RECOGNITION AND DEFENCE MECHANISMS IN THE COCKROACH *Periplaneta americana* (L.).

by

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Thesis presented for the degree of Doctor of Philosophy,
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November, 1969.

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Comparative immunology over the past decade has been confined largely to the phylogeny of the immune response within the vertebrate line. It appears that antibody formation and other 'adaptive' features (GOOD & PAPERMASTER, 1964) of the immune response are associated with the presence of lymphoid tissue, their evolution closely paralleling the development of this tissue. Studies to date have shown that the adaptive immune response is expressed at its most primitive in the Cyclostomes (FINSTAD & GOOD, 1966).

This recent research within the vertebrate line has been accompanied by a naissance of interest in immune mechanisms amongst invertebrates. The invertebrates, lacking lymphoid tissue, γ globulins, and consistently failing to show evidence of an adaptive immune response, have understandably been of secondary importance to immunologists. Much of the information relating to studies of invertebrate immunity from the early part of this century, is widely scattered throughout the literature, the studies having been undertaken for many different purposes. This lack of co-ordination must have retarded the present spate of investigations. Recent data are now co-ordinated by the Journal of Invertebrate Pathology and in April, 1967 the first symposium devoted entirely to invertebrate immunity was held in Chicago (BANG, 1967a).

Historically, the upsurge of interest in invertebrates is merely a revival. Metchnikov's classic observations in 1884 on the phagocytosis of yeast by the water flea, Daphnia, and responses of/

of other invertebrates to introduced foreign particles, led to his suggesting that ingestion by leucocytes played a role in disease resistance, and [illegible] the formation of his theory of inflammation (METCHNIKOV, 1884, 1893). The early part of the twentieth century saw a great deal of work on invertebrates, much of it carried out by two of Metchnikov's students, Cantacuzène and Metalnikov. A wide range of invertebrates were injected with a variety of bacteria and sometimes with vertebrate erythrocytes. Regrettably many of these early papers are difficult to obtain and are often of uncertain value in that claims were presented without adequate experimental data. This work has, however, been admirably reviewed by HUFF (1940) and provides an interesting background to the increasingly sophisticated studies of the past decade.

The first part of this thesis is devoted to a review of invertebrate immunity, with particular reference to recent data. The aim is that it should not only serve as background to the thesis work, but also present to the reader the present state of knowledge of an aspect of immunology that increasingly figures in immunological discussion but about which the average immunologist knows relatively little.

The elucidation of invertebrate defence mechanisms is not only of extreme interest in itself, but also of possible phylogenetic significance with respect to the evolution of the vertebrate immune response. Most invertebrate studies have been motivated by this latter aspect and have sought responses typical of those in higher animals or anything that might be construed as typical of such responses/

responses. However, it must be remembered that the invertebrates comprise an enormous group of animals, some 95% of the species in the animal kingdom; as such they embrace a multiplicity of structures and methods of functioning, which are the result of millions of years of evolutionary divergence and specialisation. One cannot therefore assume any particular species to be representative. Moreover, it is very doubtful whether any invertebrate, in its present form, can be considered ancestral to the vertebrates.

Considering this variation amongst invertebrates, it is hardly surprising that the findings of workers studying invertebrate immunity have often been diverse. On the other hand, a remarkable degree of uniformity appears to exist regarding the aspect that we shall consider first. This is the role of the cells found in invertebrate body fluids.

CELLULAR RESPONSES.

Phagocytosis of invading particles by the ameboid cells of the body fluids of invertebrates has been widely documented. The ingestion of bacteria by the hemocytes of various insects is recorded in the book 'L' Infection chez les Insectes' (PAILLOT, 1933) and the uptake of numerous other particles is reviewed by JONES (1962). Phagocytosis by mollusc cells is reviewed by FENG (1967) and the studies on the oyster (TRIPP, 1957, 1960), snail (TRIPP, 1961) and octopus (STUART, 1968) are of particular interest. Among the echinoderms, phagocytosis by the coelomocytes of the sea urchin (KINDRED, 1921), sea cucumber (HETZEL, 1965) and starfish (GHIRADELLA, 1965/

1965) has been recorded. READE (1968) has studied the uptake of carbon by crayfish (crustacean) phagocytes. CAMERON (1932) studied phagocytosis by earthworm (Annelida) coelomic corpuscles in some detail. Examples from the more 'primitive' invertebrates are the uptake of Indian ink by the hemocytes of a parasitic, digenetic trematode (platyhelminth) (CHENG & STREISFIELD, 1963) and Indian ink, carmine and vertebrate erythrocytes by sponges (porifera) (CHENG et al., 1968b, 1968c). The presence of phagocytic cells in animals of some of the major invertebrate groups is presented in table form by NELSON (1969).

What are the invertebrate cells involved? They are often loosely termed 'blood cells' and are the cellular content of hemolymph. More specifically they are referred to as leucocytes, amebocytes (from their ameboid nature), coelomocytes or hemocytes. The terms are freely interchangeable, though the term 'hemocyte' is now confined to insect blood cells by insect workers. Early cytological studies showed that the hemocytes are not a homogeneous population. A search of the literature on these early morphological studies reveals a variety of names coined to designate the various cell types, and this variety has led to considerable confusion. Insect hemocytes have recently been subjected to intensive study and the cell types have been characterised and the terminology standardised. Nine morphologically distinct cell types have been described, one of which is usually predominant, ameboid and phagocytic - the 'plasmatocyte'.(JONES, 1964).

