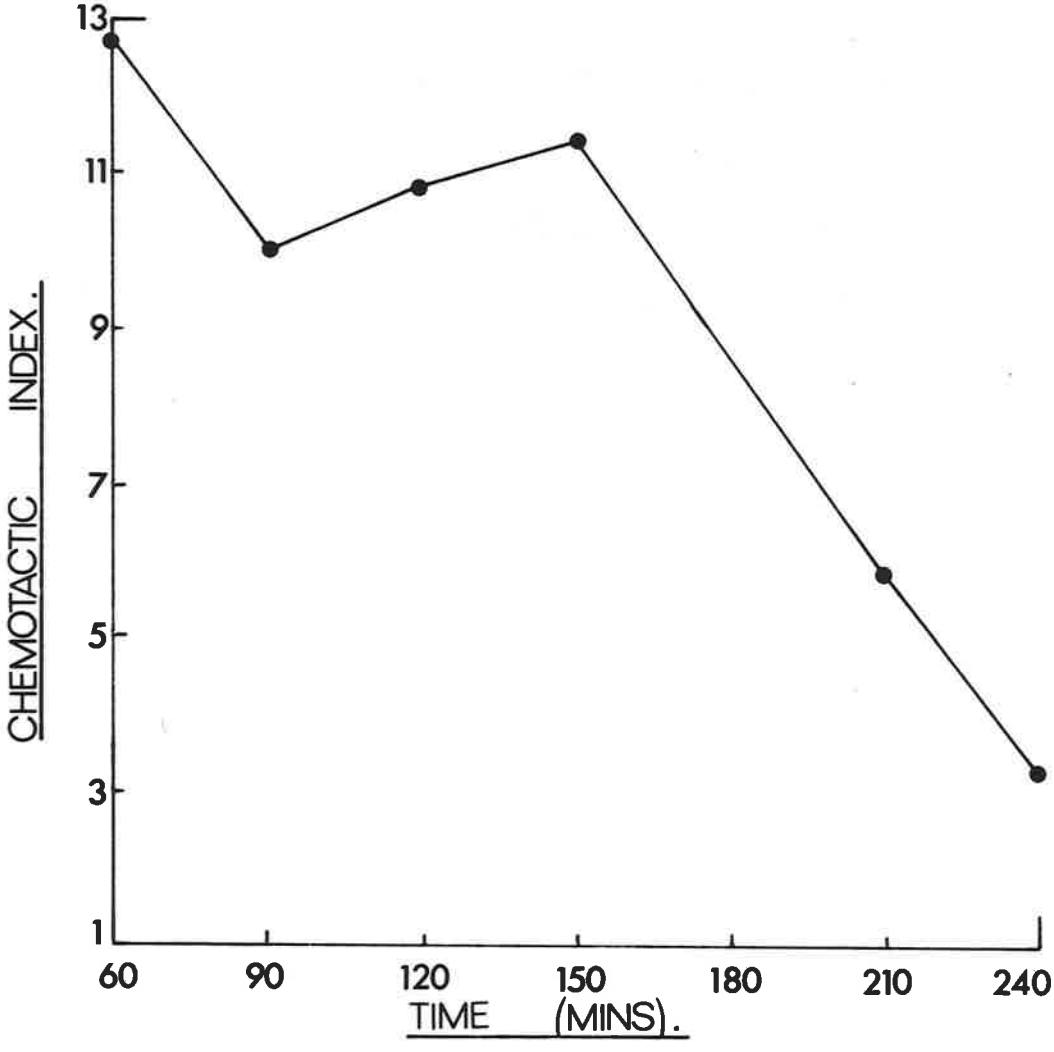
* Chemotactic index.
Concentration of H. rhysodes : 2×10^7/ml.
Test suspension : 10^{10} Pseudomonas fluorescens/ml.

FIG. 10

Variation of chemotactic index with time

Concentration of H. rhysodes : 2×10^7/ml.

Concentration of bacteria in compartment B : 10^{10}/ml.


13
11
9
7
5
3
1
CHEMOTACTIC INDEX.
60
90
120
150
180
210
240
TIME (MINS).

examination of the experimental membrane at various levels showed that the lowering of the chemotactic index was due to the fact that at a density of 150-200 amoebae/field, the cells were present in all the pores available for migration, forming a monolayer over the lower side of the membrane.

In further similar experiments it was found that the rate of migration may vary according to the age of the amoeba culture, as well as to other factors associated with their growth. In certain cases, particularly soon after subculture, migration was very slow and the chemotactic index was maximal only after a period of 4 hours. In older cultures (3-4 weeks) rapid migration occurred under a chemotactic stimulus and the chemotactic index was maximal after 50 minutes. In all subsequent experiments, unless otherwise stated, amoebae were used only in their third to fourth week after subculture. A further measure taken to ensure more constant experimental conditions was that an extra number of control chambers was set up for each experiment and one of these was removed at regular intervals during the course of the experiment to enable a membrane count to be made. The experiment was terminated when the count per field was of the order of 5-10 cells. Previous experience had indicated that the chemotactic index would be maximal at this time.

3.3b Reproducibility of results

It was important to know that under a given set of experimental conditions each experimental chamber and each control chamber behaved in a similar fashion. Ten experimental chambers were set up with a concentration of 10^{10}/ml. Pseudomonas fluorescens in the bottom compartments. Amoebae at a concentration of 2×10^{7}/ml. were inoculated on to the membranes. Ten chambers with suspension medium (P.B.S.) alone in the bottom compartments served as controls. At the end of 90 minutes the experiment was terminated and 10 fields/membrane counted. The data, given in Table 8, show that the variations between fields per membrane, and between the chambers, are small. However, it is obvious that considerable variation in the chemotactic index could occur if the result from one control and one experimental chamber were taken at random. The resulting chemotactic index could range from 8 to 20. It was found that if results from any 4 chambers in each section were averaged and the chemotactic index calculated with these figures, then the result would approach closest to that calculated if the average figures for all 10 chambers were used. In subsequent experiments it was decided to use 4 chambers for each concentration of test sample, as well as for controls.

3.3c Experiment to test the difference between chemotaxis and increased random movement

TABLE 8

Variation of results between chambers

Chamber No.	Control counts/membrane	Av./membrane
1	11, 6, 12, 7, 8, 7, 7, 9, 6, 8	8.1 ± 1.5
2	8, 8, 10, 5, 10, 7, 6, 7, 9, 7	7.7 ± 1.3
3	13,10,12,9,8,13,11,7,14,9	10.6 ± 1.8
4	4, 8, 7, 5, 4, 3, 6, 6, 5, 7	5.5 ± 1.3
5	9, 7, 7, 9, 10, 5, 7, 8, 7, 8	7.8 ± 1.0
6	10,12,7,13,9,8,11,10,10,11	10.1 ± 1.4
7	6, 8, 5, 9, 7, 7, 5, 7, 8, 6	6.8 ± 1.0
8	7, 5, 9, 5, 6, 7, 4, 7, 6, 6	6.2 ± 1.0
9	7, 8, 8, 9, 9, 10, 9, 7, 7, 10	8.4 ± 1.0
10	5, 8, 9, 9, 9, 7, 9, 6, 8, 7	7.7 ± 1.2
	Average =	7.9 ± 1.2
	Experimental counts/membrane	
1	111,94,98,108,116,101,94,100,107,114	104.3 ± 6.5
2	89,102,95,93,87,107,100,91,87,106	95.7 ± 6.4
3	109,118,113,104,108,109,113,119,111, 109	111.3 ± 3.6
4	107,119,104,108,115,119,112,110,113, 117	111.4 ± 4.7
5	87,99,97,78,101,95,99,89,100,93	93.8 ± 5.5
6	98,109,101,107,113,93,99,98,109,101	102.8 ± 5.4
7	105,101,92,98,91,102,109,102,96,100	99.6 ± 4.3
8	79,84,101,92,90,82,88,95,80,97	88.8 ± 6.2
9	107,104,108,95,99,111,109,101,96,109	103.9 ± 4.9
10	107,101,104,113,115,109,97,106,109,101	106.2 ± 4.4
	Average =	101.8 ± 5.2

The experiment was terminated at 90 minutes.
Test suspension : 10^{10} Pseudomonas fluorescens/ml.

It was possible that previous experiments using the Boyden technique merely measured the result of increased random movement of the amoebae due to the effect of chemical substances diffusing from the material under test, rather than a directional movement of these cells. The following experiment indicated that the movement through the membrane was a directional one.

Bacteria at a concentration of 10^{10}/ml. were placed in the lower compartments. Amoebae (2×10^{7}/ml.) were mixed with varying concentrations of bacteria and a sample of each mixture placed on the membranes of separate chambers. Control chambers were used for each amoeba-bacterium mixture. It was noted that when certain concentrations of bacteria had been mixed with amoebae, considerable inhibition of migration in the test chambers occurred (Table 9). If the migration had been merely due to increased random movement, one might have expected increased migration over some ranges of concentrations, rather than inhibition. That the movement was directional was further substantiated by the finding that if only P.B.S. were present in the lower compartment and amoebae were mixed with a concentration of 10^{8} bacteria/ml. before being placed on to the membrane, the resulting migration of amoebae through the membrane was not increased, compared with that occurring when normal amoebae alone were tested.

TABLE 9

Inhibition of chemotaxis

Inhibiting concent'n of bacteria /ml.	Control		Experimental		Chemo-tactic index
	Av. no. amoebae migrating/memb.	Av.for 4 cha-mbers	Av. no. amoebae migrating/memb.	Av.for 4 cha-mbers	
0	11, 9, 6, 9	9	108,97,84,96	96	10.7
10^3	7, 5, 9, 6	7	74,57,63,75	67	9.7
10^4	9, 4, 7, 7	7	58,71,64,55	62	8.9
10^5	5, 8, 3, 5	5	44,43,48,57	48	9.6
10^6	7, 7, 4, 4	5	49,36,47,36	42	8.4
10^7	2, 3, 1, 2	2	14,17,16,11	14	7.0
10^8	4, 6, 6, 3	5	13,7,15,14	12	2.4

Test suspension : 10^{10} Pseudomonas fluorescens/ml.

3.3d Effect of pH and ionic concentration of media on migration of normal amoebae

A further aspect of the kinetics of the reaction investigated was the effect of changes in pH and ionic concentration on the normal migration of amoebae.

Samples of phosphate buffered saline solution were prepared to give a series of solutions of similar ionic concentration (μ : 0.6), but of pH ranging from 9.0 to 4.0. A further series was prepared at pH 6.5, but of ionic concentrations ranging from 0.02 to 0.25. These solutions were placed in the test chambers and the migration of amoebae followed. Table 10 shows that whereas pH variations in the range tested (4.0 to 9.0) did not appreciably affect the average numbers of amoebae migrating, the variation in ionic concentration considerably influenced this result.

3.4 Discussion

As mentioned in the introduction to this chapter, the inadequacy of many of the techniques used over the last century to study chemotaxis in phagocytic cells had been largely responsible for the little progress that has been made in understanding the mechanism of this process. The analysis of three techniques which were considered suitable for demonstrating and measuring chemotaxis of soil amoebae to bacterial food, has revealed that one does not demonstrate

TABLE 10

Effect of ionic concentration and pH of medium on amoeba migration

μ	pH	No. amoebae migrating/memb.	Av.of 4 chambers
0.06	9	10, 7, 9, 10	9
0.06	6.5	14,10, 8, 6	10
0.06	5	7, 10, 12, 10	10
0.06	4	15, 7, 12, 7	10
0.02	6.5	12, 14, 11, 12	12
0.06	6.5	15, 8, 11, 6	10
0.1	6.5	1, 2, 4, 3	3
0.25	6.5	0, 0, 0, 0	0

chemotaxis at all, that the usefulness of the other appears to be very limited, leaving only one method suitable for this purpose. The Boyden chamber appears to be very suitable for assaying the chemotactic response of these phagocytic protozoans. This method will be used to analyse the mechanism responsible for chemotaxis in the soil amoeba, Hartmannella rhysodes.

CHAPTER 4

THE MECHANISM OF CHEMOTAXIS IN SOIL AMOEBAE

Savanat (1965) was the first to demonstrate that bacteria were chemotactic for soil amoebae. However, he was unable to find definite evidence for the involvement of a specific mechanism. Other studies with amoebae have suggested that the chemotactic movement of these protozoans is initiated by products released from food organisms (Jeon and Bell 1962, Konijn et al. 1968).

In this chapter the quantitative method developed as a modification of the technique originally used by Boyden (1962a) was used to analyse various aspects of the chemotactic response of the soil amoeba, Hartmannella rhysodes, to bacteria.

4.1 Chemotactic response of normal amoebae to varying concentrations of bacteria

Previous experiments (see Chapter 3) have clearly indicated that Hartmannella rhysodes exhibits chemotaxis in the presence of Pseudomonas fluorescens. It was therefore of interest to see what effect the concentration of bacteria had on this chemotactic movement. A detailed account of the experimental procedure is given in Chapter 3. Briefly, concentrations of bacteria ranging from 10^{11}/ml. to 8×10^{7}/ml. were placed in the bottom compartment of the modified Boyden chamber. Four chambers were used at each concentration, and for each

experiment there were four control chambers. Also, as mentioned earlier, trial control chambers were used to determine the time at which the experiment should be terminated.

The results of a single typical experiment appear in Table 11 and Fig. 11. The data show that there is a linear relationship between the chemotactic index and concentration of bacteria.

This result was repeated on a number of different occasions to ensure its reproducibility. Data from these experiments are given in Table 12 and Fig. 12. It may be seen that the technique gives reproducible results and that the difference between the chemotactic index for one concentration of bacteria compared with another is significant. In initial experiments it was found that marked inhibition of chemotaxis occurred using concentrations of bacteria greater than 5×10^{10}/ml. In an attempt to understand the reason for this, the following experiment was carried out.

A thick suspension of bacteria containing approximately 10^{12} organisms/ml. was mixed with a small amount of molten agar, as were several five-fold dilutions of this suspension, and the mixtures allowed to set. Moist membranes on which loopfuls of amoebae had been placed were then layered across these agar surfaces. A count of amoebae which had migrated was made at varying time intervals

TABLE 11

Chemotactic response of amoebae to varying concentrations of bacteria

Concentration bacteria/ml. *	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
0 (controls)	3, 5, 5, 2	4	
8×10^7	13, 17, 16, 9	14	3.5
4×10^8	17, 24, 17, 11	17	4.2
2×10^9	22, 33, 30, 24	27	6.8
1×10^{10}	42, 41, 45, 39	42	10.5
5×10^{10}	64, 51, 53, 45	53	13.2
1×10^{11}	5, 3, 1, 1	2.5	0.6

* Total concentration.

FIG. 11

Chemotactic response of amoebae to varying concentrations of bacteria.

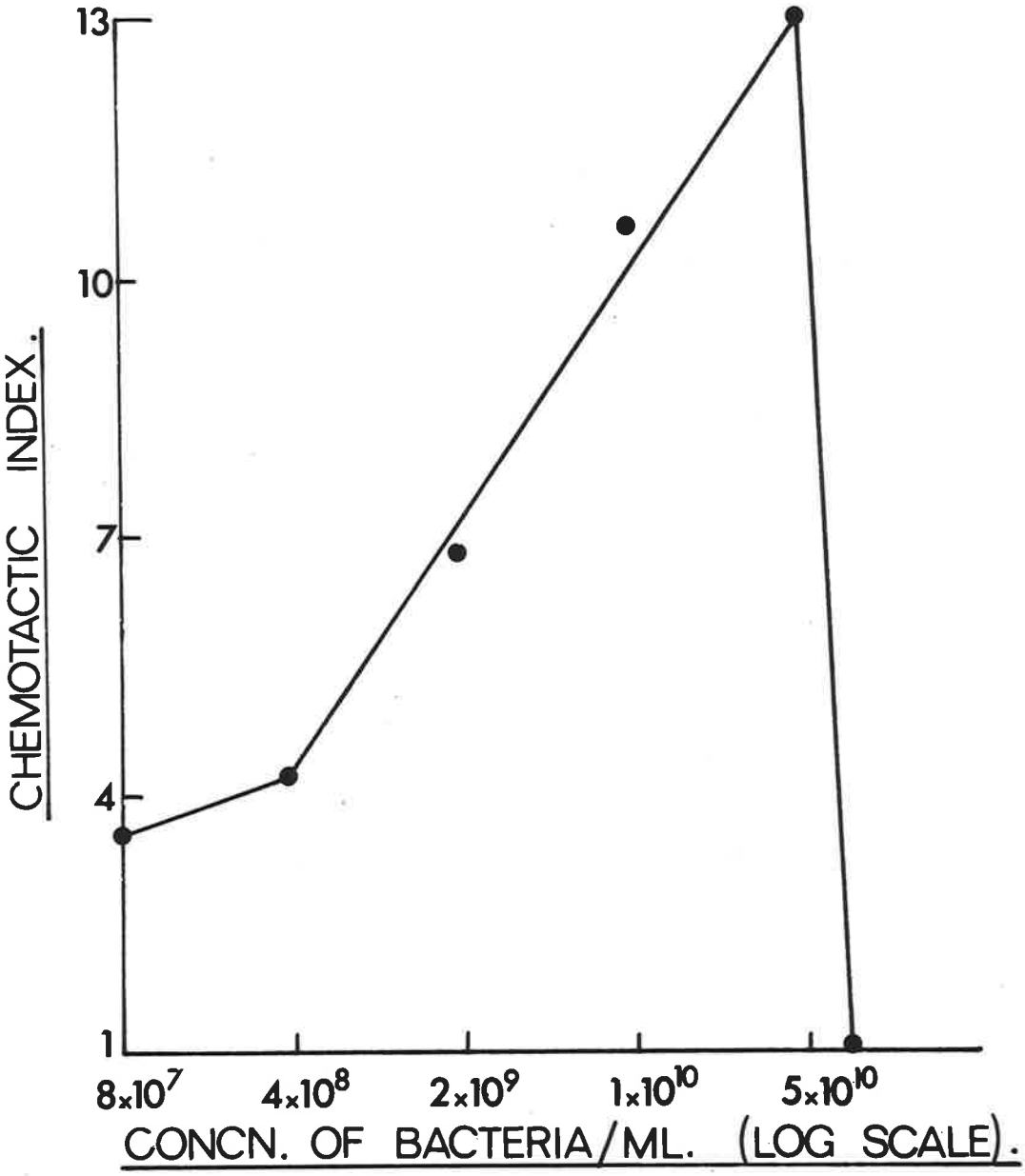

13
10
7
4
1
CHEMOTACTIC INDEX.
8x10^7
4x10^8
2x10^9
1x10^10
5x10^10
CONCN. OF BACTERIA/ML. (LOG SCALE).

TABLE 12

Significance of the results of the experiments measuring chemotaxis in the amoeba Hartmannella rhysodes

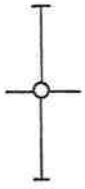
Experiment No.	Concentration bacteria/ml. in experimental chambers				
	8×10^7	4×10^8	2×10^9	10^{10}	5×10^{10}
	Chemotactic indices				
1	2.0	2.6	3.3	7.8	11.1
2	3.5	4.2	6.8	10.5	13.0
3		5.2	9.0	13.0	17.2
4		2.8	5.2	9.5	12.0
5		2.3	4.1	7.0	16.0
6	1.3	1.8	3.0	6.2	8.7
7	2.1	2.8	4.5	6.0	8.3
8		2.2	3.5	5.9	
9		3.2	5.1	9.8	13.6
10		3.8	6.8	10.4	13.3
11		5.8	7.9	12.3	
12		3.8	4.9	8.7	11.7
Mean chemotactic indices	2.225	3.375	5.342	8.925	12.490
Standard deviations	0.9	1.2	1.9	2.4	2.8
Level of * significance		10%	1%	$>0.1\%$	$>0.1\%$

* The levels of significance indicate that the mean chemotactic index from experiments using one concentration of bacteria are different from those using a five-fold higher or lower concentration of bacteria at that particular level. These figures are calculated by the student "t" test.