BOOLOOTIAN/

BOOLOOTIAN (1962) points out that fourteen distinct cell types have recently been described in various species of echinoderms. Two types, the bladder amebocyte and the filiform amebocyte occur in twenty-eight out of thirty-two species and are phagocytic. The picture is less clear in the molluscs. WAGGE (1955), reviewing the origin and functions of molluscan amebocytes, refers to WILMER (1945) who suggested three fundamental cell types; epitheliocytes and mechanocytes, both non-phagocytic, and the amebocyte - a wandering cell, markedly phagocytic. Two other recent invertebrate cell classifications, in which the phagocytic cells are characterised, are in Tunicates (ANDREW, 1962) and Sponges (CHENG, 1968a). Until the various cell homologies and analogies have been resolved, it is sufficient to say that invertebrate hemocyte populations are composed of morphologically and functionally distinct types, one of frequent occurrence being a phagocytic cell of ameboid nature.

As to the relationship of invertebrate blood cells to those found in vertebrates, there appear to be no fundamental differences either morphological or functional. However, the presence of an erythrocyte type in some invertebrates (Echiuroidea and Sipunculoidea) and the absence of even a simple form of erythrocyte in the lowest chordates, seems to favour the hypothesis of parallel evolution rather than direct continuity (JORDAN, 1965).

In insects, not all cells are circulating and some are often found adhering to the internal tissue surfaces. Phagocytosis by these/

these fixed cells has been reported and some groups of Orthopterans possess localised phagocytic 'organs' situated near the heart and composed of phagocytic cells (NUTTING, 1952). HUFF (1940), in his review, refers to descriptions of phagocytic organs in Nematodes, Annelids, Crustaceans, Insects and Molluscs. Recent carbon clearance studies have been carried out by READE (1968) in the snail and crayfish, and in his histological studies he describes a highly active system of phagocytic cells associated mainly with the digestive diverticulae of both species.

Leucocytosis, an increase in the number of blood cells under conditions that constitute an immunological challenge, has been reported, as far as I could discover, in the Platyhelminths, Molluscs and Insects. Since a proportion of these cells are phagocytic, the response has been interpreted as a mechanism whereby the number of cells is increased for defence purposes. CHENG & STREISFIELD (1963) observed a slight increase in the number of hemocytes in trematodes challenged with carbon particles. FENG & CAZONNIER (1949) found an increase in the number of oyster leucocytes after introduction of foreign materials; even an injection of sterile sea water induced an increase within 24 hours.

The phenomenon is better documented amongst the insects. Increase in the total hemocyte counts (T.H.C.) (cells/mm^3) of some insects in response to bacterial infection, injection of materials, implantation of foreign tissue and wounding have been reported (see JONES, 1962). BRADY (1967) has shown that wounding raises the/

the hemocyte density four times in the American cockroach; also, 24 hours after wounding there is a significant increase in the number of circulating hemocytes/mm^3 in the wax moth (SHAPIRO, 1968). Wounding in insects constitutes an immunological challenge in that the integrity of the internal tissue surface is broken, exposing 'foreign' surfaces which evoke an encapsulation response (see Encapsulation Studies). The origin of these cells in insects is not fully understood. Three possibilities are - division of existing hemocytes, release of hemocytes from tissue surfaces into the circulation, and the release of hemocytes from hemocytopoietic organs (JONES, 1962). In his study on the wax moth, SHAPIRO (1968) reported that a peak of mitotic activity (6 x control) was associated with a significant increase in T.H.C. 24 hours after wounding. At this time, however, there were four times as many degenerating cells as there were in mitosis. Thereafter, a decrease in mitotic activity occurred but the maximum T.H.C. was recorded 96 hours after wounding. There being no strong evidence for the release of hemocytes from hemocytopoietic organs into the hemolymph in lepidopterous larvae, he attributed the initial increase to mitotic activity and release from tissue surfaces, the peak number of cells being due to further release from tissue surfaces.

When considering any quantitative data concerning insect hemocytes, or indeed cells of any invertebrate, some points must be borne in mind. JONES (1962) has reviewed the variation in insect/

insect T.H.C. due to technique, developmental stage, sex and nutritional status. WHEELER (1963) considers that the calculated number of hemocytes in the entire insect (absolute count) gives far more reliable information concerning changes in hemocyte numbers than T.H.C.'s, since the latter obviously varies with hemolymph volume. In the American cockroach he finds that, despite an increase in T.H.C. before ecdysis, the absolute number of hemocytes does not increase, the effect being due to decrease in hemolymph volume. The reverse situation is true with the decrease in T.H.C. at ecdysis. WITTIG (1966) in a detailed, comprehensive study, reports on the influence of certain variations in technique on T.H.C.'s. She also considers the choice of control insects, the size of experimental groups and the number of replications necessary to achieve statistical significance in comparing cell counts. Her data on caterpillars show that injection of latex and ink particles at low concentrations causes an increase in T.H.C., and a decrease with high concentrations. Control insects injected with distilled water showed a slight increase in T.H.C., merely due to the increase in volume caused by the distilled water. FENG (1965a) too, urges caution in interpretation of apparent leucocytosis in molluscs and other animals. He demonstrates that in oysters the number of circulating leucocytes is strongly influenced by heart rate, which in turn is dictated by temperature as well as mechanical stimuli such as injection of particulate and soluble material.

The/

The third kind of cellular response is encapsulation. This is the response to a target particle too large to be phagocytosed by a single hemocyte. It involves the aggregation of hemocytes around the particle, and the formation by them of a capsule which effectively isolates it from the circulation. It has been described in Arthropods, Annelids, Molluscs, Crustaceans, Arachnids and larval Echinoderms (SALT, 1963a), and in sponges (Porifera) (CHENG, 1968c). The accounts of the way in which the blood cells come together to form capsules are in general agreement (SALT, 1963a), but it is only in insects that encapsulation has been studied in any detail.

In insects, the response is said to be mounted against the eggs and larvae of parasitic insects not normally associated with the host in question, heterologous organ implants, the insect's own damaged tissue and various particles experimentally introduced into the hemocoele (SALT, 1961). The eggs or larvae of the ichneumonid parasite Neritis injected into the hemocoele of their normal caterpillar host Ephestia remain unencapsulated. However, when injected into a foreign host Diatarixia, they were completely encapsulated within 4 hours with a hemocyte layer 50-60μ thick. Hemocytes continued to accumulate and after 6 hours the capsule was 60-100μ thick. Up to 24 hours there was no further increase in thickness but a considerable change in structure. At first the hemocytes were subspherical, but at 10 hours the cells comprising the inner layer were seen to be flattened. After 24 hours the bulk/

bulk of the capsule consisted of flattened hemocytes, densely packed in planes parallel to the surface of the parasite. Only the superficial cells retained a rounded appearance (SALT, 1960). Encapsulated parasites were usually killed within 48 hours, the process sometimes being accompanied by a deposition of melanin onto the parasite surface (SALT, 1963b). Persistance of capsules around the remains of dead parasites was followed through metamorphosis of the host and up to 33 days (SALT, 1965), although a decrease in capsule size with shrinkage of the contained parasite would seem to indicate a loss of cells (SALT, 1960). Similarly, polythene rods were removed fully encapsulated after 7 days. In neither case were there any apparent ill effects on the host.

What happens at the parasite-hemocyte interface is not entirely clear. Light microscopy studies suggested that the inner cell layer became membranous (SALT, 1961) but recent E.M. studies show that capsules around implanted araldite remain cellular throughout for at least 20 days. The cytoplasm of the innermost cells penetrates every crevice and fissure in the araldite surface (SALT, 1967). Presumably these cells are involved in the disposal of degenerating material and possibly in melanin deposition.

We do not know yet whether the principles outlined for insect encapsulation are true for most invertebrates. CHENG *et al.* (1968c) found that, in the sponge, the capsules around implanted trematode cercariae disintegrated after decomposition of the cercariae. Some of the mollusc data are based on observations where foreign material is/

is actually implanted into the host tissue (see FENG, 1967). The responses are typified by those described by TRIPP (1961) in a fresh water snail Australorbis glabratus to implanted pollen, polystyrene and heterologous tissue. The result was a well defined lesion consisting of a central mass of densely packed particles and amebocytes surrounded by one or more layers of fibroblasts. Implanted tissue eventually became a fibrotic mass. BROOKS (1953) noted collagenous fibres towards the edge of such lesions in the same snail's response to miracidia not normally associated with them. This focal fibrotic reaction in molluscs would appear to be a characteristic tissue form of encapsulation, the purpose again being the isolation and eventual disposal of offending material.

STAUBER (1950) has studied the disposal of foreign material in the oyster. He observed a migration of carbon laden phagocytes through the epithelium to the exterior of the oyster. TRIPP (1957) studied the disposal, by the oyster, of injected vertebrate red cells. He noted five phases in the oyster's response.

1. Large masses of red cells virtually occlude many arteries.
2. These particles are then phagocytosed.
3. Phagocytes migrate into the tissues.
4. Phagocytes containing red cells migrate through the epithelium eliminating the particles from the body.
5. Those red cells within phagocytes retained in the body are digested.

A similar study in the fresh water snail Australorbis glabratus was carried out by TRIPP (1961). Here, particles small enough to be/

be phagocytosed, were removed from the tissue by migration of the host amebocytes through the epithelial layer to the exterior, or by intracellular degredation. In the sponge Terpios zeteki, ink and carmine laden archeocytes moved through the mesoglea, entering excurrent canals by migrating through the lining pinacocytes (CHENG et al., 1968b). Encapsulated erythrocytes were eliminated in the same way, the migration being somewhat less rapid (CHENG et al., 1968c). A different method of elimination is described by GHIRADELLA (1965): heterologous tissue implants in the starfish coelom began to collect within 1-3 days in the dermal branchiae of the host. The distal halves of the branchiae, swollen with donor tissue, rounded up and pinched off from the base, in effect undergoing autotomy. In other cases, the branchiae merely ruptured without autotomising, the tissue simply passing out through the distal opening.

With terrestial invertebrates the surrounding medium would seem not to be so conducive to the removal of any 'loaded' cells that might be extruded. Here the disposal of phagocytosed material seems to be by intracellular digestion or by disintegration and digestion within capsules. Encapsulated inert particles and non-metabolizable objects are not digested and may be carried in the phagocytes for long periods of time (SALT, 1965). In most of the earthworms a system of dorsal pores connects each coelomic compartment with the exterior. Coelomic fluid can be extruded through these pores, possibly to moisten the body surface.