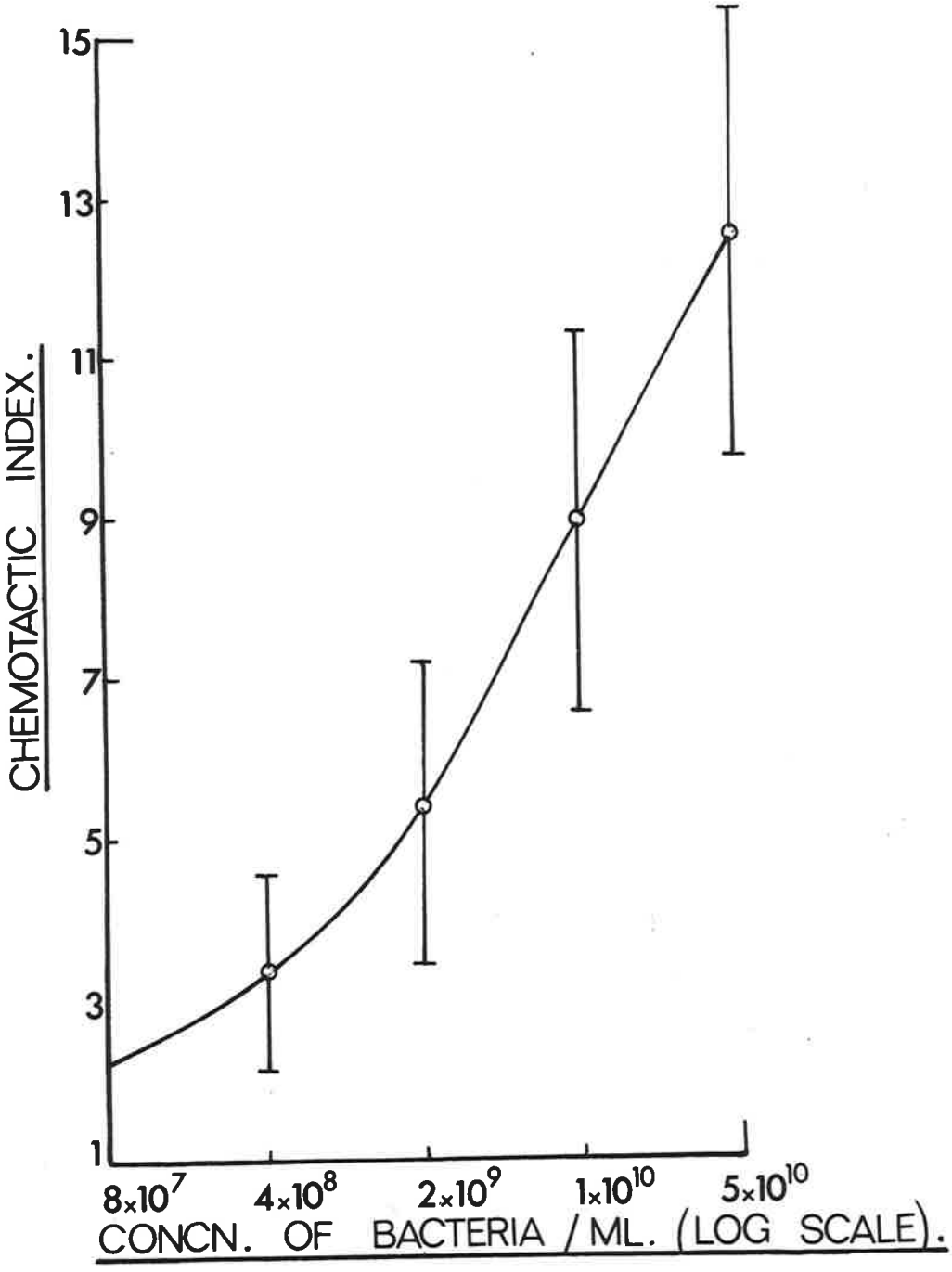
FIG. 12

The mean variation of the chemotactic index in experiments using different cultures of amoebae.

Mean chemotactic indices from 12 experiments for each concentration of bacteria.



Standard variation from the mean.


15
13
11
9
7
5
3
1
CHEMOTACTIC INDEX.
8×10^7
4×10^8
2×10^9
1×10^10
5×10^10
CONCN. OF BACTERIA / ML. (LOG SCALE).

and the results showed that under these conditions no inhibition occurred. (See Table 13). Agar controls showed that this substance was not chemotactic. These results suggest that the inhibition of migration observed in the chambers was due to the movement of numbers of bacteria into the pores of the membrane in such concentrations as to set up local gradients of chemotactic material sufficient to prevent further migration of amoebae through the membrane.

4.2 Chemotactic response of amoebae to killed bacteria

In view of the evidence favouring products of food organisms as initiating the chemotactic response in certain protozoans, experiments were designed to test if this was so in the case of the soil amoebae. Bacteria (Pseudomonas fluorescens) were killed by various means, namely heating for 60 minutes at 100°, suspending in 70% ethanol at 4° overnight, or incubation with 3,000 units of Streptomycin/ml. at 30° overnight.

Viable counts after this treatment revealed that only in the latter case was killing incomplete. However, only 3×10^4 organisms/ml. had survived and it was obvious from the previous data that this concentration of live bacteria would, of themselves, be unable to initiate a chemotactic response. After these treatments bacteria were washed twice in P.B.S. and the concentration of each sample was

TABLE 13

Chemotaxis of amoebae towards bacteria incorporated into agar

Concentration bacteria/ml. in agar *	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
0 (controls)	4, 7, 5, 7	6	
2.6×10^{8}	32, 37, 33, 23	31	5.1
1.3×10^{9}	42, 48, 57, 62	52	8.7
8×10^{9}	78, 72, 73, 72	74	12.3
4×10^{10}	97, 78, 94	90	14.4
2×10^{11}	82, 93, 94, 65	84	14.0
1×10^{12}	80, 94, 84, 86	86	14.3

* Total concentration.

adjusted to 5 x 10^{10}/ml. which was diluted in the usual way for chemotactic tests. It is clear from the results (Table 14A,B,C Fig.13) that amoebae responded to the presence of killed bacteria as well as they did to live samples.

4.3 Chemotactic response of amoebae to bacterial cell wall preparations

Cell walls were prepared from Pseudomonas fluorescens as described in Chapter 2.7. When these were diluted and the various concentrations tested for chemotactic activity, the results (Table 15, Fig. 14) showed that these induced a similar response in amoebae as did live bacteria. This result, as well as that obtained using killed well-washed bacteria, was not consistent with the suggestion that bacterial metabolic by-products initiate the chemotactic response in soil amoebae.

4.4 The effects of starvation on the ability of amoebae to respond to chemotactic stimuli

In some earlier experiments on coverslip cultures it was noted that amoebae which had been maintained in saline or P.B.S. in the absence of nutrient medium for a few days appeared unable to respond chemotactically to the presence of bacteria. For example, if a clump of bacteria was placed immediately behind a moving, starved amoeba, it was ignored. This was in marked contrast to the behaviour of normally fed amoebae. These immediately reorientated their movement towards

TABLE 14A

Chemotactic response of amoebae to bacteria killed by heat *

Concentration bacteria/ml. **	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
0 (controls)	7, 10, 5, 9	8	
8×10^7	21, 21, 14, 13	17	2.1
4×10^8	21, 29, 28, 19	24	3.0
2×10^9	39, 50, 42, 55	46	5.8
1×10^{10}	77, 76, 64, 65	70	8.8
5×10^{10}	101, 91, 79, 88	90	11.2

* 100° for 60 minutes.

** Total concentration.

TABLE 14B

Chemotactic response of amoebae to bacteria killed by 70% alcohol*

Concentration bacteria/ml. **	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
0 (controls)	11, 7, 8, 13	10	
8×10^7	52, 39, 44, 37	43	4.3
4×10^8	49, 46, 57, 56	52	5.2
2×10^9	69, 65, 60, 65	65	6.5
1×10^{10}	95, 103, 102, 87	97	9.7
5×10^{10}	129, 111, 104, 128	118	11.8

* At 4° overnight.

** Total concentration.

TABLE 14C

Chemotactic response of amoebae to bacteria killed by Streptomycin (3,000 μ/ml.)*

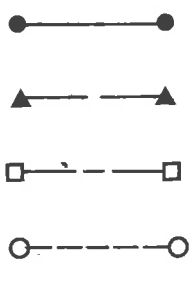
Concentration bacteria/ml. **	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
0 (controls)	2, 4, 5, 4	4	
8×10^7	10, 7, 13, 15	11.2	2.8
4×10^8	12, 17, 18, 14	15.2	3.8
2×10^9	26, 21, 27, 15	22.2	5.5
1×10^{10}	34, 30, 40, 29	33.2	8.3
5×10^{10}	51, 39, 33, 44	42	10.4

* At 30° overnight.

** Total concentration.

FIG. 13.

The chemotactic response of amoebae to killed bacteria.



Living bacteria (control)

Heat-killed bacteria (100° for 60 minutes)

Alcohol-killed bacteria (70%, overnight at 4°).

Streptomycin killed bacteria (3,000 μ units/ml. at 30° overnight).

The bacteria were washed thoroughly following these treatments.

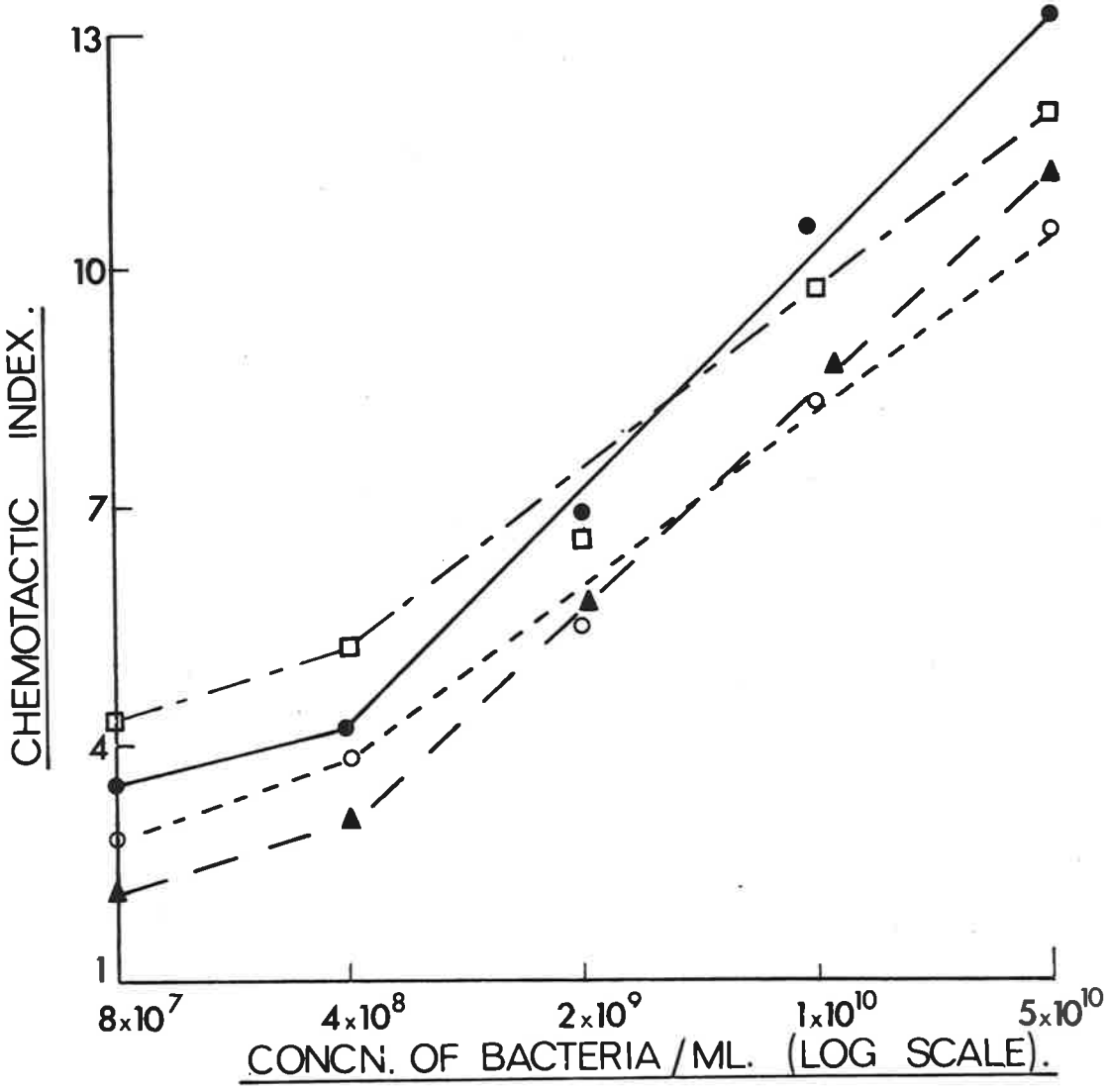

13
10
7
4
1
CHEMOTACTIC INDEX.
8x10^7
4x10^8
2x10^9
1x10^10
5x10^10
CONCN. OF BACTERIA / ML. (LOG SCALE).

TABLE 15

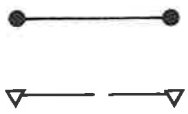
Chemotactic response of amoebae to bacterial cell walls

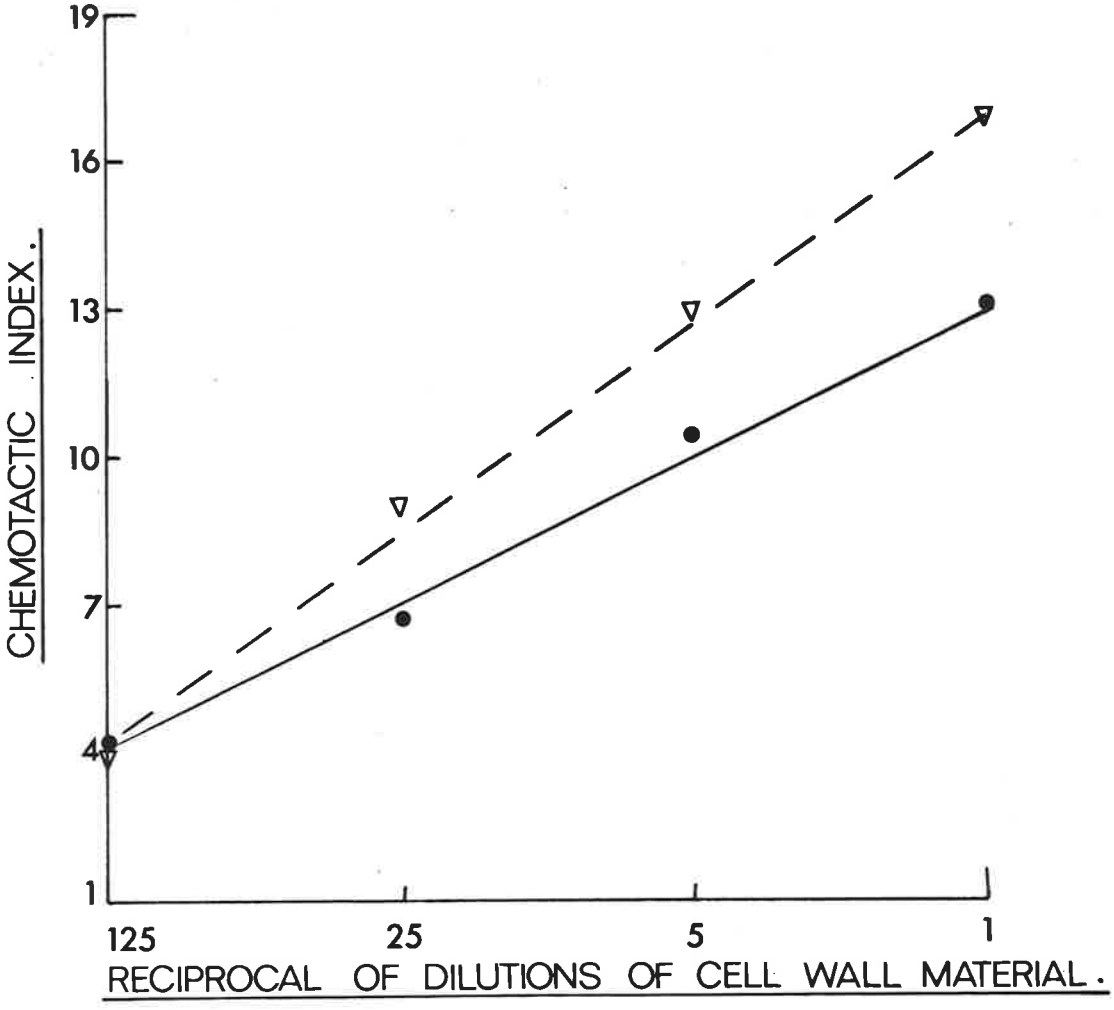
Reciprocal of dilutions of cell wall preparation*	Av. no. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
No cell walls	5, 7, 8, 5	6	
125	27, 31, 20, 19	24.2	4.0
25	47, 58, 56, 55	54	9.0
5	92, 71, 69, 80	78	13.0
1	114, 91, 103, 105	103.2	17.2

* The preparation of cell walls was equivalent in concentration to that which would be obtained from 5×10^{10} bacteria/ml. The dry weight of the preparation was 10 mg./ml.

FIG. 14.

The chemotactic response of amoebae to bacterial cell walls.




19
16
13
10
7
4
1
CHEMOTACTIC INDEX.
125
25
5
1
RECIPROCAL OF DILUTIONS OF CELL WALL MATERIAL.

the source of food.

It was decided to test this observation quantitatively. Samples of freshly washed cell walls were used in the same concentrations as in the previous experiments. Amoebae were washed in P.B.S. under aseptic conditions by centrifugation at 300 g., using a small M.S.E. centrifuge, and resuspended in this medium at an approximate concentration of 5×10^7 amoebae per 20 ml. of P.B.S. Samples were left in this saline solution for up to 5 days. Visual observation of these cultures showed that the normal random movement of the cells increases markedly even after one day in this medium. After three days, whilst most cells remained very active, a small number appeared to round up and become sticky. These adhered to any moving cell which came in contact with them. This pre-encystment phase proceeded until most cells had encysted on day 5. Chemotactic tests were carried out using amoebae which had been starved for varying periods up to 4 days, using cell walls as the test substance. The results are presented in Table 16, Fig. 15. In this and future experiments, the term "normal" amoebae refers to cells removed directly from P.P.G. being untreated in any way except for washing. It will be noticed that the ability of amoebae to respond chemotactically to the presence of cell wall material progressively decreases with the time of starvation. This is not due to any decrease in activity of the

TABLE 16

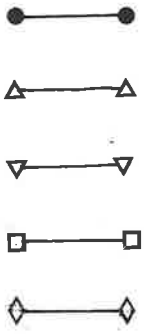
Chemotactic response of starved amoebae to bacterial cell walls

Recip. diln of cell wall prepn.	Normal amoebae		One day starved		Two day starved		Three day starved		Four day starved	
	Av. No. amoebae migrating/ membrane	C.I.*	Av. No. amoebae migrating/ membrane	C.I.	Av. No. amoebae migrating/ membrane	C.I.	Av. No. amoebae migrating/ membrane	C.I.	Av. No. amoebae migrating/ membrane	C.I.
No cell walls	5 7,8,5.		7,12,4,8.		2,4,3,1.		6,5,4,4.		8,6,7,11	
125	27 31,20,19.	4.0	29,41,52,38.	5.0	7,5,3,9.	3.0	17,13,11,9.	2.5	10,18,15,20.	2.0
25	47,58,56,55.	9.0	37,39,53,47.	5.5	10,5,7,7.	3.5	17,14,21,9.	3.0	22,13,19,16.	2.2
5	92,71,69,80.	13.0	104,79,57, 81.	10.0	10,17,12, 13.	6.5	22,9,16,23.	3.5	27,13,15,24.	2.5
1	114,91,103, 105.	17.2	52,53,39, 33.	5.5	7,3,9,6.	3.0	14,9,17,10.	2.5	16,23,15,9.	2.0

* Chemotactic index.

FIG. 15.

The chemotactic response of starved amoebae to bacterial cell walls.



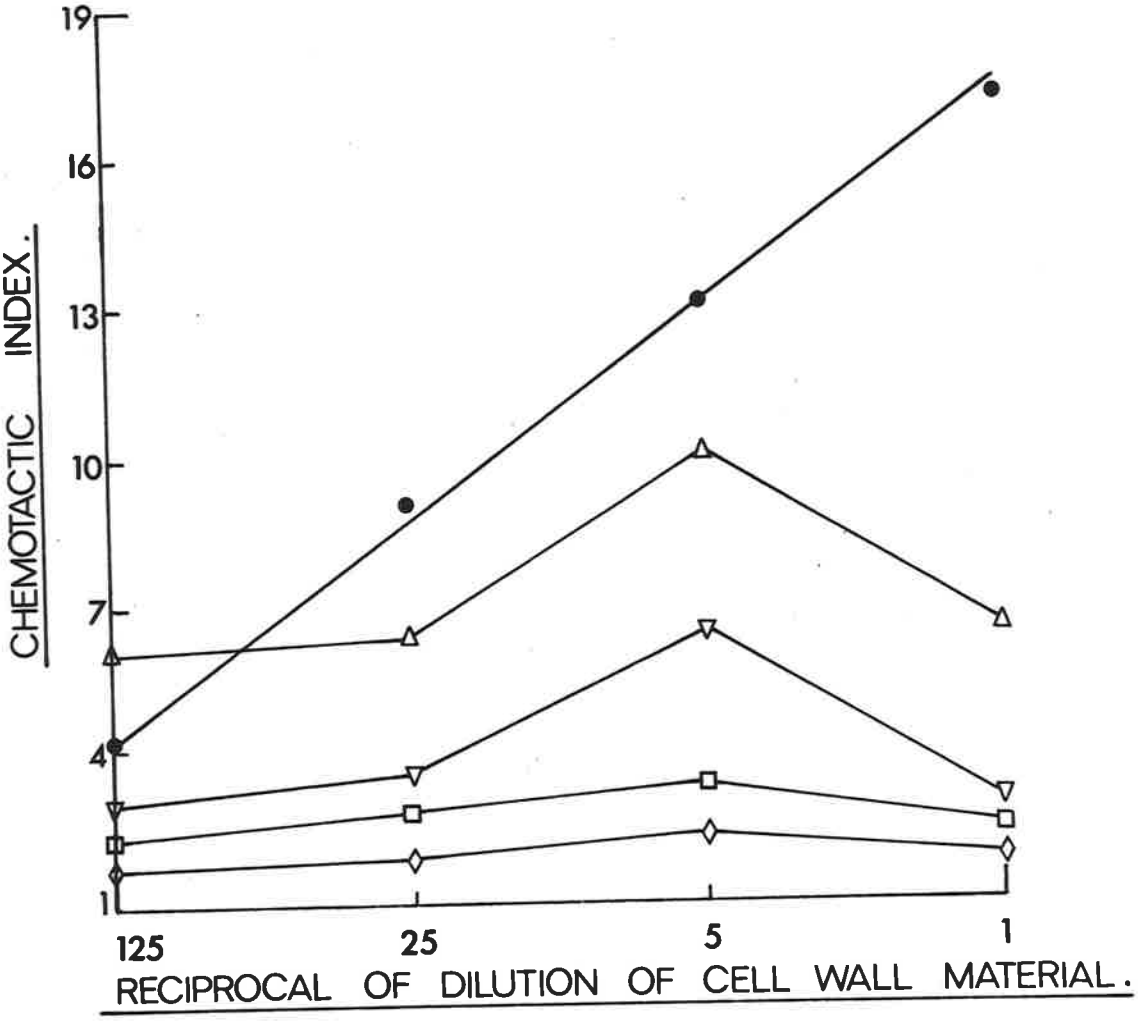
Normal amoebae.

One day starved amoebae.

Two day starved amoebae.

Three day starved amoebae.

Four day starved amcebae.


19
16
13
10
7
4
1
CHEMOTACTIC INDEX.
125
25
5
1
RECIPROCAL OF DILUTION OF CELL WALL MATERIAL.

cells. As was explained previously activity increases considerably during the early period of starvation.

4.5 The restoration of chemotactic responsiveness to starved amoebae

If amoebae which had been starved for three days were suspended in P.P.G. solution for a period of time longer than 24 hours, their chemotactic response to food particles began to return. After three days in this nutrient medium a considerable proportion of the original response was restored (Table 17, Fig. 16). However, after four days starvation, very little responsiveness was demonstrable, even after four days in P.P.G. Apparently the process of encystment had reached a 'stage of no return.' These observations parallel the findings of Weismann and Moore (1969). They found that when Acanthamoebae were placed in a starvation medium, their ability to take up latex beads progressively decreased as they proceeded toward encystment. A stage of this progression was reached when, even if replaced in growth medium, the cells were unable to regain their ability to phagocytose the beads, but proceeded to encyst. It was obvious that the process of differentiation had been initiated, and after this stage was not able to be reversed.

Apart from the ability of starved amoebae to regain normal chemotactic behaviour after a period of time in a nutrient medium,

TABLE 17

Restoration of chemotactic responsiveness to starved amoebae **

Recip. dilu of cell wall prepn.	Normal amoebae		Three day starved amoebae				Four day starved amoebae			
			Starved		Re-fed		Starved		Re-fed	
	Av. No. amoebae migrating/ membrane	C.I.*	Av. No. amoebae migrating/ membrane	C.I.	Av. No. amoebae migrating/ membrane	C.I.	Av. No. amoebae migrating/ membrane	C.I.	Av. No. amoebae migrating/ membrane	C.I.
No cell walls	5,7,8,5.		6,5,4,4.		2,5,3,6.		8,6,7,11.		10,13,6,8.	
125	27,31,20, 19.	4.0	17,13,11,9.	2.5	13,7,9,8.	2.3	10,18,15,20.	2.0	25,25,17,16.	2.3
25	47,58,56, 55.	9.0	17,14,21,9.	3.0	13,15,9,8.	2.8	22,13,19,16.	2.2	15,22,20,11.	1.9
5	92,71,69, 80.	13.0	22,9,16,23.	3.5	24,32,31, 28.	7.2	27,13,15,24.	2.5	19,37,24,24.	2.9
1	114,91,103, 105	17.2	14,9,17,10.	2.5	45,31,29, 42.	9.7	16,23,15,9.	2.0	17,29,21,19.	2.4

* Chemotactic Index

** Amoebae resuspended in P.P.G. for up to 4 days.

FIG. 16

The restoration of chemotactic responsiveness to starved amoebae following their resuspension in P.P.G.

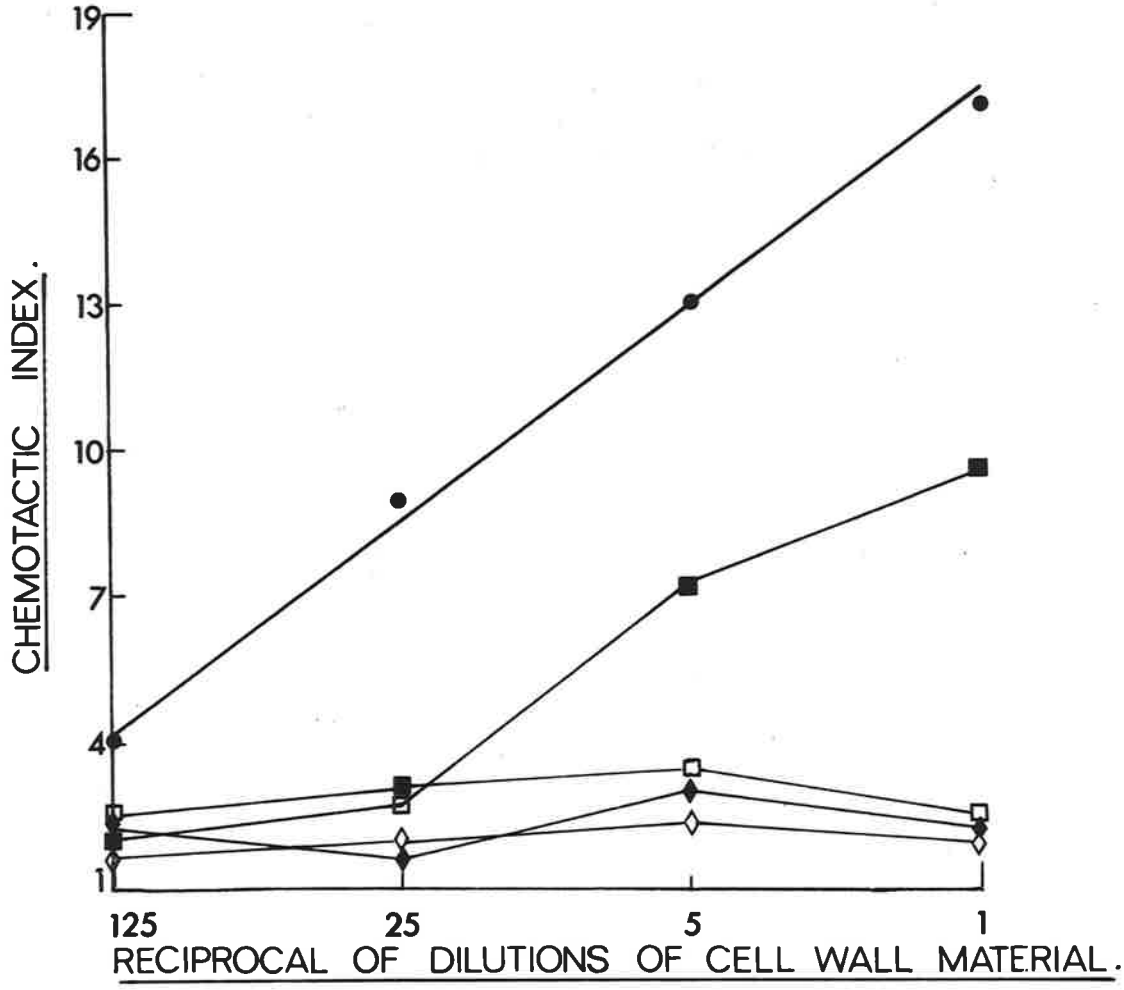
Normal amoebae.

Three day starved amoebae.

Three day starved amoebae after being resuspended in P.P.G. for three days.

Four day starved amoebae.

Four day starved amoebae after being resuspended in P.P.G. for four days.


19
16
13
10
7
4
1
CHEMOTACTIC INDEX.
125
25
5
1
RECIPROCAL OF DILUTIONS OF CELL WALL MATERIAL.

the normal chemotactic response may be restored in the following manner. Normal amoebae at a concentration of 5×10^6 ml. were washed twice in P.B.S. by centrifugation at 120 g. for 10 minutes, cooled to 4°, and disrupted by treatment in a small M.S.E. ultrasonic disintegrator for 45 seconds. Following this treatment, 2 ml. of the ultrasonicate were added to 10^{11} heat-killed well-washed bacteria and a suitable aliquot of the mixture placed in the test chamber. The supernatant of the mixture, after centrifugation at 12,000 g. for 20 minutes, was also tested for activity. The ultrasonicate of amoebae and the heat-killed bacteria at similar concentrations to those used in the experimental chambers, were tested separately in control chambers. The fact of major interest revealed by the data (Table 18) was that the response of starved amoebae was enhanced significantly compared with that in the controls. These findings indicated that the chemotactic principle was released as a result of an interaction between substances present in normal amoebae and heat-killed bacteria.

4.6 Production of the chemotactic factor

Normal, washed amoebae, at a concentration of 2×10^7/ml. were mixed with 5×10^{10} heat-killed well-washed bacteria/ml. and the mixture left for 10 minutes at 20°. Following this the mixture was centrifuged at 12,000 g. for 10 minutes and the supernatant filtered through millipore filters (Millipore Filter Corp., Bedford, Massachusetts,

TABLE 18

The chemotactic response of starved amoebae to products resulting from the interaction of an ultrasonicate of normal amoebae with heat-killed bacteria

Test substance	Av. no. of amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
Controls (P.B.S.)	4, 6, 2, 3	4	
5×10^{10} heat-killed bacteria per ml.	7, 4, 7, 5	6	1.5
Last washings of 5×10^{10} bacteria per ml.	3, 3, 1, 2	2	0.5
Ultrasonicate of 5×10^{6} amoebae per ml.	7, 13, 13, 11	11	2.7
Bacteria + ultrasonicate.	49, 40, 39, 35	41	10.2
Supernatant from bacteria + ultrasonicate.	27, 18, 23, 19	22	5.5

U.S.A.) of pore size 0.45 μ. The filtrate was then tested for chemotactic activity against starved amoebae. (Table 19). It is apparent from these results that the supernatant which contained neither bacteria nor amoebae was chemotactic to starved amoebae. Further tests confirmed that the ratio of amoebae to bacteria used in the above experiment resulted in the most efficient production of chemotactic factor.

Previous experiments suggested that starved amoebae could not elaborate the enzymes which reacted with the food source. It was therefore of interest to see if mixing starved amoebae with the food source resulted in the production of chemotactic factor. A sample of starved amoebae was mixed in a similar way with heat-killed bacteria at the same concentrations. Absolutely no chemotactic activity was induced in normal cells (Table 20). One aspect of the results from this section, which was a little disturbing, was the relatively poor response produced by what were presumed to be liberated chemotactic substances. The chemotactic index was only 3. This result became even more puzzling when it was found that the solution could be diluted 10^{-3} before the amoebae no longer responded (Table 21, Fig.17). One reason for this drop might possibly be due to the fact that a concentration gradient exists for only a short time. The concentration in the lower chamber is uniform and therefore a gradient would only be

TABLE 19

Effect of the products of the interaction of normal amoebae and heat-killed bacteria on the chemotactic response of starved amoebae

Test substance	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
Control	10, 13, 7, 13	11	
Last washings from 5×10^{10} heat-killed bacteria/ml.	15, 10, 10, 16	13	1.2
Last washings from 2×10^{7} amoebae/ml.	12, 13, 15, 9	12	1.1
Supernatant from mixture of bacteria and amoebae	33, 41, 32, 33	35	3.1

TABLE 20

Effect of the products of the interaction of 3-day starved amoebae* and heat-killed bacteria on the chemotactic response of normal amoebae

Test substance	Av. No. amoebae migrating/membrane	Av. for 4 chambers	Chemotactic index
Control	3, 6, 4, 3	4	
Last washings from 5×10^{10} heat-killed bacteria/ml.	7, 4, 4, 8	6	1.5
Last washings from 2×10^{7} starved amoebae/ml.	4, 2, 2, 3	3	0.7
Supernatant from mixture of starved amoebae + bacteria	3, 5, 4, 7	5	1.2

* At a concentration of 2×10^{7}/ml.

TABLE 21

Response of starved and normal amoebae to varying concentrations of chemotactic factors

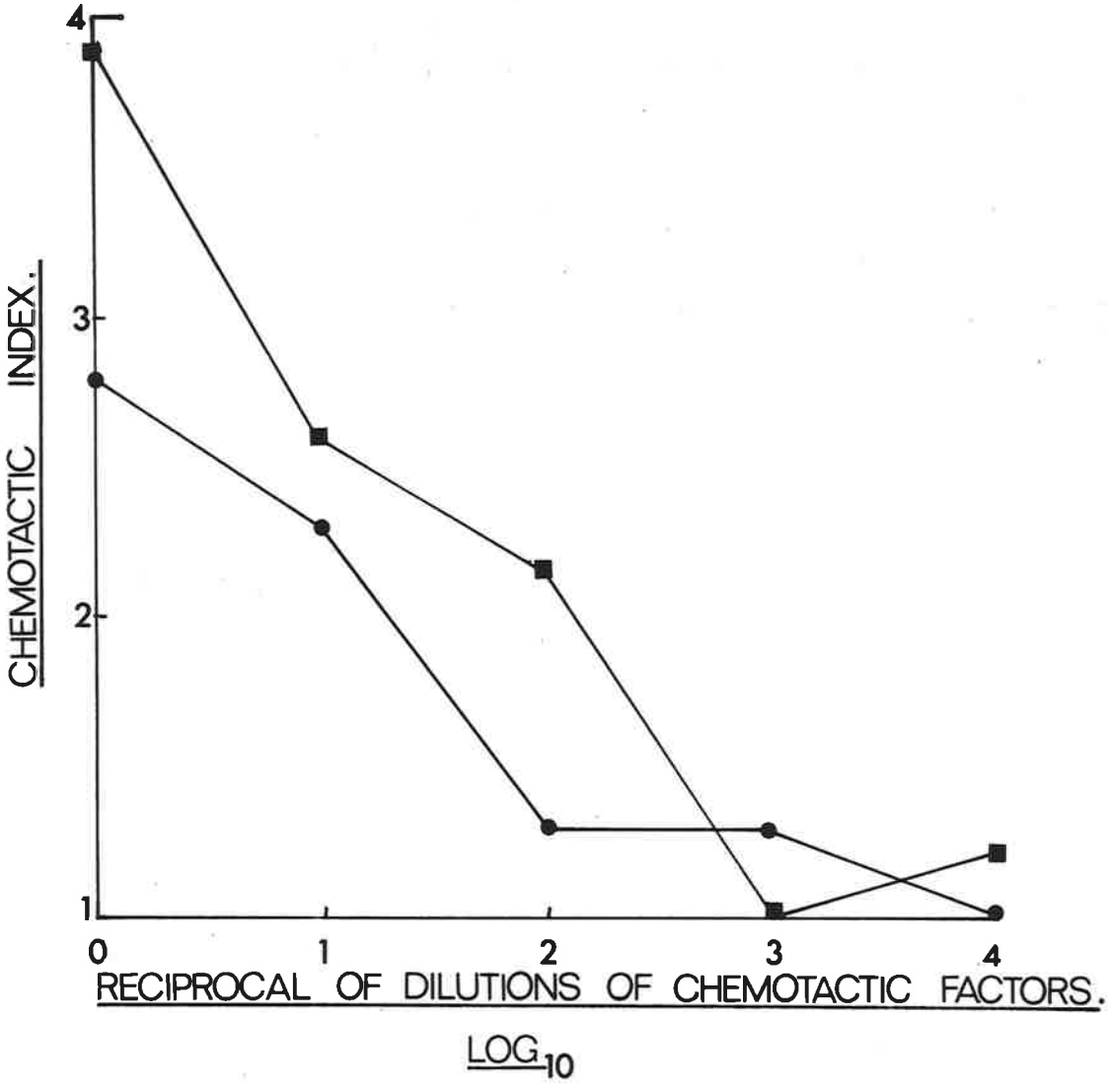
Reciprocal of dilutions of chemotactic factors	Normal amoebae	3 day starved amoebae
	Chemotactic index	
10^0	3.9	2.8
10^1	2.6	2.3
10^2	2.1	1.6
10^3	0.9	1.6
10^4	1.2	0.9

FIG. 17

Response of starved and normal amoebae to varying concentrations of chemotactic factors.

■——■ Normal amoebae.

●——● Three day starved amoebae.


4
3
2
1
CHEMOTACTIC INDEX.
0
1
2
3
4
RECIPROCAL OF DILUTIONS OF CHEMOTACTIC FACTORS.
LOG10

expected within the pores of the membrane. This is in contrast to the situation where one uses bacteria. Here one would expect varied gradients to be set up as the chemotactic factor was released from the bacteria and diffused into the culture fluid.

4.7 Other sources of chemotactic factor(s)

It would appear most improbable from the foregoing results that by-products of bacterial metabolism are able to initiate chemotaxis in amoebae. The chemotactic factor(s) appear to be produced only as the result of the interaction of substances from amoebae (probably enzymes) with the food source. However, during some of the experiments involving bacterial cell walls as a food source, it was noted that if the cell wall preparation was allowed to stand at room temperature for 24 hours, and then centrifuged at 27,000 g. for 30 minutes, the supernatant was chemotactic to starved amoebae. Further investigation showed that if viable bacteria suspended in P.B.S. at a concentration of 10^{10}/ml. were allowed to stand at room temperature for periods of up to 7 hours, then the supernatant of this suspension also was able to initiate chemotaxis in starved bacteria (Table 22). The supernatant from a similar concentration of well-washed, heat-killed bacteria did not possess the same property.