CAMERON/

CAMERON (1932) injected earthworms intracoelomically with colloidal iron and smears of the dorsal pore secretions were then examined at intervals. Phagocytes containing iron and free iron particles were easily found in the secretions. Such material is presumably readily removed by the worm's passage through the soil. In the same study a definite tendency was found for the transference of waste products, bacteria and foreign substances to the terminal segments of the worm which are subsequently autotomised, regeneration following rapidly.

Phagocytosis is therefore firmly established as a primary defence mechanism in invertebrates. As will be seen from the following review of the humoral aspects of invertebrate immunity, it is also probably the only defence mechanism common to both vertebrates and invertebrates. For phylogenetic reasons then, studies of various invertebrate phagocytic systems are of interest. Some quantitative data on carbon clearance in the fresh water crayfish and land snail are presented by READE (1968). Phagocytic indices (K) are calculated as 0.15 and 0.01 for the crayfish and snail respectively. In comparison with data for equivalent carbon doses in mice, rats, guinea pigs and rabbits (BENACERRAF et al, 1957) the crayfish figure is high. That of the snail is lower than that of the mouse, guinea pig and rat, but about the same as the rabbit. The clearance of T_1 phage in the land snail, Achatina and the shore crab, Carcinus meanas, has been followed (NELSTROP et al., 1968). In the shore crab, clearance following a/

a second dose was seen to be faster than after the first. This was not attributed to an immune reaction since at no time was phage neutralising activity found in the hemolymph. This apparent, purely cellular secondary response is interesting but, as suggested by the authors, the repeated bleedings may have had some effect on the phagocytic response. From our earlier discussion of leucocytosis and the effect of wounding in insects this might indeed be true. T4 phage clearance studies in sipunculid worms failed to reveal any secondary response (CUSHING & McNEILY, 1967). Again using a sipunculid, TRIPLETT *et al.* (1958) found no enhancement of phagocytosis or encapsulation following secondary implants of tissue into the coelom.

Before concluding this section on invertebrate phagocytes, it should be pointed out that they also have their limitations as is shown by reports of the persistence of injected particles. In the lobster, unlike mammals, T4 phage has been reported to persist up to 168 days (CUSHING & McNEILY, 1967). Circulating øX174 phage was detectable up to 88 days in the crayfish (TEAGUE & FRIOU, 1964). There is little phagocytosis of *S. aureus* phage 80 in the oyster (FENG, 1966). BANG (1961), studying the *in vitro* phagocytosis of marine bacteria by oyster leucocytes, concluded that phagocytosis, though readily demonstrable, was by no means an invariable phenomenon. STEPHENS (1959) reported that most cells of *Pseudomonas aeruginosa* were not phagocytosed by hemocytes in wax moth larvae, even after the bacteria had been heat/

heat killed. This organism is highly pathogenic in the wax moth and it seems that pathogenicity and this resistance to ingestion might be related. Further correlation of pathogenicity and non-phagocytosis in other invertebrates would be interesting. The other extreme of phagocytosis and pathogenicity is seen in the Honey Bee where phagocytosis of spores of Nosema apis is followed by the rupture of the containing hemocyte and eventual death of the host (GILLIAM & SHIMANUKI, 1967).

From gross observations, phagocytosis by invertebrate and mammalian cells appears similar, but as yet nothing is known in detail about the phagocytic process, i.e. attachment, engulfment and the intracellular events which follow engulfment. Such comparative studies should be rewarding in view of the fact that the phagocytic hemocyte might be the evolutionary precursor of the vertebrate phagocyte.

HUMORAL REACTIONS.

Once again, for work carried out in the early part of the century, the reader is referred to HUFF (1940). Here the presence of natural and acquired bacterial agglutinins, hemagglutinins, hemolysins, antitoxins and precipitins are reported for some members of most invertebrate phyla. Most of this work was done by Cantacuzene who summarised most of his findings himself (CANTACUZÈNE, 1923). The previous caution as to the early cellular work again applies here, but enough confirmations exist for it to warrant/

warrant serious consideration (BANG, 1967b).

Thus although invertebrate body fluids have been consistently shown to lack gamma globulins (ENGLE et al., 1958), mixing some of them in vitro with 'antigen' results in reactions characteristic of mammalian antigen-antibody reactions. The most prominent of these antibody-like substances are the naturally occurring heteroagglutinins. TYLER (1946) demonstrated the presence of agglutinins for various vertebrate erythrocytes and invertebrate sperms in the body fluids of some species of Annelids, Echinoderms, Molluscs and Tunicates. BROWN et al. (1968) screened fifty-four species of invertebrates and found agglutinins for human erythrocytes in five invertebrate phyla. One coelenterate and seven echinoderm species showed hemolytic activity. BERNHEIMER (1952) examined forty-six species of lepidopterous larvae and found hemagglutinins in seven different families. In his study TYLER (1946) showed that, with one exception, no agglutination occurred between body fluid and cells from taxonomically related animals. Absorption tests with body fluid from the starfish Patiria miniata yielded results similar to those found previously in the lobster (TYLER & METZ, 1945), namely, the presence of a number of distinct agglutinins, each with broad group specificity.