It was quite clear that the production of this chemotactic principle(s)

TABLE 22

The chemotactic properties of bacterial products for starved amoebae

Supernatant from 10^{10} viable bacteria/ml.	Chemotactic index
From a freshly washed suspension	0.8
After standing 1 hour	1.4
After standing 3 hours	1.9
After standing 5 hours	2.9
After standing 7 hours	2.7

Supernatant from 10^{10} heat-killed bacteria/ml.	Chemotactic index
From a freshly washed suspension	1.2
After standing 1 hour	0.7
After standing 3 hours	1.2
After standing 5 hours	1.5
After standing 7 hours	1.6

was dependent on time, since if freshly prepared cell walls, or freshly washed bacterial suspensions were again washed, the last washings were not chemotactic.

If the washed cell walls were heated at 56° or 100° for 30 minutes and left to stand at room temperature for three days, together with a similar sample that had not been heated, the supernatant of the sample heated at 100° did not contain chemotactic activity to starved amoebae. However, heating at 56° had not prevented the formation of chemotactic factors (see Table 23). These data suggested that an enzyme(s) present in the cell wall preparation was responsible for this result.

4.8 Discussion

The former results suggested a model of chemotaxis in the soil amoebae which involved the active participation of this protozoan. It appeared that chemotaxis was initiated by the release of certain factors, probably enzymes, from the amoeba, and these reacted with the food material to cause the release of a chemotactic factor(s) from the bacteria. The amoebae were thus able to move along an increasing concentration gradient of this substance to its source. The latter results appeared to be inconsistent with this hypothesis, suggesting that chemotactic substances may be produced over a period of time

TABLE 23

The effect of temperature on the production of chemotactic factors from bacterial cell walls

Treatment of cell wall preparation*	Chemotactic index**
No treatment	2.9
Heated at 56° for 30 minutes	3.4
Heated at 100° for 30 minutes	1.2

* Incubated for 3 days at room temperature following these treatments

** For 3-day starved amoebae.

even in bacterial cell wall suspensions of themselves. However, the earlier evidence that washed, killed bacteria were as chemotactic to normal soil amoebae as were viable organisms, though they were not so to starved amoebae, argued strongly in favour of the proposed model being of considerable significance. The further demonstration that these amoebic enzymes were capable of releasing this chemotactic material from heat-killed bacteria during a brief interaction between the two organisms also lent support to this model. Heat-killed bacteria, of themselves, were unable to generate chemotactic material over a considerable time period. However, the latter data do indicate that the enzymes involved in the release of the chemotactic factor(s) are not specific for amoebae. It is clear that chemotactic factors may be produced as the result of bacterial autolysis. In succeeding chapters some attempt will be made to purify and compare the properties of the chemotactic factor(s) produced by these two different processes.

CHAPTER 5

CHEMICAL AND PHYSICAL PROPERTIES OF THE CHEMOTACTIC FACTOR(S)

In the preceding chapter, substances chemotactic for the soil amoeba, Hartmannella rhysodes, were found to be produced as the result of the interaction of amoebae with heat-killed bacteria, the autolysis of bacteria, or from bacterial cell wall preparations.

Products of bacteria have also been found to be chemotactic for polymorphonuclear leucocytes (Ward, Lepow and Newman, 1968, Keller and Sorkin, 1967a) and for amoebae of the genus Dictyostelium (Konijn 1961). The former substance was described as being fairly heat-stable (50% loss in activity on heating at 56^{o} for 30 minutes) and with a molecular weight of about 3,000. Konijn et al. (1967) found evidence that the active substance in the latter case was cyclic 3'5' adenosine monophosphate.

In this chapter, the purification and some of the physical and chemical properties of the chemotactic factor will be described.

5.1 Stability to heat and freezing

The chemotactic factor was prepared by the interaction of amoebae with heat-killed bacteria as described in Chapter 4.7. After removing the cells by centrifugation, the supernatant containing

the chemotactic factor(s) was treated as follows. Starved amoebae were used throughout these experiments to test for chemotactic activity, since these amoebae were unable to react with the bacteria or their products to produce the chemotactic factor(s), but would respond to its presence if it was produced by other means.

One ml. samples of the supernatant were heated for 60 minutes at 100°, or repeatedly frozen and thawed several times. Table 24 and Fig. 18 show that no loss of activity occurred.

5.2 Effect of dialysis on chemotactic activity of the supernatant

Several 2 ml. samples of the supernatant were placed in dialysis tubing (Visking Dialysis Tubing, size 18/32) which allowed the passage of molecules of approximately 5,000 molecular weight. Dialysis took place in P.B.S. (μ = 0.06) at 4°, each sac being dialysed against 4 litres of phosphate buffered saline.

The P.B.S. was changed at 1 hour, 6 hours, and at 12 hours. Whereas at 5 hours no apparent loss in activity was measurable, at 16 hours, considerable activity had been lost from inside the bag, and at 30 hours no activity could be demonstrated in the original material. (Table 25). To show that this loss was due to dialysis and not to further degradation by enzymes, or to adsorption to the membrane, a further experiment was carried out. Five ml. of the supernatant in a

TABLE 24

The effect of heat and freezing on the chemotactic activity in the supernatant

Treatment of supernatant	Reciprocal of dilution		
	10^0	10^1	10^2
	Chemotactic index		
Untreated	2.6	2.1	1.5
Heated at 100^o for 60 minutes	2.9	2.6	1.6
Frozen and thawed	3.1	2.5	1.8

FIG. 18.

The effect of heat and freezing on the chemotactic activity in the supernatant.

Untreated supernatant.

Supernatant heated at 100^{o} for 60 minutes.

Supernatant subjected to repeated freezing and thawing.

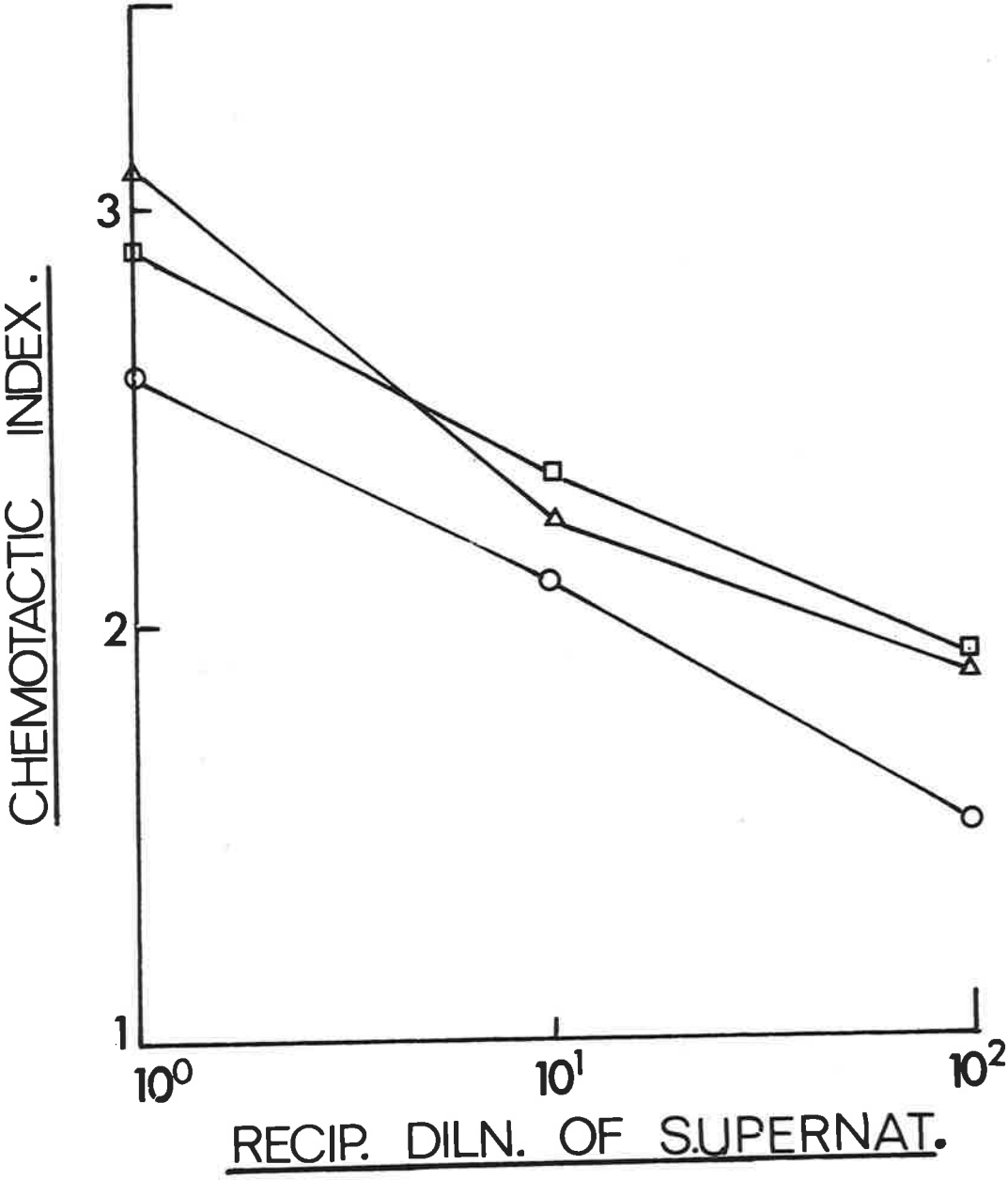

CHEMOTACTIC INDEX.
3
2
1
10^0
10^1
10^2
RECIP. DILN. OF SUPERNAT.

TABLE 25

The effect of dialysis on the chemotactic activity in the supernatant

Treatment of supernatant	Reciprocal of dilution		
	10^0	10^1	10^2
	Chemotactic index		
Untreated	2.7	2.3	1.7
Dialysis 5 hours (inside bag)	3.2	3.1	1.4
Dialysis 16 hours (inside bag)	1.7	1.0	1.0
Dialysis 30 hours (inside bag)	1.2	0.9	0.8
Dialysis for 24 hours with an equal volume of P.B.S.			
Inside bag	3.0	2.5	-
Outside bag	3.3	2.1	-

dialysis bag were placed in a large test tube containing 5 ml. of P.B.S. Chemotactic activity inside and outside the bag was found to be similar after 24 hours at 4°. This indicated that the loss of activity in the previous experiment was due to dialysis.

5.3 Gel filtration chromatography

Sephadex G-25.

It appeared from the preceding result that the active substance in the supernatant following the interaction between the organisms was of low molecular weight, possibly less than 5,000. Thus it appeared reasonable to use Sephadex G-25 for gel filtration studies of the substance. From such studies it was hoped to gain an approximate idea of the molecular weight. A sample of 10 ml. of the chemotactic factor(s) was concentrated by freeze drying, as described in Chapter 2.9a. After drying, water was added to give a volume of 1.5 ml. This solution contained a high concentration of salts. However, on diluting a small sample 10-fold, and testing its biological activity, it was found that the high salt concentration did not affect the chemotactic material. The concentrated material was placed on a column of Sephadex G-25 using P.B.S. as the buffer, as described in Chapter 2.9b. The eluate was collected in 2 ml. samples and these were examined in the ultraviolet range (220-300 mμ)

using a Hitachi Perkin-Elmer recording spectrophotometer with quartz cells (1 cm, volume 0.8 ml). Each sample from the column was tested for its chemotactic activity. It was quite clear from this work that it was important to test each sample over a series of dilutions, since in those tubes containing high concentrations of activity marked inhibition of chemotaxis occurred if these were tested undiluted. This aspect will be commented on later in the discussion. The results of this experiment are given in Table 26. It was clear from the ultraviolet spectrum of each fraction that where absorption occurred, it did so in the range 260-280 mμ. Absorption at these wavelengths indicates the presence of either certain peptides or nucleotides. A chart depicting absorption at these wavelengths and corresponding chemotactic activity (Fig. 19) showed that the peak of the chemotactic response coincided with the shoulder of one of the 260 mμ peaks. The spectrum of the most active fraction exhibited a peak of absorption at 256 mμ. This may have indicated that the chemotactic factors contained nucleic acid. However, as this separation was only on the basis of molecular weight, it was realised that the active material could be present in the nucleotide peak as a contaminant, and may perhaps be a completely unrelated compound.

5.4 Probable molecular weight of the chemotactic material

In view of the retardation of the chemotactic substance(s) on

TABLE 26

Chemotactic activity of eluate samples from the Sephadex G-25 column

Tube No. *	Reciprocal of dilutions		Tube No.	Reciprocal of dilutions	
	10^0	10^1		10^0	10^1
	Chemotactic Index			Chemotactic Index	
10	1.0	1.1	21	0.1	3.0
11	1.0	0.9	22	0.4	3.2
12	1.1	1.1	23	2.9	1.9
13	0.9	1.0	24	2.4	1.9
14	1.1	1.2	25	1.8	1.3
15	1.3	0.9	26	1.7	1.4
16	1.8	1.1	27	1.2	0.9
17	2.0	1.4	28	0.9	1.1
18	3.4	2.5	29	1.2	1.1
19	0.2	3.1	30	1.0	1.0
20	0	3.5			

* 2 ml. samples.

In the experiment, tubes 1-45 were examined for chemotactic activity, but only tubes on either side of the activity peak are considered in the above Table.

FIG. 19.

The elution of the concentrated sample of supernatant from a Sephadex G-25 column.

●------● Optical density of each sample at 260 mμ.

●——— ● Optical density of each sample at 280 mμ.

●———● Chemotactic Index (from a 1/10 dilution of the eluate sample - see Table 26).

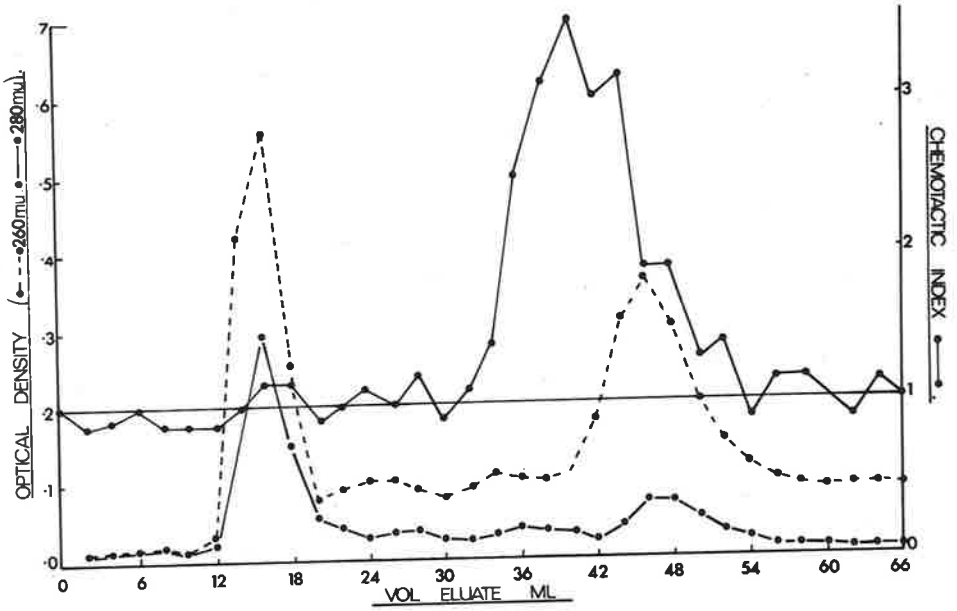
FIG. 20.

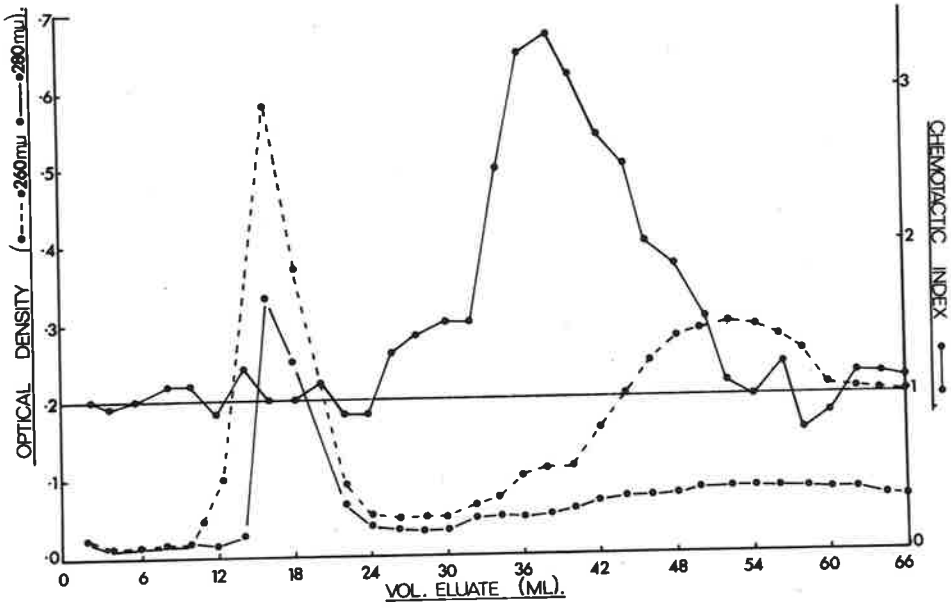
The elution of the concentrated sample of supernatant from a Biogel P-2 column.

●------● Optical density of each sample at 260 mμ.

●——— ● Optical density of each sample at 280 mμ.

●———● Chemotactic Index (from a 1/10 dilution of the eluate sample).


OPTICAL DENSITY (•---•260mμ •——•280mμ)
CHEMOTACTIC INDEX
VOL ELUATE ML
0
6
12
18
24
30
36
42
48
54
60
66


OPTICAL DENSITY (•---•260mμ •——•280mμ)
CHEMOTACTIC INDEX
VOL. ELUATE (ML).
0
6
12
18
24
30
36
42
48
54
60
66

Sephadex G-25, the column was calibrated according to the method given in Chapter 2.9b. From the pattern of elution of the chemotactic substance(s) compared with that of known molecular weight substances, they would appear to have a molecular weight of 400 (Fig. 21). However, it was conceivable that the material was retarded abnormally on the gel as was, for example, dinitrophenol. In order to check this possibility, the experiment was repeated using P2-Biogel. The results of this experiment are given in Fig. 20, where it may be seen that the chemotactic material again behaved as if it had a molecular weight of approximately 400 (Fig. 22).

5.5 Expression of chemotactic activity in terms of units

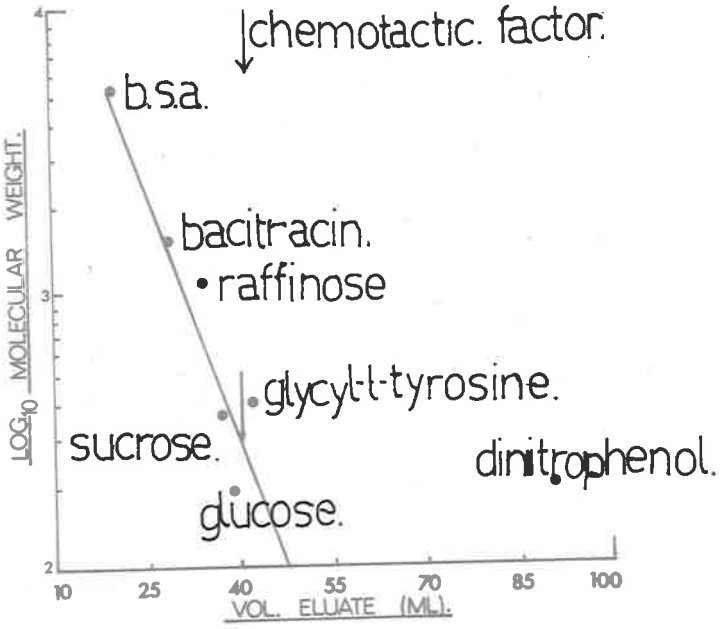
In order to quantitate recoveries of chemotactic activity following column chromatography, it was desirable to express activity in terms of units. It was clear that this presented some difficulties, since high concentrations of chemotactic material in the bottom of the chambers inhibited migration of amoebae. An experiment was designed to measure the chemotactic response of amoebae to various concentrations of the chemotactic material. Data are presented in Fig. 23. From the profile of the response it is obvious that one could choose two widely differing concentrations and obtain a similar response, e.g. 2^2 as compared with 2^7. The reason for this being that the higher concentrations are inhibitory and therefore as the

FIG. 21.