The hemagglutinins in planorbid snails (GILBERTSON & ETGES, 1967), the lobster (TYLER & METZ, 1945), the oyster (TRIPP, 1966) and the crayfish, clam and mussel (McKAY et al., 1969) are each capable of agglutinating a wide range of vertebrate erythrocytes whereas/

whereas a hemagglutinin in the snail Viviparus malleatus was found to be specific for rabbit erythrocytes (CHENG & SANDERS, 1962). Reciprocal absorption tests showed the lobster hemagglutinin to be completely nonspecific with respect to fish, frog, mouse and rabbit erythrocytes (TYLER & METZ, 1945). Similar tests with the clam mussel and crayfish against a broad spectrum of vertebrate erythrocytes revealed the presence of specific hemagglutinins, some of which cross reacted with a variety of erythrocytes (McKAY et al., 1969). This specificity was reflected, although to a lesser extent, in the oyster (TRIPP, 1966).

The hemagglutinins from some invertebrates exhibit a specificity for human blood group antigens. Blood group reactive substances in invertebrates have been reviewed and discussed by CUSHING et al. (1963), CUSHING (1964, 1967). The snails Otalea lactea and Helix pomatia both possess strong specific anti-A agglutinins (BOYD & BROWN, 1965; PROKOP et al., 1965), and the butter clam a specific anti-A_1 agglutinin (JOHNSON, 1964). Apparently the occurrence of strong specific agglutinins is uncommon; among the fifty-four species of invertebrate tested by BROWN et al. (1968) fifteen agglutinated nonspecifically, six contained weak anti-H activity, one contained a possible, but very weak anti-A, and one a probable anti-M. A substance in the octopus body fluid which specifically inhibits the agglutinination by anti-A serum of human A erythrocytes was demonstrated by CUSHING et al. (1963). In the same paper the hemerythrins of a sipunculid were demonstrated to possess antigens that/

that react with human anti-A, human anti-B and anti-Forsmann antisera. The human P blood group antigen has been reported in earthworms and the nematode Ascaris (PROKOP & SCHIESINGER, 1966). CUSHING & McNEILY (1967) have demonstrated that the body fluids of different Hermit crabs are either positive or negative for an agglutinin for human erythrocytes and that agglutination by positive serum appears to be inhibited on addition of negative serum. KIM et al. (1966) report that a snail which produces an agglutinin against human A cells contains a soluble B substance in its tissue. These last two observations are of obvious immunological interest, but to date, the physiological significance, if any, of this wide distribution of blood group reactive substances remains unclear.

Some data are now available concerning the nature of these naturally occurring hemagglutinins. That in the oyster is described as a heat labile (65°C), non-dialysable protein (TRIPP, 1966; McDADE & TRIPP, 1967a). All their attempts at purification were unsuccessful but, in the snail, hemagglutinating activity was again found to be protein and associated with all five electrophoretic fractions of normal hemolymph (CHENG & SANDERS, 1962). Preliminary reports on the mussel and crayfish hemagglutinins show them also to be heat labile (57°C), non-dialysable protein (JENKINS & ROWLEY, 1969). Exhaustive studies designed to elucidate the structure of the naturally occurring hemagglutinin in the Horse shoe crab Limulus polyphemus have been carried out by/

by MARCHALONIS & EDELMAN (1968). The hemagglutinin was purified and found to be a protein of relatively low electrophoretic mobility. The intact molecule had a sedimentation constant of 13.5S and a molecular weight of around 400,000. From the physico chemical data and the electron microscope studies of the purified fraction, (FERNANDEZ MORAN et al., 1968) a hypothetical structure of a ring shaped molecule comprising about 18 subunits was postulated.

The vertebrate red cell can hardly be considered a potential pathogen likely to be encountered in the natural state. Its use as an antigen has of course derived from the tendency to search for and define invertebrate immune responses within a vertebrate framework, but it is questionable whether these agglutinins have an immune role or whether their activity is merely fortuitous. Some evidence that they might indeed function as antibodies comes from the demonstration that sensitisation of erythrocytes with oyster and crayfish hemagglutinins facilitates their uptake by oyster and crayfish hemocytes respectively (McDADE & TRIPP, 1967a; McKAY et al., 1969). This opsonic function, and the fact that agglutinins of different specificities appear to exist in the same animal, has interesting implications with respect to the possible mechanism underlying phagocytic discrimination in invertebrates. This subject will be discussed later.

It would seem sensible to study target particles more likely to be encountered in the natural state. TRIPP (1966) failed to demonstrate/

demonstrate any bacterial agglutinins in oyster hemolymph against nine species of bacteria, although heat stable bacterial inhibitors were described in oyster tissue extracts by LI (1960). McDADE & TRIPP (1967b) found lysozyme activity in normal oyster hemolymph. This bacteriolytic agent was not identical to the hemagglutinin and its heat stability suggested identity with the bacterial inhibitor of LI (1960). CHADWICK (1967) lists the virulence of various organisms for wax moth larvae and their susceptibility to natural bactericidal effects; it is not surprising to find that the hemolymph was not bactericidal for any of the pathogenic bacteria, although activity could be demonstrated against a few of the non-pathogenic species. The large spider crab Maia squinado is resistant to infection with the large carnivorous ciliate Anophrys sarcophaga, whereas the shore crab Carcinus is susceptible, its amebocytes being swallowed and digested by the ciliate (POISSON, 1930). BANG (1962) examined the serum of Maia and found that, in some individuals, the serum strongly agglutinated the parasite, whereas others showed no activity. Injection of the parasite into the latter group of animals led to death from overwhelming infection whereas those possessing the agglutinin survived. It was suggested that the agglutinin was a genetically determined factor for resistance, or that it was acquired during the course of exposure to the parasite.