Determination of the molecular weight of the chemotactic factor(s) by chromatography on Sephadex G-25.

FIG. 22.

Determination of the molecular weight of the chemotactic factor(s) by chromatography on Biogel P-2.


chemotactic. factor.
b.s.a.
bacitracin.
raffinose
glycyl-l-tyrosine.
sucrose.
dinitrophenol.
glucose.
LOG10 MOLECULAR WEIGHT.
4
3
2
10
25
40
55
70
85
100
VOL. ELUATE (ML).

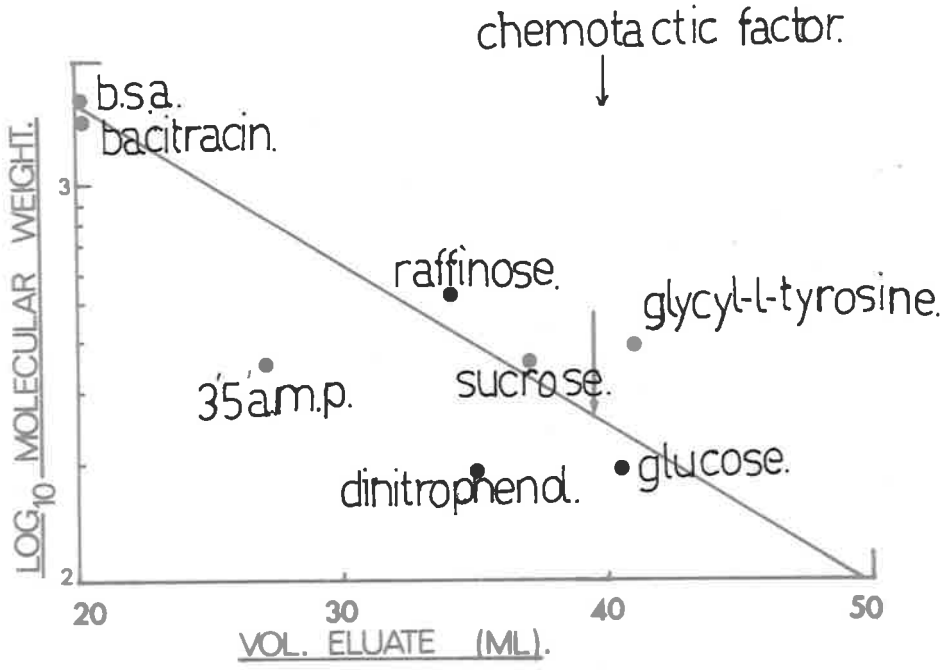
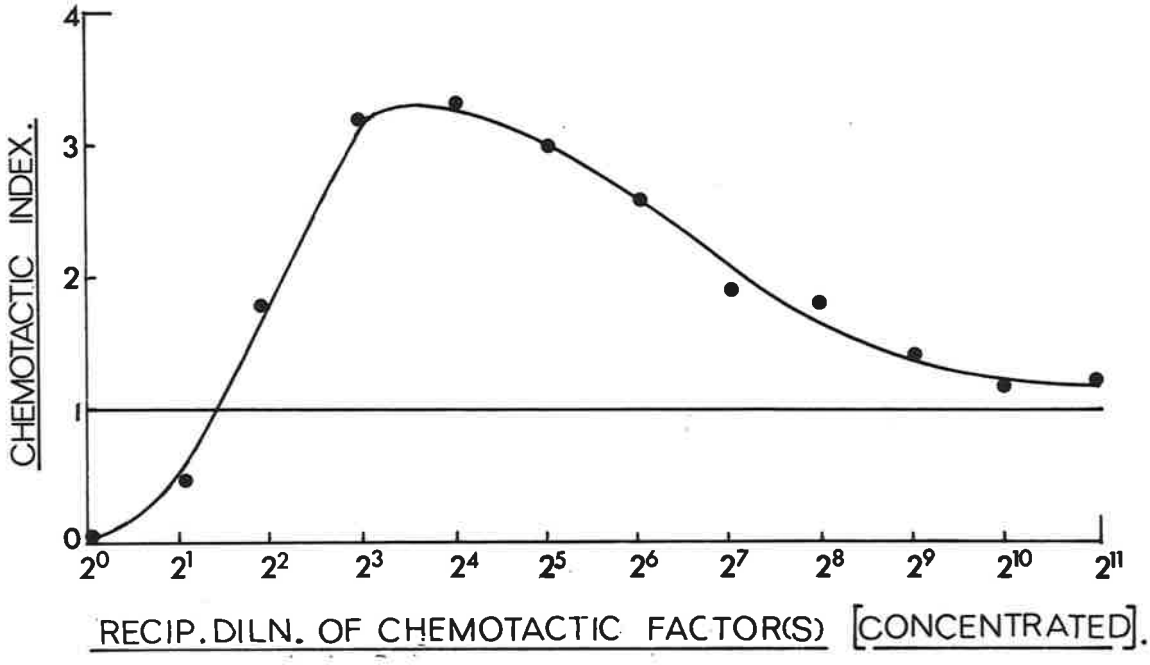

chemotactic factor.
b.s.a.
bacitracin.
raffinose.
glycyl-l-tyrosine.
3'5'a.m.p.
sucrose.
glucose.
dinitrophenol.
LOG10 MOLECULAR WEIGHT.
3
2
20
30
40
50
VOL. ELUATE (ML).

FIG. 23.

The response of starved amoebae to varying dilutions of chemotactic factor(s).


CHEMOTACTIC INDEX.
4
3
2
1
0
2^0 2^1 2^2 2^3 2^4 2^5 2^6 2^7 2^8 2^9 2^{10} 2^{11}
RECIP. DILN. OF CHEMOTACTIC FACTOR(S) [CONCENTRATED].

concentration falls the response reaches a maximum, to decline again as the concentration of chemotactic material becomes limiting. All the subsequent fractions were therefore tested over a wide range of dilutions and 1 unit of chemotactic activity defined as the amount of material that would give a chemotactic index of 2 following the decline from the maximum response. If one examines the profile in Fig. 23, this would occur at a dilution of 2^7, so that one could say that the original mixture contained 128 units of chemotactic activity.

5.6 Preparation of large amounts of chemotactic material for further purification studies

It was realized that considerable quantities of chemotactic material would be required for various purification procedures. Furthermore, it was necessary that the salt be removed from these preparations. To enable this to be achieved, a larger P2 Biogel Column was prepared as described in Chapter 2.9b, with the exception that the phosphate-saline buffer was replaced with water.

Whole amoebae, at a concentration of 2×10^7/ml. were mixed with well-washed heat-killed Pseudomonas fluorescens at a concentration of 5×10^{10}/ml. as described in Chapter 4.7. Two litres of the resulting supernatant containing approximately 6 units/ml. was concentrated by rotary evaporation to 30 ml. This resulted in the precipitation of a considerable amount of salt. The remaining fluid

was passed in batches of 5 ml. through the large P2-Biogel column, the salt being retarded more than the active material. Following each elution, the salt was washed from the column with excess water. Samples containing the active material (from each elution) were concentrated and again passed through the column. The 5 tubes containing the highest concentrations of chemotactic substances were concentrated by freeze drying to 2 ml. This preparation contained approximately 6,000 units of activity/ml. and was diluted to give lower concentrations as required.

At this point, while the approximate molecular weight of the chemotactic factor(s) present in these preparations was known, no evidence had been obtained regarding its chemical structure. Further experiments were designed to investigate this aspect.

5.7 Effect of acid and alkaline treatment on activity of the chemotactic factor

The molecular weight of the chemotactic material suggested the possibility that the active molecule may be a simple peptide, oligosaccharide, or a nucleotide. Such molecules are known to be hydrolysed with hot concentrated acid. The following tests were made on the chemotactic fraction.

Volumes of 0.5 ml. concentrated hydrochloric acid (11.2N) were added to three 0.5 ml. samples of the chemotactic substance

containing approximately 3,000 units. This high concentration of chemotactic material was used due to the dilution of this material required later in the experiment to bring the high salt concentration to a level suitable for the biological test. This high salt concentration resulted from the neutralization of the acid with sodium hydroxide. These samples were added to pyrex tubes and a stream of nitrogen gas used to remove the air. The tubes were then sealed under vacuum and heated at 110° overnight. On cooling, the samples were removed, neutralized with 12M sodium hydroxide, and diluted 125 times. It was obvious from Chapter 3.3d that salt concentration was a critical factor influencing the movement of amoebae. A sample of distilled water to which acid and base had been added at the same concentrations as above was also diluted 125 times and served as the control.

Chemotactic activity comparable to that in the original sample remained in the treated samples. (Table 27). This result indicated that the active molecule had an exceptionally stable structure. It was unlikely, therefore, that the chemotactic material was a peptide, oligosaccharide, glycopeptide, or a nucleotide.

Further 1 ml. samples of the chemotactic factor(s) at pH 11 (concentration 128 units) retained their activity after boiling for

TABLE 27

Effect of acid and alkali on the activity of the chemotactic factor(s)

Treatment of the chemotactic factor(s)	Units of activity
Heated with 6N HCl at 110° overnight	3,150
Untreated sample	3,125
Heated at pH 11 for 60 minutes at 100°	134
Untreated sample	125

These results are the average from 3 separate experiments.

60 minutes at 100° in sealed tubes (Table 27). In this case the alkali was neutralized and the solution diluted 8 times to give the right salt concentration for the biological assays. Again, the control consisted of a sample of distilled water to which acid and base at similar concentrations had been added, and this was diluted 8 times.

5.8 Ion exchange chromatography

It was clear from the results of gel chromatography using P2-Biogel that this method resulted in a considerable purification of the chemotactic material, though only on the basis of molecular weight. It was decided to further investigate whether the chemotactic substances were charged, and if so, to exploit this property in further purification work.

A 5 ml. sample of the chemotactic material containing approximately 640 units was passed through a D.E.A.E. A-25 column, which was prepared as described in Chapter 2.9c. A step-wise gradient of volatile buffers of decreasing pH and increasing molarity was used to elute adsorbed materials. A description of the buffers used is also given in Chapter 2.9c. Volatile buffers were used since the biological test necessitated the presence of low concentrations of salts ($u = 0.06$). The buffers could be completely removed by evaporation on a rotary evaporator, and the resultant solid matter redissolved in water.

In preliminary tests, due to the necessity of this procedure, the eluate was bulked into batches corresponding to the volumes of buffers used and each batch dried as described above. The solid matter resulting from each batch elution was redissolved in 5 ml. of P.B.S. The chemotactic activity was found to be eluted with 1M acetic acid (Table 28), indicating that it adsorbed quite strongly to the cellulose.

The experiment was repeated but this time the 1M acetic acid eluate was collected in a series of 10 ml. fractions. The contents of the individual tubes in this batch were dried and redissolved as described above and tested for chemotactic activity. These results are also given in Table 28. In each case for both experiments the fractions were examined for absorbance in the 220-300 mμ range. The resulting pattern of absorption at 260 mμ is presented in Fig. 24, together with the number of units of chemotactic activity in the various samples. Spectrophotometric analysis of the original sample of chemotactic material had shown that it absorbed most strongly in the 256-260 mμ range. Negligible absorption occurred in the 280 mμ area. Thus it is apparent from Fig. 24 that a considerable degree of purification may be achieved by this method. It resulted in the complete separation of the peak of chemotactic

TABLE 28

Ion exchange chromatography of the chemotactic factor(s)

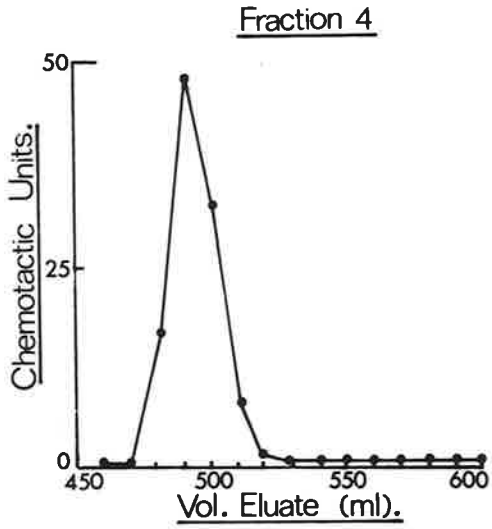
Batch elution		Activity in each sample of the active batch	
Batch No.	Units of activity	Tube No.	Units of activity
1		46	0
(0.025M, pH 6.5)	0	47	0
		48	80
2		49	240
(0.1M, pH 5)	0	50	160
		51	40
3		52	5
(0.5M, pH 4)	0	53	0
		54	0
4		55	0
(1M, pH 3.8)	560	56	0
		57	0
5		58	0
(2M, pH 3.2)	0	59	0
		60	0

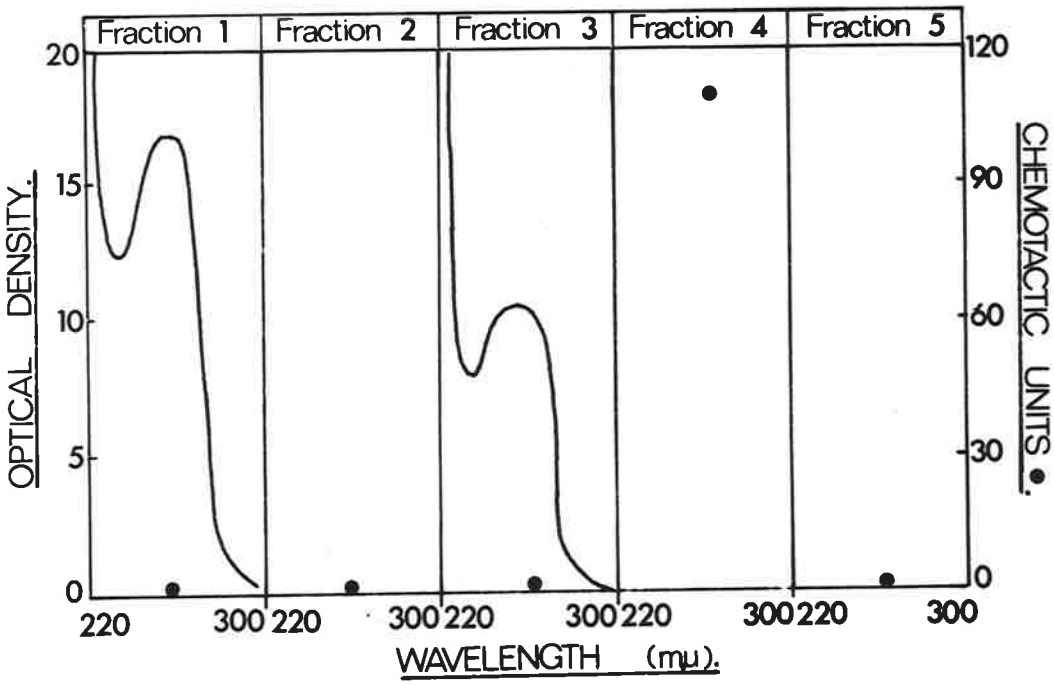
Concentration of original sample : 640 units.

FIG. 24.

Ion exchange chromatography of the chemotactic factor(s).

The ultraviolet absorption spectra (220-300 mμ range) for the individual batch fractions are presented in the lower figure, together with the number of units of chemotactic activity assayed in each fraction. The activity present in each 10 ml. sample of the active fraction following further purification is given in the upper graph.


Fraction 4
Chemotactic Units.
50
25
0
450
500
550
600
Vol. Eluate (ml).


Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
OPTICAL DENSITY.
CHEMOTACTIC UNITS ●.
20
15
10
5
0
120
90
60
30
0
220
300
WAVELENGTH (mμ).

activity from the nucleotide peak. A negligible loss of chemotactic activity occurred during this process (resultant activity approximately 560 units). The fact that 1M acetic acid was required to elute the adsorbed material suggested that the active molecule was strongly negatively charged.

5.9 Solubility of the chemotactic factor(s) in various organic solvents

A number of methods used for the purification of small molecules rely on the variable solubilities of these molecules in organic solvents. In the following experiments the solubility of the chemotactic molecule(s) in various organic solvents was determined.

Samples of 2 ml. of the chemotactic material dissolved in water at pH 2 and pH 10 (256 units) were extracted with either 20 ml. of ether or with a similar volume of chloroform. However, no evidence of chemotactic activity could be found in the ether or chloroform phases following evaporation and dissolving the extracted substances in 2 ml. of P.B.S. All the activity was recovered in the water phase. This result indicated that the active substance was unlikely to be a fatty acid.

Further 2 ml. samples of the chemotactic substance at a similar concentration were completely dried, using a freeze dryer (as described in Chapter 2.9a). Extractions of the dried samples were

carried out with 2 ml. volumes of either warm ethanol, methanol, butanol, acetone, benzene, or ethyl acetate. Particulate matter in the solvents was removed by centrifugation at 12,000 g. for 20 minutes in a Servall refrigerated centrifuge. After evaporation, the extracted materials were dissolved in 2 ml. of P.B.S. and chemotactic properties of each extract tested. Table 29 shows the amount of activity extracted in each case compared with that left in the tube. All the solvents except ethyl acetate extracted the chemotactic activity. Butanol appeared to be the solvent in which the substance(s) was most soluble.

Each solvent extract was analysed in the spectrophotometer over a range of wavelengths from 220-300 mμ. These data indicated (Fig. 25) that in all cases with the exception of methanol, the solvents had extracted material that absorbed in the ultraviolet. Since butanol extracted all the active material, leaving behind a considerable quantity of solid matter, this solvent was used in preliminary crude extractions of the chemotactic material. The results suggested, also, that it would be a useful solvent to use in paper chromatography. Methanol, on the other hand, did not extract as much active material under the above conditions but appeared to be more selective in that none of the extracted material absorbed in the ultraviolet. These two solvents were considered

TABLE 29

Solubility of the chemotactic factor(s) in organic solvents

Solvent used in extraction	Activity extracted (Units of activity)	Activity remaining (Units of activity)
Methanol	128	128
Ethanol	144	120
Butanol	248	0
Acetone	64	104
Benzene	72	116
Ethyl Acetate	0	250

Concentration of original sample : 256 units.

FIG. 25.

Absorption spectra of extracted materials following extraction of the chemotactic factor(s) with various organic solvents.

Extracted materials were dried and redissolved in water (2 ml.) before examination in the spectrophotometer. The spectra are of 1/25 dilutions of the individual 2 ml. samples.

A. Initial sample

B. Butanol extract

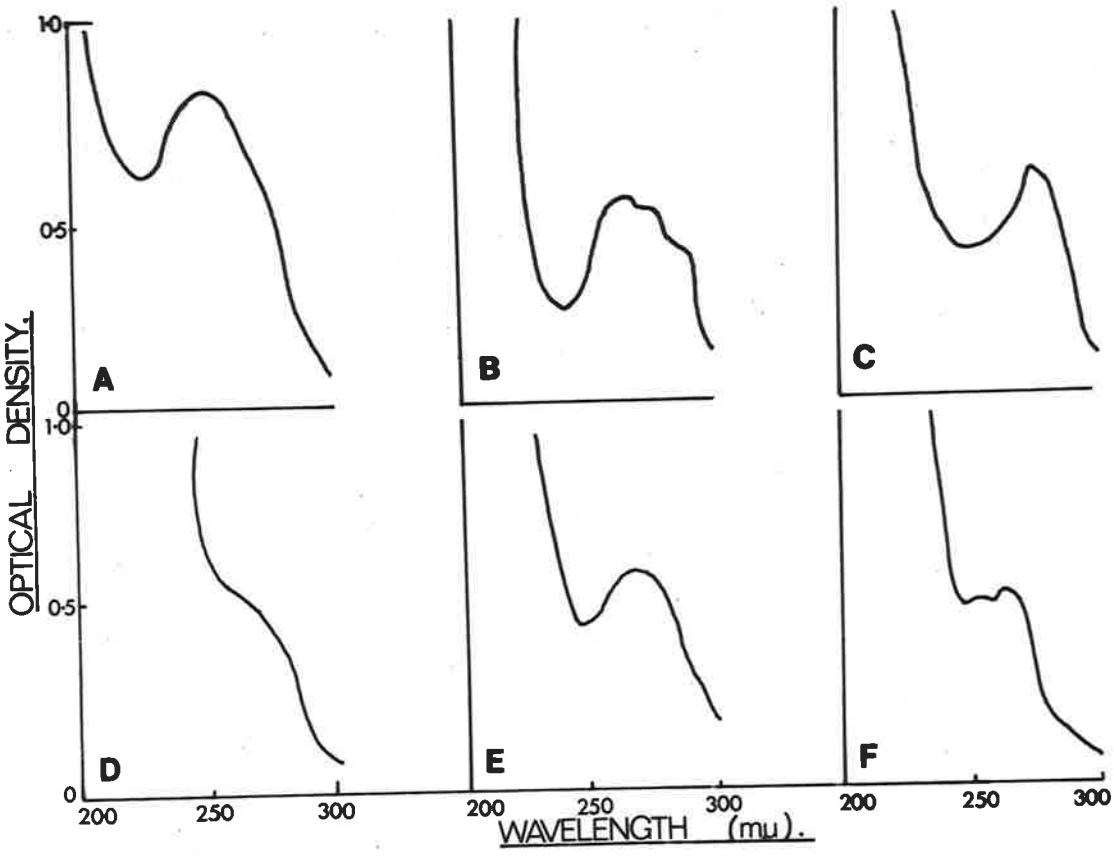
C. Ethanol extract

D. Benzene extract

E. Acetone extract

F. Ethyl acetate extract

No absorption could be detected over this range in the methanol extract.


OPTICAL DENSITY.
1·0
0·5
0
A
B
C
1·0
0·5
0
D
E
F
200
250
300
200
250
300
200
250
300
WAVELENGTH (mμ).

to be most potentially useful of the solvents for further purification work.

5.10 Paper chromatography

This method is used frequently in the final purification steps of small molecules. As the chemotactic factor was very soluble in butanol, two of the most commonly used solvent systems involving this solvent were used in preliminary experiments. These were :

(1) butanol, acetic acid, water (65 : 10 : 25)

(2) butanol, methyl ethyl ketone, formic acid, water (222 : 220 : 13 : 55)

Samples of 1 ml. containing 128 units of activity were freeze dried, suspended in 0.1 ml. of the solvents and these solutions placed on strips of chromatography paper (Whatman No. 3) as described in Chapter 2.9d. The strips were placed in the solvent tanks and removed when the solvent had reached within 1 cm. of the top of the paper strip. They were dried overnight at room temperature, and cut into ten sections of equal length. Phosphate buffered saline was used to elute the material from each section and the resultant solutions tested for chemotactic activity. The results (Table 30) showed that in both cases the activity was present at the solvent front.

These data suggest that ionization of the chemotactic molecule had been suppressed, resulting in the lypophilic properties which

TABLE 30

Migration of the chemotactic factor(s) in paper chromatography

Fraction No.	Solvent system		
	Butanol-acetic acid-water (65: 10 : 25)	Butanol-methyl ethyl ketone-formic acid-water (222:220: 13: 55)	Isopropanol-ammonia-water (7 : 2 : 1)
	Units of activity		
1	0	0	0
2	0	0	0
3	0	0	8
4	0	0	28
5	0	0	64
6	0	0	24
7	0	0	0
8	0	0	0
9	0	0	0
10 (Solvent front)	136	128	0
	Rf: 0.9 - 1.0	Rf: 0.9 - 1.0	Rf: 0.5

Concentration of original sample : 128 units.

caused its movement with the fast moving butanol phase. This suppression of ionization in the presence of acetic acid would suggest that the molecule possessed active acidic groups. Such a result would agree with the findings from ion exchange chromatography. If the molecule was acidic, one would expect its migration to be retarded in a solvent system containing alkali, where it would readily ionize. The solvent system chosen was :-

Isopropanol : conc. ammonium hydroxide : water (7 : 2 : 1).

When treated in a similar fashion as above the activity was found to have been retarded considerably as compared with that in the former systems (Table 30).

Spectrophotometric data using one of the solvent systems (butanol, acetic acid, water) indicated that a considerable degree of purification may also be achieved by this method (Fig. 26). The bulk of the ultraviolet absorbing material remained at the origin, a negligible amount of this material occurring in the active fraction.

5.11 Purification of the chemotactic factor

Based on the preceding evidence concerning the physical and chemical properties of the chemotactic factor, the final purification procedure was as follows. It involved four basic steps.

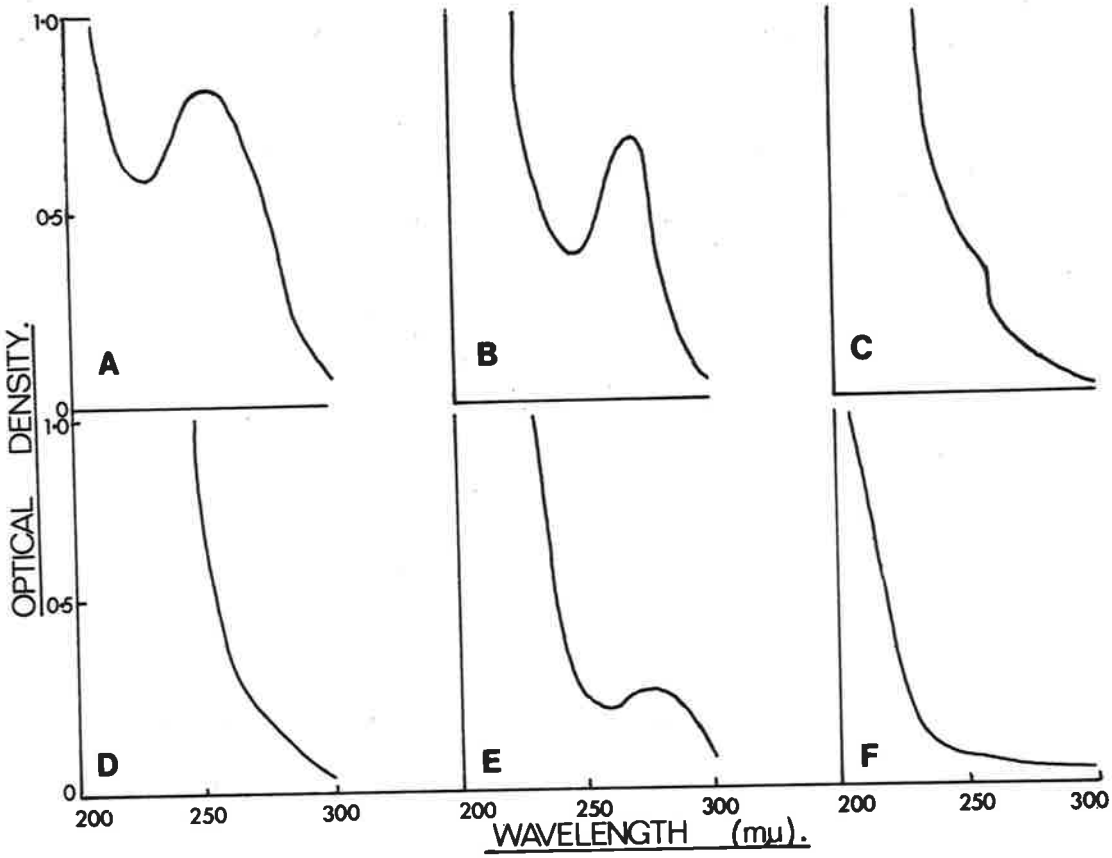
FIG. 26.

Absorption spectra of the fractions resulting from paper chromatography of the chemotactic factor(s).

Range of wavelengths for each spectrum : 200-300 mμ.

Each spectrum results from a 1/25 dilution of a fraction or combined fractions.

A.	Initial sample
B.	Fractions 1 - 3.
C.	Fractions 4, 5.
D.	Fractions 6, 7.
E.	Fractions 8, 9.
F.	Fractions 10.


OPTICAL DENSITY.
1·0
0·5
0
A
B
C
D
E
F
200
250
300
WAVELENGTH (mμ).

(i) Gel chromatography using P2-Biogel

↓

(ii) Active fractions dried and extracted once with butanol

↓

Butanol extract dried and extracted three times with methanol

↓

(iii) Methanol extract dried, dissolved in water and subjected to DEAE-A25 ion exchange chromatography

↓

(iv) Active fractions subjected to paper chromatography

Chemotactic materials were prepared as described in Chapter 4.7. Amoebae were washed with P.B.S. and suspended in this medium at a concentration of 2×10^7/ml. Bacteria (Pseudomonas fluorescens) which had been killed by heat (100° for 60 minutes) were washed several times in P.B.S. and suspended in the same medium at a concentration of 5×10^{10}/ml. Samples of 24 ml. of the bacterial suspension were centrifuged at 10,000 g. for 15 minutes and the supernatant discarded. A volume (24 ml.) of the suspension of amoebae was added to this pellet and the two organisms thoroughly mixed. The mixed suspension was allowed to stand at room temperature (20°) for 10 minutes, following which time the cells were removed by centrifugation at 10,000 g. for 20 minutes. The resulting supernatant was then subjected to filtration using a 0.45μ pore size filter (Millipore membrane Corp., U.S.A.). A volume of 600 ml. of the supernatant was prepared in this way, containing 6 units of chemotactic activity/ml.

(1) Gel filtration

The supernatant was concentrated by rotary evaporation to 30 ml. This was desalted in the preparative column (see Section 5.6), the active fractions concentrated again by rotary evaporation, and the resultant material passed through the large P2-Biogel column. The ultraviolet absorption pattern of the concentrated active fractions (in 5 ml. water) may be seen in Fig. 27. The resulting sample had a dry weight of 5.6 mg./ml. and an activity of approximately 640 units/ml. (Total 3,200 units).

(2) Extraction with Butanol and Methanol

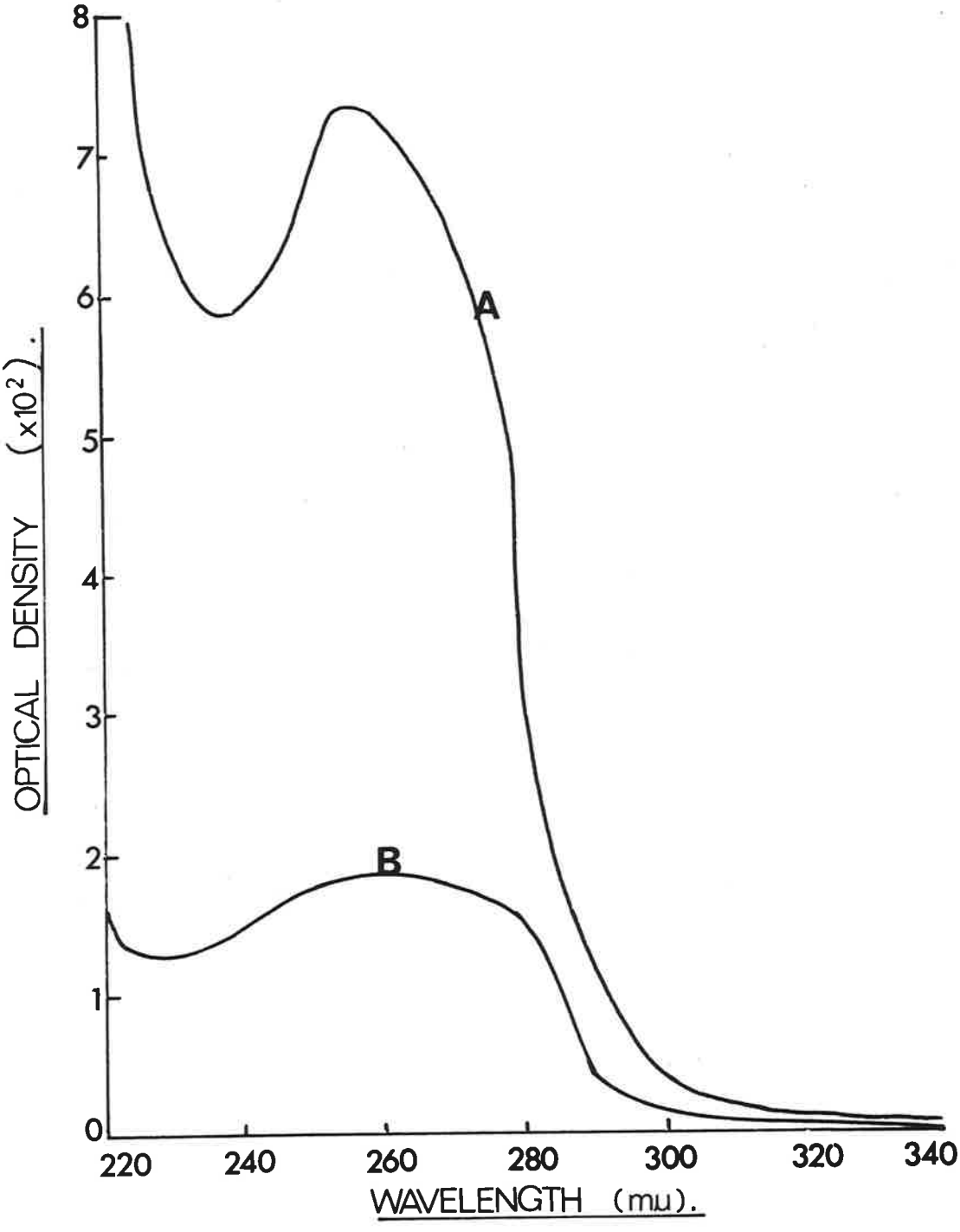
The active fraction was again dried by rotary evaporation to complete dryness and extracted with 30 ml. of hot butanol. The butanol extract was dried by rotary evaporation and the resulting solid matter extracted three times, each with 30 ml. of methanol. The methanol extracts were pooled and dried by evaporation on a warm plate. The dried material was then dissolved in 5 ml. of water. This material had an activity of 620 units/ml. (Total 3,100 units). It was apparent from the ultraviolet spectrum that there was a considerable reduction in the amount of ultraviolet absorbent material (Fig. 27). The dry weight of the sample was 1.5 mg./ml. thus indicating a four-fold increase in purity.

FIG. 27.

Absorption spectra of the solutions of chemotactic factor(s) following gel filtration chromatography and extraction with butanol and methanol.

A. Spectrum following gel filtration with P-2 Biogel.

B. Spectrum following further purification by extraction with butanol and methanol.


8
7
6
5
4
3
2
1
0
A
B
OPTICAL DENSITY (x10²).
220
240
260
280
300
320
340
WAVELENGTH (mμ).

(3) DEAE A-25 chromatography

The solution of chemotactic material (5 ml.) was placed on the DEAE A-25 ion exchange column and eluted as described earlier in this chapter (5.8). The samples in which maximum activity was found, i.e. samples 48 to 52, were pooled, the acetic acid removed by rotary evaporation, and the resultant material again dissolved in 5 ml. water. During this process all signs of absorption in the ultraviolet range were removed from the active fractions, while activity still remained high (600 units/ml. in 5 ml.). No dry weight of the active material could be detected in 1 ml. of the sample using a sensitive balance. However, it was noticed that if the sample was placed in a quartz cell in front of an ultraviolet light (together with a similar cell containing water), then the chemotactic material fluoresced strongly. Analysis of the fluorescence in a Farrand Optical Spectrofluorimeter showed a strong peak of fluorescence at between 400-420 mμ. Although only one peak of fluorescence appeared to be present in the purified sample, it was not certain whether the chemotactic molecule was present in pure form, and whether it was therefore this molecule which exhibited the fluorescent properties. To gain further evidence as to how pure the sample was, it was subjected to paper chromatography.

(4) Paper chromatography

Paper chromatography was carried out using the three solvent systems described earlier (Chapter 5). These were :

(i) Butanol, acetic acid, water (65 : 10 : 25)

(ii) Butanol, methyl ethyl ketone, formic acid, water (222 : 220 : 13 : 55)

(iii) Isopropanol, ammonium hydroxide, water (7 : 2 : 1)

The chemotactic material was concentrated to 1 ml. by boiling, and samples of 0.05 ml. placed on paper strips. After the chromatographs were run, the strips were cut into ten equal pieces and the material eluted with distilled water. Analysis with the Farrand Optical Spectrofluorimeter showed that for each solvent system two areas of fluorescence could be detected. The chemotactic activity coincided with one of these fluorescent fractions, having a fluorescent peak at 400 mμ in each case (Fig. 28). The other fluorescent peak was at 415 mμ and possessed a widely differing activation peak from that of the chemotactic material (see Fig. 28).

5.12 Discussion

Although the chemotactic factor was not chemically identified, it appeared that the foregoing procedures resulted in a considerable purification. Further, a certain amount of information was gained on the nature and properties of the chemotactic substance. Its

FIG. 28.

Fluorescent spectra and chemotactic activity of the various fractions resulting from paper chromatography.

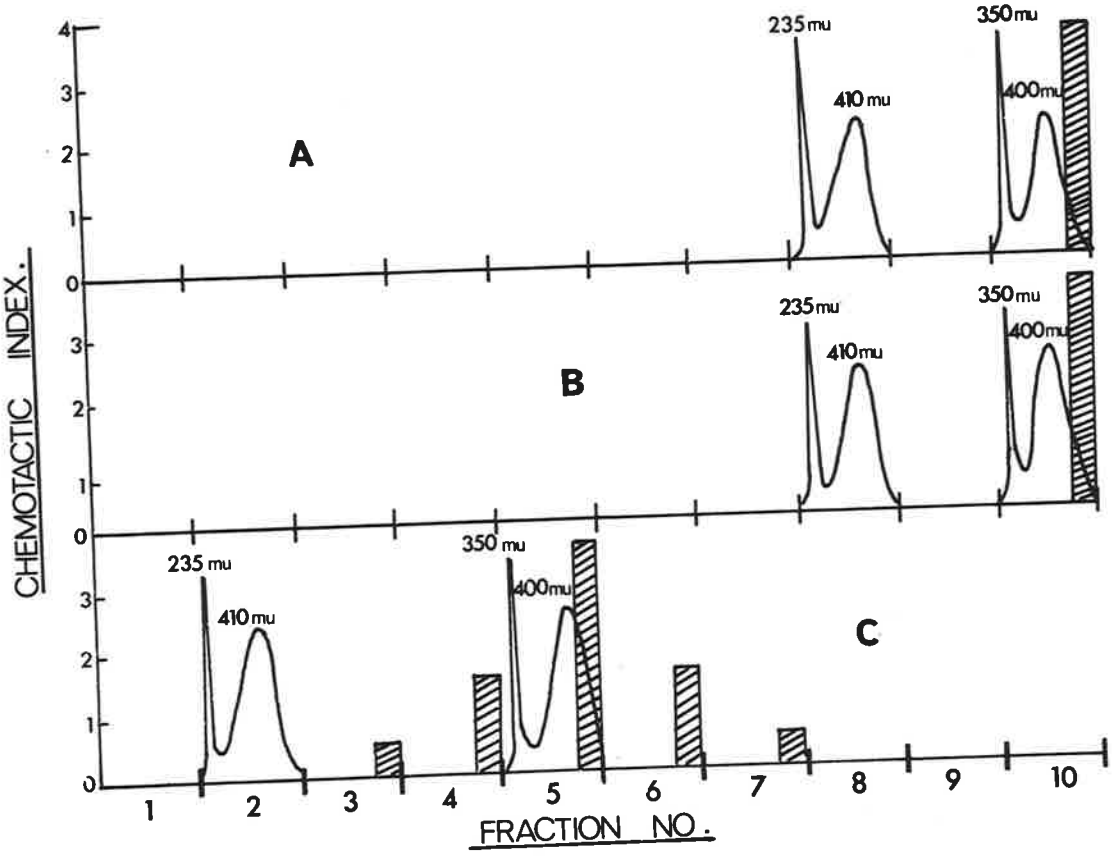
Chemotactic activity : 

A. Butanol-acetic acid-water (65 : 10 : 25)

B. Butanol-methyl ethyl ketone-formic acid-water (222 : 220 : 13 : 55)

C. Isopropanol-ammonium hydroxide-water (7 : 2 : 1)

The initial sharp peak in each fluorescent spectrum represents the activation peak, the latter blunt peak the fluorescent peak.