Acquired activity.

Although the term 'natural activity' implies the unimmunised state/

state, it is of course impossible to state, unequivocally, that an invertebrate is naive when dealing with microbes in its natural environment. Thus the division between acquired and natural immunity must be considered less sharply defined than may appear in this discussion. Nevertheless, the following data are grouped together under the theme of acquired humoral responses. By an acquired response is meant the de novo production, or enhancement, in response to previously injected material, of substances which react with this material.

There are many reports of the failure of invertebrates to produce antibody-like activity following the injection of purified materials known to be antigenic in mammals. TRIPLETT et al. (1958) was unable to demonstrate precipitins against numerous antigens in a sipunculid worm. TEAGUE & FRIOU (1964) could show no activity against human and bovine serum albumin or phage øX174 in the crayfish Cambarus virilis. Lack of anti-phage activity was reported also in the shore crab (NELSTROP et al., 1968). TRIPP (1966) was unable to increase the amount of naturally occurring hemagglutinin in the oyster Crassostrea virginica by injection of erythrocytes. Similar results are reported for the crayfish Parachaeraps bicarinatus by ROWLEY (1967). In contrast with some of these results is the work of PHILLIPS & YARDLEY (1960) who considered that the failure to detect antibody-like activity by conventional serological techniques might be due to non-optimal assay conditions for invertebrate fluids. Sea anemones injected with/

with bovine serum albumin (BSA) gave negative precipitation results, but when assayed for anti-BSA activity by measuring the ability of body fluid extract to inhibit precipitation of BSA and rabbit anti-BSA, the results appeared to indicate the production of an inhibitory substance about 18 days after injection. Such extracts did not affect human serum albumin (HSA) precipitation. Despite the implications of these results, no further work with this system or similar systems has been reported.

What then is the fate of these soluble antigens if indeed no antibody like material is formed against them? BSA and HSA are taken up both in vivo and in vitro by sea urchin Strongylocentratus purpuratus coelomocytes (HILGARD & PHILLIPS, 1968). Likewise, oyster leucocytes are capable of pinocytosing both mammalian and crab serum proteins (FENG, 1965b). Studies on the clearance of radio-labelled HSA in the crayfish failed to show immune clearance typical of mammals, the kinetics being indicative of simple catabolism (TEAGUE & FRIOU, 1964).

Once again, a great deal of work has been done on insects. BERNHEIMER et al. (1952) failed to ellicit a response against T2 phage, E. Coli and streptolysin-O in caterpillars, although some hemagglutinating activity was detectable following injection of erythrocytes or egg albumen. BRIGGS (1958) studied eleven species of caterpillar and failed to demonstrate any agglutinating activity against bacteria, both pathogenic and non-pathogenic, and erythrocytes. Precipitin studies with egg albumin were also negative/

negative, as were complement fixation and antitoxin tests. However, a naturally occurring, heat stable (121°C) bacterial inhibitory principle was found in the hemolymph of all species except one. Following vaccination the principle appeared if not already present, or the existing level of activity rose. This rise paralleled an increase in tolerance to minimum mortal doses of pathogenic bacteria. Acquired resistance to certain pathogenic bacteria in insects is becoming well documented (CHADWICK, 1967). Pseudomonas aeruginosa is pathogenic for wax moth larvae Gallera mellonella and hemolymph from normal larvae contains no bactericidal activity against it. Following immunisation with heat killed organisms, bactericidal activity appeared in the hemolymph and was correlated with developing acquired immunity as evidenced by resistance to a challenge dose (STEPHENS, 1962). Activity was found between 4-72 hours, with the peak at 24 hours, and appeared to be fairly specific with respect to a related pathogenic Pseudomonas and non-pathogenic S. dysenteria. No activity against Pseudomonas aeruginosa was detectable using gel diffusion, precipitation or agglutination techniques (STEPHENS, 1959). Preparations of endotoxin from P. aeruginosa and other bacteria were compared for ability to induce resistance against P. aeruginosa. All preparations induced significant resistance, the homologous vaccines appearing to be only slightly more effective than the heterologous (CHADWICK & VILK, 1969). GINGRICH (1964) described a bacteriolytic substance elaborated in the large milkweed bug/

bug Oncopeltus fasciatus following a single injection of P. aeruginosa, the kinetics of which were similar to those described for the wax moth caterpillar. The response appeared to be relatively specific although saline and needle injury provoked demonstrable responses.

All the above reports indicate that the bactericidal factors appear to be non-protein and stable at high temperatures (around 100°C). HINK & BRIGGS (1968) recently studied the bactericidal factors in normal and immune wax moth larvae using gel filtration. Two fractions with bactericidal activity were found. The first of molecular weight around 7000, heat stable (100°C) and not of protein polypeptide nature was present in both normal and immune insects and was capable of passively transferring immunity. A second fraction was present only in immune hemolymph and was unable to confer passive immunity.

Bactericidal factors in both normal and 'immune' insects are thus well established, but appear not to be the only humoral substances involved in insect immunity. SEAMAN & ROBERT (1968) found that hemolymph from male American cockroaches, previously injected with living Tetrahymena pyriformis (pathogenic for male cockroaches) immobilised washed ciliates. Again activity appeared to peak at 24 hours but was heat labile and associated with a non-reactive protein component in hemolymph from normal insects. No data on specificity were presented.