CHEMOTACTIC INDEX.
A
B
C
235 mu
410 mu
350 mu
400 mu
0
1
2
3
4
1
2
3
4
5
6
7
8
9
10
FRACTION NO.

resistance to hydrolysis by concentrated hydrochloric acid suggests that it is not a complex molecule with long side chains. Peptides or nucleotides are unlikely to survive such treatment. The anionic property suggested by ion exchange chromatography was also of significance, pointing to the presence of a strong carboxylic acid group in the structure of the molecule(s). Lack of solubility in ether or chloroform made it unlikely that a fatty acid could be involved. One property not previously discussed was that the chemotactic molecule adsorbed to a sample of Polyclar AT (insoluble polyvinyl-pyrrolidone manufactured by General Alinine and Film Corp., Dyestuff and Chemicals Division, N.Y., U.S.A.). This substance is known to irreversibly adsorb phenols (Gustavson, 1963). While this property may not be a very reliable criterion, it suggests (considering the other properties) that the chemotactic molecule was phenolic in nature. If this were so, the active substance might be expected to have a saturated or unsaturated ring structure with, among other short side chains, a carboxylic acid.

CHAPTER 6

THE OCCURRENCE OF SUBSTANCES CHEMOTACTIC TO SOIL AMOEBAE AND ASPECTS OF THEIR PRODUCTION

In a previous chapter (Chapter 4) it was found that if suspensions of viable bacteria were allowed to stand at room temperature for a period of time, chemotactic material was released into the suspending medium. In this chapter, the nature of this material will be examined and compared with that produced by the interaction of substances from amoebae with bacteria.

6.1 Production of the chemotactic factor(s) from Pseudomonas fluorescens

A suspension of viable Pseudomonas fluorescens (concentration 10^{10}/ml.) in distilled water was incubated for 48 hours at 30^{o}. The mixture was centrifuged at 27,000 g. for 30 minutes to remove bacteria and the supernatant concentrated tenfold. A sample of 1.5 ml. was placed on the analytical Biogel-P2 column. The results (Fig. 29) show the existence of two peaks of activity, one to which only the normal amoebae would respond (16-32 ml.) and the other to which both normal and starved cells responded (38-44 ml.).

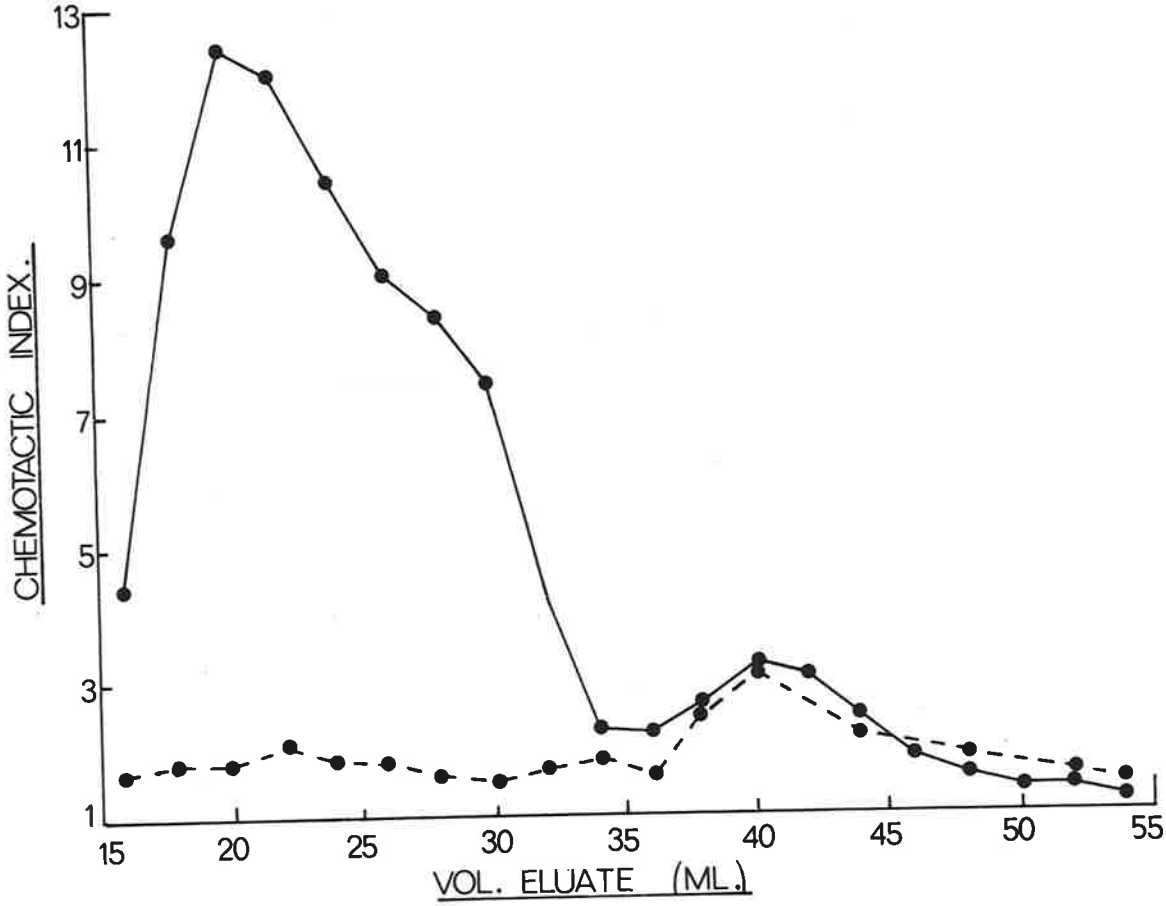
It was noted that the samples of eluate which contained material chemotactic for starved amoebae (38-44 ml.) corresponded to those in which chemotactic factor(s) produced as a result of the interaction

FIG. 29

The chemotactic response of normal and starved amoebae to bacterial products eluted from a column of P2-Biogel.

●——● Normal amoebae.

●– – –● Three day starved amoebae.


CHEMOTACTIC INDEX.
13
11
9
7
5
3
1
15
20
25
30
35
40
45
50
55
VOL. ELUATE (ML.)

of amoebae with heat-killed bacteria were eluted (see Chapter 5.3). Furthermore, it was necessary to dilute the most active fraction (40-42 ml.) 10^3 times before one could no longer detect a chemotactic response to this material (compare Chapter 4.6). Chemical tests on this chemotactic factor(s) showed that it resisted hydrolysis with strong hot acid, was eluted from DEAE A-25 cellulose with 1M acetic acid, and had the same solubility pattern in certain organic solvents as the factor(s) produced by the interaction of amoebae with heat-killed bacteria. Thus it appeared possible that factors produced directly by viable bacteria, and those produced as a result of the interaction of amoebae with heat-killed bacteria, were similar chemical substances.

6.2 "Substrate" for the production of chemotactic factor(s)

Since the large molecular weight factor was chemotactic only for normal amoebae, it suggested the possibility that this might contain the "substrate" from which the smaller material was split, the rationale behind this being dependent on the earlier experiments (Chapter 4). These experiments suggested that substances, possibly enzymes, from normal amoebae reacted with the bacteria to release the chemotactic material. Starved amoebae would not be able to react with the "substrate." To test this possibility 2×10^7 normal amoebae/ml. were suspended for 10 minutes at 20° in a sample of

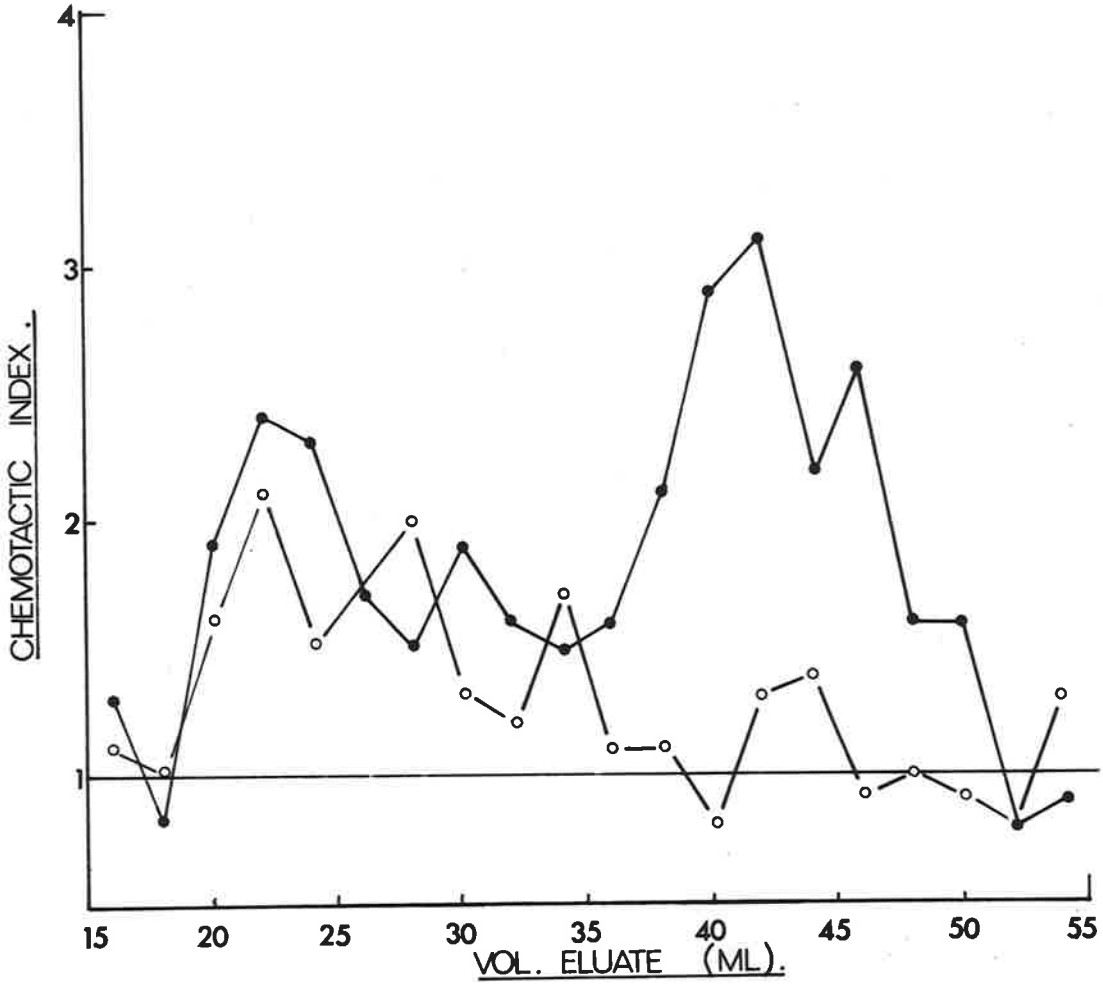
10 ml. of the high molecular weight material obtained from eluate samples 18-26 ml. in the previous experiment. These fractions had been previously heated at 100° for 60 minutes to inactivate any enzymes that may be present. Gel filtration (on Biogel P-2) of a sample of 1.5 ml. of the supernatant from this reaction resulted in the elution of small molecular weight material which was chemotactic for starved amoebae (Fig. 30). None of this material was produced when a sample of the large molecular weight fraction alone was eluted from the column. Also, the supernatant from a suspension of 2×10^{7} normal amoebae/ml. in P.B.S. which had been standing at 20° for 40 minutes, induced no chemotactic response in starved amoebae. It follows that the release of the small molecular weight chemotactic factor(s) as a result of enzymatic digestion of bacterial components following lysis is accompanied by the production of chemotactic "substrate" in a soluble form. Similar results were obtained when cell wall preparations were incubated at 20° for 24 hours. In contrast, no "substrate" appeared in the supernatant following the interaction of amoebae with heat-killed bacteria, indicating a certain degree of specificity in the action of the amoebic enzymes that split chemotactic factor(s) from its "substrate." (It appears from Fig. 30 that the samples of high molecular weight

FIG. 30

The chemotactic response of starved amoebae to factor(s) released by the interaction of normal amoebae with chemotactic "substrate".

●——● Substrate + amoebae (supernatant).

○ —— ○ Substrate.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material are to a limited extent chemotactic for starved amoebae. It is most probable that while starved amoebae have almost exhausted their ability to secrete extracellular enzymes, they still are able to produce small amounts of these substances until they eventually encyst. These would be able to split minute quantities of chemotactic factor(s) from substrate present in the test compartment of the Boyden chamber, resulting in negligible migration as compared to that detected using normal amoebae (see Fig. 29)).

6.3 Ability of other bacteria and their products to induce a chemotactic response in H. rhysodes

The only organism tested so far for its ability to induce a chemotactic response (using the Boyden chamber) in the soil amoeba, H. rhysodes has been Pseudomonas fluorescens. From the former results, it appeared that the substrate on which the amoebic or bacterial enzymes act to produce the chemotactic factor(s) was in the cell wall of the bacterium. To test the influence of cell wall surface antigenic characteristics on the chemotactic nature of a bacterium, a selection of representative chemotypes of Salmonellae from chemotype groups B, C, E and H (Kauffman, 1961) was made. Suspensions of these organisms in P.B.S. (concentration 10^{10}/ml.) were all chemotactic to a similar extent irrespective of their chemotype (Table 31).

TABLE 31

Chemotactic response of normal H. rhysodes to strains of Salmonellae possessing varying antigenic determinants

Chemo-type	Name of organism	Antigenic structure	Chemotactic index for normal amoebae
B	S. budapest	1, 4, 12	10.1
	S. typhimurium M206	1, 4, 5, 12	11.3
	S. typhimurium C5	1, 4, 5, 12	10.4
	S. typhimurium	4, 5, 12	9.7
C3	S. virginia	8	9.8
	S. kentucky	8, 20	10.1
E1	S. uganda	3, 10	11.2
	S. london	3, 10	11.4
H	S. onderstepoort	1, 6, 14, 25	10.6
	S. carrau	6, 14, 25	11.2

No. of organisms tested : 10^{10}/ml.

Further tests showed that a selection of gram-positive organisms, including Streptococcus pneumoniae, Staphylococcus albus, and Micrococcus lysodeikticus, which had been washed in P.B.S. and suspended in this medium at the same concentration, were also able to elicit a similar chemotactic response in normal soil amoebae.

Whether the chemotactic substance(s) liberated from gram-positive bacteria was similar to that from gram-negative, was not determined. However, a sample of chemotactic factor was made by interacting 2×10^7 amoebae/ml. with 5×10^{10} heat-killed Micrococcus lysodeikticus/ml. for 10 minutes at 20°. Gel filtration of this factor(s) on the Biogel-P2 column showed that it was eluted at the same region as that from Pseudomonas fluorescens. While the chemical characteristics of this molecule(s) were not examined, it seems not unreasonable to expect that the same, or similar, substances may be liberated from both gram-positive and gram-negative bacteria.

6.4 Demonstration of the production in lysates of amoebae of a factor(s) chemotactic for starved amoebae

During the investigation into the mechanism responsible for the chemotactic process (Chapter 4), it was discovered that products from lysed amoebae were chemotactic to normal and starved amoebae. To determine the approximate size of the factor(s) responsible for this

chemotaxis, 10 ml. of washed H. rhysodes (10^7/ml.) were subjected to ulstrasound for 45 seconds in an M.S.E. ultrasonic disintegrator. The resulting ultrasonicate was centrifuged at 27,000 g. for 30 minutes to remove remaining particulate matter. The remaining supernatant was concentrated by freeze drying, and placed on the P2-Biogel analytical column. The activity for starved amoebae peaked in the same region as did that of the original factor formed by the interaction of amoebae with bacteria (Fig. 31). Further properties of this factor were not investigated. Molecules having similar properties may have been responsible. If this was so, it would appear that the possession of a cell wall was not a necessary requirement for the production of this factor, amoebae having only a cell membrane. The most interesting aspect of this result was in the ability of lysed amoebae to produce substances to which whole specimens of the same cell responded chemotactically. This aspect will be discussed further in Section 6.7.

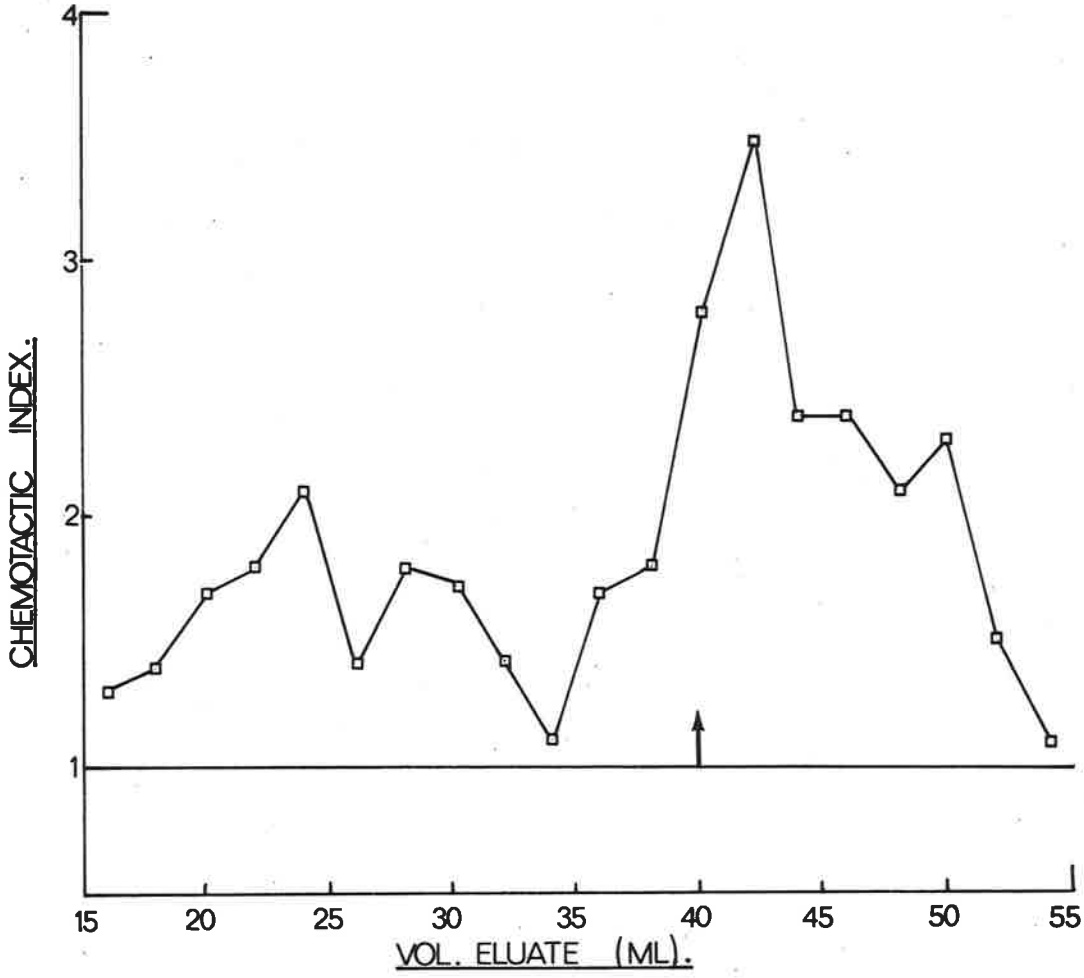
6.5 Substances tested for chemotactic properties during the course of the investigation

The finding that the chemotactic molecule must be a very fundamental unit, due to its resistance to concentrated acid hydrolysis, suggested that an amino acid, or simple sugar may be

FIG. 31

The chemotactic response of starved amoebae to lysates of normal amoebae.