Studies with micro-organisms in invertebrates other than insects have also proved profitable. An induced bactericidin has/

has been reported in the Spiny lobster Panulirus argus by EVANS et al. (1968). Injection with living or killed bacteria induced synthesis of a heat labile (65°C), non-dialysable substance, detectable within 12 hours and peaking at 24-48 hours. Varying results indicated a low degree of specificity, the highest titres occurring with a bacterium, EMB-1, isolated from the normal intestinal flora of the Spiny lobster. The same bacterium has been shown to evoke a bactericidin in the sipunculid worm Dendrostomum zostericolum (EVANS et al., 1969). In the sipunculids, however, there was no significant response during the first week after primary immunisation. Significant titres were detected after 60 days, the response being considerably enhanced following secondary immunisation at 60 days. The crayfish, Parachaeraps bicarinatus, developed resistance to a bacterial pathogen, Pseudomonas CP, following immunisation with whole organisms or endotoxin (McKAY & JENKIN, 1969). Immunity was able to be induced by a variety of unrelated bacteria and was found to be both dose and temperature dependent. No bactericidal or bacteriolytic activity was detectable in vitro, the growth rate of Pseudomonas CP in both normal and 'immune' hemolymph being similar to that in nutrient broth. MICHELSON (1964) has demonstrated the presence of a miracidia-immobilising substance in fresh water snails (Australorbis glabratus - the normal host) infected with Schistosoma mansonii. The potency increased with the age of infection and constantly higher activity was found in tissue extracts from infected/

infected snails than from non-infected. The substance was heat stable at 56°C and does not appear therefore to be related to the naturally occurring hemagglutinins found in the hemolymphs of planorbid snails (GILBERTSON & ETGES, 1967).

An aspect that has to date been neglected in invertebrate humoral studies concerns the origins of the reported factors. The sipunculid worm Sipunculus produced a lysin for a parasitic marine ciliate following the injection of various foreign substances and such activity was demonstrated to be released from the animals' cells in vitro (BANG, 1966, 1967b). Some experiments with ligatured wax moth larvae Galleria mellonella suggested that the organs or cells that function in the immune mechanism, probably producing antibacterial substances, exhibited maximum activity in the posterior portion of the larva, but appeared to be located and functioned throughout the insect (HINK & BRIGGS, 1969).

Most of the data concerning the humoral responses of invertebrates have been relatively isolated cases, and considerably more and fuller data are required before we are able to judge how much the responses of the different invertebrates have in common. The antibacterial activity of insects is highly heat stable, whereas that of the lobster is heat labile. In the oyster it again appears to be heat stable. This diversity may seem to argue against any unified hypothesis of invertebrate humoral immunity, but it may be argued that there is a single class of bactericidal factors throughout the invertebrates and that the differences are due/

due to the fact that, in some invertebrates, they are normally bound to a protein carrier whose denaturation results in loss of activity, whilst in others they are not.

RECOGNITION OF FOREIGNESS.

The ability to discriminate between 'self' and'non-self' is basic to immune systems. Before discussing the mechanics of discrimination, a preliminary question is asked - what constitutes foreigness to an invertebrate? Vertebrates are capable of a fine degree of discrimination. The homograft reaction (i.e. the recognition of another genetically different individual of the same species as foreign) is exhibited throughout the vertebrate line above the hagfish (GOOD & FINSTAD, 1964) and also recently in the hagfish itself (HILDEMAN & THOENES, 1969).

Discrimination in earth worms was reported briefly by CAMERON in 1932. Heterologous worm sperm, injected into the coelom, were phagocytosed within 48 hours. Homologous sperm were detectable in the body fluid 3-4 days after injection and were not seen to be phagocytosed. Mammalian sperm was apparently phagocytosed no more rapidly than heterologous sperm. TRIPLETT et al. (1958) inserted severed bits of tentacle into the coelom of sipunculid worms and found that both auto- and homo-transplants were encapsulated with hemocytes at the same rate. Encapsulated tentacles were viable up to 70 days, but implanted, encapsulated sea anemone tentacles were found to be dead and almost completely digested within 5 days. Experiments on the transplantation of scallop/

scallop eyes revealed little difference in survival times between auto- and homografts (CUSHING, 1957). Similar experiments with octopus skin showed that the octopus was unable to discriminate between auto- and homografts (CUSHING, 1962). In a gastropod mollusc, the snail, Australorbis glabratus, implants of homologous tissue elicited no fibroblast response and only transient amebocytic infiltration. Such grafts usually fused with host tissue. However, both formalin-fixed homologous tissue and heterologous snail tissue implants were encapsulated within 24 hours (TRIPP, 1961). Host reactions to implants were studied in the starfishes Patiria miniata and Astenas forbesi by GHIRADELLA (1965). Homologous material in the coelom was tolerated and no discrimination between this and autologous implants was noted. Heterologous starfish material was eliminated, the host being able to discriminate between simultaneous homo- and heterotransplants. The overall results revealed no discrimination between two closely related species, but discrimination between two different families of the same order and between members of two different orders.