The arrow indicates the position where the chemotactic factor resulting from the interaction of amoebae with heat-killed bacteria is eluted.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the active principle. Numerous small molecular weight substances were tested for their ability to induce chemotaxis in starved amoebae. However, none of the naturally occurring (L) amino acids, simple sugars, or sugar acids was active. Starch, which has been found to be chemotactic to other phagocytic cells (Harris, 1954) was also not active. The fern sperm attractant, maleic acid, also produced a negative result. During the course of the investigation it was reported that cyclic 3'5' adenosine monophosphate was the substance liberated by E. coli which was chemotactic to the slime mould amoebae (Konijn et al. 1967). Although it was realized that this molecule would be unlikely to withstand hydrolysis by concentrated acid, it was possible that it could be broken down by amoebae to substances chemotactic for soil amoebae. However, this was found not to be so. A further substance tested was the plant hormone, 3' indolylacetic acid (auxin) which is chemotrophic to plant cells at very low concentrations. This substance at concentrations of 10 mg./ml. inhibited the migration of the amoebae through the membrane, but it was found that dilution of the solution merely resulted in a return of the migration to a normal control level.

6.6 The lack of species specificity in the response of soil amoebae to the chemotactic factor(s)

Shaffer (1957) reported that the ability of cellular slime mould amoebae to respond to chemotactic substances demonstrated a genus specificity, while lacking species specificity.

Savanat (1965) found that H. astronyxis and Acanthamoeba would respond chemotactically to Pseudomonas fluorescens to the same extent as H. rhysodes, using the coverslip culture method described in Chapter 3. This was further confirmed using the modified Boyden chamber technique (Table 32), although it was observed that the larger cells H. astronyxis responded very slowly. It was found that these species also responded to the chemotactic factor(s) produced as a result of interaction between H. rhysodes amoebae and heat-killed Pseudomonas fluorescens (Table 32). Furthermore, a sample of the chemotactic factor(s) produced by the interaction of 2×10^7 Acanthamoebae with 5×10^{10} heat-killed Pseudomonas fluorescens/ml. also was chemotactic for all the species of amoebae to the same extent. P2-Biogel filtration of this factor(s) showed that it was also of a similar molecular weight to the factor(s) produced by the interaction of H.rhysodes with bacteria.

Although Acanthamoebae were originally classed in a genus differing from that of the Hartmannellid amoebae, they are obviously able to respond to similar chemotactic substances as the two

TABLE 32

Lack of specificity in the chemotactic response of three species of soil amoebae to chemotactic substances

Test substance	H. rhysodes	Acanthamoeba	H.astronyxis
	Chemotactic index (normal amoebae)		
Ps.fl. 5×10^{10}/ml.	13.3	14.7	14.9
10^{10}/ml.	10.1	11.4	12.2
2×10^{9}/ml.	7.5	7.9	8.4
H. rhysodes + bact. chemotactic factor(s).	3.2	3.1	3.2
Acanthamoeba + bact. chemotactic factor(s).	3.5	2.9	3.4
	Chemotactic index (starved amoebae)		
H. rhysodes + bact. chemotactic factor(s).	2.9	3.0	3.3
Acanthamoeba + bact. chemotactic factor(s).	3.2	3.1	3.4

Hartmannellids tested. However, this species is remarkably similar in taxonomic characteristics to H. rhysodes, and recently attempts have been made to re-classify it to the genus Hartmannella(Page, 1967). Thus this result may not indicate that similar substances are chemotactic to cells of two differing genera.

6.7 Discussion

The experiments concerned with the release of chemotactic factor(s) from lysed bacteria have shown that two substances differing in molecular weight are chemotactic to normal amoebae. However, the observation that only one, the small molecular factor(s), is chemotactic to starved amoebae, suggests that normal amoebae are able to produce this factor(s) from the larger molecular weight compound. Therefore, one could regard the larger molecular weight compound as the substrate from which the smaller factor(s) is split. Starved amoebae probably lack suitable concentrations of enzymes to perform this task. This suggestion is supported by the experiments showing that normal amoebae, when mixed with this 'substrate' produce a small molecular weight factor(s) which is chemotactic to starved amoebae. Starved amoebae are unable to do this. It is also interesting to note that if the normal amoebae are mixed with bacteria only the small molecular weight factor(s) appears to be released.

The fact that amoebae are able to respond chemotactically to products of the lysis of cells of their own genotype is of considerable interest in relation to the ability of phagocytic cells to discriminate between foreign matter or altered self, and functional self. Hurley (1964) has presented good evidence that mammalian phagocytes are also able to react chemotactically to disrupted tissues from their host organism, though only in the presence of fresh serum. The process whereby such chemotactic recognition between healthy amoebae of similar genotype is prevented, despite the possession by each individual cell of the substrate for the production of chemotactic factors, may provide an intriguing model for the study of the ability of phagocytic cells in general to discriminate self from foreign matter.

CHAPTER 7

DISCUSSION

Despite the apparent similarities in the ability of metazoan and protozoan phagocytic cells to recognize and take up foreign matter, the use of a species of soil amoeba as a model for metazoan phagocyte activity may seem extravagant. However, studies of the physiology of primitive metazoans have revealed that the transition of the phagocytic process during evolution, from one of nutrition and defence to one solely of defence, was a very gradual one. In many primitive metazoans, phagocytosis of microorganisms appears to be both a means of food intake and protection against invasion. Metchnikoff (1893) was the first to recognize this fact. He was able to trace the continuity of phagocytic and digestive activity from the amoeba, in which nutrition and destruction or elimination of potentially harmful microorganisms are inseparable functions within a single cell, to the sponges, which possess two types of phagocytic cells. The inner food canal in sponges is lined by a loose layer of endodermal phagocytes (choanocytes) which capture food, including bacteria, and these pass the captured material on to the mesodermal phagocytes (amoebocytes) which either digest or reject the material. The evolution of the digestive system to a tract whose lumen is essentially outside the organism has progressively decreased the importance of the role of the phagocytic cell in nutrition. The liberation of

enzymes into this space enables, as it were, extracellular digestion to occur. Thus the amoeboid phagocytes within the body cavities and tissues have been left purely a protective role with respect to the rest of the organism. It seems reasonable to expect that there may be common processes involved in the recognition of pathogens and other foreign matter by metazoan phagocytes and in the recognition of food material by protozoan phagocytes.

The part played by chemotaxis in the recognition of foreign matter has been studied for some time. In the metazoans, the accumulation of large numbers of phagocytes at sites of inflammation led earlier workers to believe that some attractive force emanating from these sites was responsible. Metchnikoff (1893) proposed that the ability of phagocytes to move towards and engulf foreign matter endowed them with the primary responsibility of defending the animal against infection. However, despite a considerable amount of investigation into the phenomenon during the first half of the century, little positive information on the substances or mechanisms involved in chemotaxis was available by the beginning of the 1960s. As will be evident from the review of literature on chemotaxis (Chapter 1), difficulties have arisen from the lack of a suitable reliable quantitative technique. The introduction of such a technique by Boyden (1962a) gave

considerable stimulus to the *in vitro* study of chemotaxis in vertebrate phagocytic cell systems, but to date no studies of this process have been carried out on the haemocytes and amoebocytes of invertebrates using this method. Despite the use of the Boyden technique, it is evident that a complete understanding of the phenomenon of chemotaxis has not yet been achieved. One of the disturbing aspects of these studies has been the conflicting results arising from investigations into supposedly similar processes (Ward, Cochrane and Muller-Eberhard, 1966; Stecker and Sorkin, 1969).

The study of the ability of protozoan phagocytes to recognize the presence of foreign material in their environment has received comparatively little attention. In the present study, modification of the Boyden chamber technique has provided a useful and reliable method to study chemotaxis in soil amoebae. This technique has considerable advantages over others in which movement of amoebae towards food material either in liquid culture (on glass) or on an agar surface has been studied. Quantitation of the latter systems has only been obtained by varying the distances between the amoebae and the stimulus. These methods allow only very coarse measurements of chemotaxis to be made. It is also very difficult to test soluble substances using these systems, although Konijn in his studies on

the chemotaxis of slime mould amoebae (Konijn et al. 1967) has developed a method using hydrophobic agar which enables him to attain a reasonable degree of quantitation. It is interesting to note that even in the study of chemotaxis in the slime moulds, which Bonner began to investigate during the 1940s, one of the main factors responsible for the slow progress was the unreliability of the techniques used to demonstrate the phenomenon in these protozoans. Bonner himself alludes to this fact in his reminiscences on the researches which led over a period of 20 years to the discovery of cyclic 3'5' adenosine monophosphate as an acrasin for amoebae of the genus Dictyosteliae (Konijn et al. 1968).

In the preliminary stages of this investigation, a technique involving food selection by amoebae was studied in the hope that it would be useful for the demonstration and measurement of chemotaxis. However, it was concluded that this method merely measured the cytotoxic effects of bacterial by-products on the protozoans. At the outset of the experiments using the Boyden chamber, it was considered that increased migration of amoebae through the membranes may result from stimulation of random movement (chemokinesis) by the test substances. However, that one was demonstrating chemotaxis of amoebae, i.e. the directional movement of cells along a concentration

gradient of chemical substances, rather than chemokinesis, was confirmed by the following experimental results. It was found that placing increasing concentrations of the chemotactic material in the top chamber caused progressive inhibition of migration through the membrane. If the effect of the chemotactic factor(s) was merely to increase random movement of the amoebae, then the addition of this material to the top chamber should have increased migration.

Reproducible results were obtained with the modified Boyden chamber providing certain precautionary measures were taken in the experiments. These measures included the use of cultures of amoebae which had been maintained only three to four weeks since the last subculture, the maintenance of constant temperature (20^{o}) and constant humidity, the careful estimation of concentrations of test samples and suspensions of amoebae, and the use of additional control chambers to determine the rate of progress of each particular experiment, and thus the time at which the experiment should be terminated. The resulting constant migration figures from the controls enabled a meaningful comparison to be made between results from different experiments. The fact that the result from each concentration of test substance and from the controls was the average from four chambers, also contributed to this reproducibility. The adoption of

the chemotactic index, in which migration towards the test substance was considered relative to that in the control, enabled the detection of inhibition of migration.

In some experiments during this investigation, it was found that very high concentrations of chemotactic substances in the test compartments would inhibit the migration of amoebae through the membranes. This was seen with both the chemotactic "substrate" and the isolated chemotactic factor(s). It has been stated in Chapter 4 that inhibition was probably due to high concentrations of test substances passing through the pores, causing local concentrations of chemotactic factor(s) of such strength as to inhibit the movement of amoebae through the membrane. Such concentrations may saturate receptors on the amoeba surface so as to render the cell unable to detect a gradient. Ward and Becker (1968) found that suspensions of leucocytes lost their ability to react to gradients of chemotactic substances after they had been suspended in a solution of these substances for 30 minutes and subsequently washed in normal culture medium. Bell and Jeon (1962) have reported that heparin was chemotactic to Amoeba proteus up to a certain concentration, and above this their movement was inhibited.

Perhaps the most significant observation in this present study has

been that this species of soil amoeba may release substances, probably enzymes, which react with the food material to release a chemotactic factor(s). This appears to be the first evidence for such a system in the amoebae. Evidence indicating this reaction followed the observation that killed bacteria and bacterial cell walls are able to initiate a chemotactic response in normal amoebae similar to that given by viable bacteria. The data from studies with starved amoebae gave stronger support to this possibility. Starvation progressively reduced the ability of amoebae to release chemotactic factor(s) from the "substrate". However, they still retained the ability to respond to these factor(s) formed as a result of the interaction of whole normal amoebae with the "substrate".

The reason is not known why amoebae lose their ability to respond chemotactically to food bacteria when they are suspended in saline solutions for certain periods of time. It is probable that the cells gradually exhaust the supply of materials needed for the synthesis of the enzymes which cause the liberation of chemotactic substances from bacteria. Phosphate ions are known to increase considerably the rate of mitosis in soil amoebae and it could be argued that cellular activity in this medium is directed more fully to mitosis than to any other activity. However, it is clear that the presence of

phosphate ions in the medium does not in any way account for the loss of the responsiveness of amoebae to the presence of bacteria, since similar results were obtained when the cells were suspended in a saline solution at the same ionic concentration as the phosphate buffered saline.

The physical and chemical properties of the chemotactic substance(s) released from bacteria indicate a molecule or a small group of molecules which have a molecular weight of about 400. They have a stable structure and are negatively charged, probably due to the possession of strong carboxylic acid groups. The substance is certainly not volatile, and would appear to have a characteristic fluorescent absorption peak. The most commonly occurring amino acids and sugars are not chemotactic. It is quite clear that the substance is very active at low concentrations, and possibly phenolic in nature. With these properties in mind, one is immediately led to think of substances formed as the by-products of aromatic amino acid metabolism. Examples of such by-products are indolylacetic acid (the plant hormone, also called auxin) and a number of animal hormones which are very active at the site of the cell membrane. Substances such as indolylacetic acid are chemotrophic to plants at concentrations of less than 1 mμ M. The fact that the chemotactic substance is

produced in culture filtrates and that it may be released from all bacteria tested, as well as from amoeba lysates, suggests that it may be a common metabolic product of these organisms. If the substance were a phenolic compound, as was suggested by its absorption to Polyclar AT, then such compounds are known to adsorb non-specifically to proteins and other large molecular weight substances. It is, therefore, possible that the active molecule(s) may be a metabolic product adsorbed to components of the bacterial cell wall. The strong negative charge would facilitate such adsorption. Enzymes may be necessary to release this substance by disrupting the molecule on which the chemotactic factor is adsorbed. The fact that the substances responsible for the release of the chemotactic factor from bacterial cell walls were temperature-sensitive suggested that enzymes were involved. If bacteria are of themselves able to effect the release of this factor(s), though over a much longer time period than taken by the amoebae, then it would seem that the enzymes required for this release are common to both bacteria and amoebae. However, these facts do not argue against the chemotactic process being mediated by a specific substance. This is supported by the fact that a large number of purified products tested for chemotactic properties were not active.

One disturbing aspect of the results obtained using this modified Boyden technique was their disagreement with the results obtained by Savanat (1965) using the coverslip culture technique for demonstrating chemotaxis (see Chapter 2). Savanat found that killing bacteria by heat, alcohol, or streptomycin completely eliminated their chemotactic properties for amoebae. In this present work, bacteria treated in any of these ways were found to elicit as strong a chemotactic response as did viable cells. Disagreement has also occurred between Harris (1954, 1961) and Hurley (1964) in the study of chemotaxis in leucocytes. Harris claimed that vertebrate phagocytes would not respond by chemotaxis to products of disrupted or injured tissue, following his tests using coverslip cultures of these phagocytic cells. In contrast, Hurley, using Boyden's technique, demonstrated that such damaged tissues initiated a chemotactic response in these same cells. In the case of the amoebae it is possible that the Boyden chamber technique may be more sensitive than others, enabling the detection of low levels of chemotactic activity, while only a very strong response can be discerned using the more qualitative techniques. In the Boyden technique, chemotaxis results from the interaction at fairly close quarters of a large number of amoebae with a large number of bacteria. The distance between the two masses of cells is determined only by

the thickness of the membrane. In the coverslip culture technique, only a very small number of amoebae are involved, and these are at varying distances from the mass of test organisms. Thus the concentration of chemotactic factor(s) produced may be very low. The conditions of the Boyden test chamber allow many amoebae to interact with the food source, with a consequent increase in the concentration of chemotactic substance(s). Another important aspect of this technique is that chemotactic substances will be produced from a number of point sources, such that the whole population of amoebae will be involved in the chemotactic response compared with the few in the vicinity of the food source in the other technique.

In the experiments on the response of polymorphonuclear leucocytes to injured tissue, it is difficult to suggest possible explanations for the discrepancies between the results from both test systems. Harris does not describe in very great detail the quantities of test substance used, or the manner in which they were introduced to the cultures of granular or agranular phagocytes. From the present studies it is clear that the concentration of materials used is extremely important. If high concentrations of chemotactic substances were produced or dissipated throughout the culture system, then the movement of the cells would be inhibited.

It is very possible that the discrepancies between certain results obtained by Ward et al. and Keller and Sorkin may be due to the lack of appreciation by both groups of workers that inhibition of chemotaxis may occur with high concentrations of chemotactic substances. For example, Keller and Sorkin find that no substances normally chemotactic for polymorphonuclear leucocytes are chemotactic for monocytes with the possible exception of casein (Keller and Sorkin, 1967b). However, Ward (1968a) has found that almost all the substances chemotactic for the polymorphonuclear cells are attractive to macrophages. There is a further discrepancy in the report by Stecker and Sorkin (1969) that sera of rabbits deficient in C'6 will release a chemotactic substance from antigen/antibody complexes, which is contrary to the findings of Ward, Cochrane and Muller-Eberhard (1966). Stecker and Keller claimed that the discrepancy was a result of Ward's inadequate technique. Ward has consistently used membranes of 0.16μ pore size for polymorphonuclear cell studies. Such pore sizes are too small to allow the migration of the cells through the membrane. In order to estimate the chemotactic response of these cells, Ward counts the number of cytoplasmic processes which have penetrated a certain distance into the membranes. However, neither group has tested their suspected chemotactic materials over a wide range of concentrations.

Hence some of the discrepancies arising from their results might be merely due to the fact that one group has tested chemotactic materials at such a concentration that migration is inhibited. Therefore, negative results from these experiments may be quite meaningless. In many experiments both groups use only one experimental chamber and one control chamber. We have found that this is quite inadequate if one desires to quantitate the reaction and compare the activity of fractions at various stages during purification procedures. There is no reason to believe that the polymorphonuclear leucocyte is a more standard cell in its behaviour than the amoeba. Indeed, looking at the results obtained by various workers it is quite clear that variation does exist from one experiment to another in the numbers of cells migrating in the control chambers over a particular time. Variations may be as great as from zero to 40 cells per given area. Ward (1968c) has also claimed that a number of substances such as hydrocortisone, prednisolone, chloroquine and a variety of phosphonate esters are able to inhibit the ability of polymorphonuclear leucocytes to respond to chemotactic substances. While this may well be true, it is very likely that they act primarily by completely inhibiting normal leucocyte mobility, rather than the ability of these cells to respond to chemotactic substances. Ward used the "trypan blue exclusion test" to determine

that the cells remained viable with the concentrations of drugs used. However, he did not test the effect of these drugs on the migration of polymorphonuclear leucocytes when tissue culture medium alone was present in the chambers. The lack of such a control would appear to lessen the significance of work by Ward and his colleagues on the mechanism of chemotaxis in leucocytes (reviewed in Chapter 1.1a). Other workers have shown that substances such as hydrocortisone do inhibit the normal mobility of polymorphonuclear leucocytes in tests involving the migration of these cells from buffy coat layers in capillary tubes (Ketchel, Favour and Sturgis, 1958).

The apparent similarities between metazoan and protozoan amoeboid phagocytic activity have led some workers, including Boyden (1962b, 1963) to propose that the basic mechanism behind the recognition of foreign material by organisms, both unicellular and multicellular, is similar. He suggested that as antibody is required to mediate this recognition in vertebrates, "recognition factors," either humoral or cell bound, are necessary for this process in invertebrate metazoan phagocytic cells. While Boyden appeared to have in mind substances such as the haemagglutinins in invertebrate serum, Phillips (1966) has suggested that since the phagocytes of many invertebrates have a nutritional function, an enzymic system

of recognition could occur in these animals. Such molecules may act as extracellular complexing agents. Their activity would be specific, enabling them to act only on heterologous molecules and not those of the host organism. One wonders if a recognition system, involving the extracellular release of certain enzymes as occurs in amoebae, may be used by the amoebocytes in some of the more primitive metazoan forms. In fact, it is not impossible that a system such as this may be the precursor of the antibody system in the vertebrates.

Conclusions

The model of chemotactic recognition discovered in the soil amoeba may have a widespread significance. Similar reactions may be involved in the chemotaxis of invertebrate metazoan phagocytic cells.

The Boyden technique may be used to considerable advantage in the study of chemotaxis.

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