SALT (1961) searched the literature concerning insect transplantation, regardless of the purpose for which it was undertaken, and looked for reports of any host hemocyte reaction against the transplant. Although there were exceptions, it appeared that in general the hemocytes of an insect do not react against their own healthy tissue, most intraspecific implants, many (?) interspecific implants and some intergeneric implants. In his own experiments

SALT/

SALT (1960) found that intraspecific implants of nerve cord in lepidopterous larvae remained unencapsulated save for damaged surfaces. Implants from another lepidopteran species were completely encapsulated.

Some recent studies on transplantation immunity in invertebrates have been carried out on earthworms. DUPRAT (1964) presented evidence for a homograft reaction between earthworms (E. Foetida) from the same stock. Four days after grafting pieces of body wall, ameboid and chlorogogenous cells were observed to mass at the internal surface of the graft; they disappeared at day 5 and the graft became integrated with the host. These cells were not observed beneath autografts. If a second graft was performed 4-8 days after the first, cells again massed but this time a phagocytic attack was mounted against the graft leading to its destruction. First set reciprocal homografts between individuals from different geographical regions were rejected and replaced by host tissue. PASQUIER et al. (1966) studied heterografts between some species of earthworm and found that grafts were accepted between certain species, whilst, with other species combinations, rejection occurred. Comparative studies of the antigens of body wall extracts from the different species were carried out using immuno-diffusion. Species which were compatible with respect to grafting, were found to be antigenically identical, whereas antigenic differences were evident between incompatible species. COOPER (1968) confirmed autograft tolerance and heterograft rejection/

rejection in earthworms; both grafts healed during the first 24 hours but subsequent events always led to the destruction of heterografts. He also reported that most second set heterografts were destroyed by accelerated reactions, although a significant number survived longer than the first set grafts.

A relatively sophisticated demonstration of discrimination in invertebrates has been carried out in the sea urchin by HILGARD & PHILLIPS (1968). They followed the clearance of radio-labelled BSA and HSA from the coelomic fluid and also its uptake by the coelomocytes. The clearance of these foreign molecules was seen to be considerably faster than that of native molecules (coelomic fluid substances). The coelomocytes were also demonstrated to be able to discriminate between the two closely related albumins both in vivo and in vitro. There is then definite evidence that invertebrate hemocytes have a capacity (however limited) to recognise foreigness.

Mammalian phagocytosis is known to be largely serum-dependent and similarly, phagocytic discrimination is considered to be due largely to specific humoral components of both normal and immune sera (BOYDEN, 1963, 1966; VAUGHAN, 1965a). In such a system the absence of serum factors directed against healthy self components would prevent uptake of such material. Clearance studies in mice have shown that the more distantly related are erythrocytes, the faster they are cleared from the circulation (HOLLINGSWORTH, 1958; HALLIDAY, 1967). Mouse erythrocytes showed a prolonged period of slow/

slow clearance (7-10 days). Rat erythrocytes, after a similar initial slow clearance rate, were relatively rapidly removed after about 24 hours, this phase being correlated with the appearance of specific antibodies. Passive transfer of immune anti-rat erythrocyte sera also caused a rapid increase in the clearance of rat cells (HOLLINGSWORTH, 1958; HALLIDAY, 1967).

BOYDEN (1960, 1962, 1963) suggested that the mechanism underlying invertebrate phagocytic discrimination might be similar and postulated the presence of humoral recognition factors in invertebrate hemolymph. Assuming that the specificities of such factors are randomly generated, then factors having an affinity for self must be eliminated. Short of a form of tolerance to self components induced during neonatal life, as with mammals, one must assume that such factors exist. Boyden points out that they would quickly be absorbed out of the hemolymph and would be ineffective in promoting opsonisation of host cells, since the number of molecules adsorbed onto any given host cell is likely to be small. In contrast, recognition factors with foreign affinities would tend to accumulate, either free in the hemolymph or possibly like cytophilic antibodies attached to phagocytes. BURNET (1968) discussing the evolution of the immune response, echoes and elaborates Boyden's ideas. His recognition factors are globulin-like proteins (protoglobulins) associated with the hemocyte surface and probably leaking into the hemolymph. Diversification of globulin pattern is provided at the cellular level, that is, each hemocyte/

hemocyte produces globulins of one pattern only, presumably with phenotypic restriction extending to any descendant clone. Both Boyden and Burnet point out the presence of substances in invertebrate body fluid with antibody-like activity and suggest that these might serve as recognition factors.

The finding that the naturally occurring hemagglutinins in the oyster (TRIPP, 1966) and the crayfish (McKAY et al. 1969) appear to be opsonic for the in vitro uptake of red cells by the hemocytes is interesting in the light of the above, and a degree of specificity has been reported for these agglutinins. Whether they were actually produced by the hemocytes was not investigated. More evidence in support of the existence of specific receptor sites comes from the ability to block differentially the uptake, by sea urchin coelomocytes, of HSA and BSA following pretreatment with BSA, suggesting that the receptor sites for BSA and HSA are different (HILGARD & PHILLIPS, 1968).

At present the phagocyte-recognition factor hypothesis is the only one that has been committed to print. PHILLIPS (1966) discussed the concept of complexors. Briefly these were specific extracellular factors that complexed with incoming foreign material, the resulting complex triggering off cellular uptake and eventual degradation of themselves. Such complexors are essentially the recognition factors of Boyden and Burnet. Phillips pointed out that enzymes possess the required degree of specificity for such a recognition system i.e. enzymes that do not catalyse the breakdown of/

of homologous tissue but are functional with respect to heterologous or modified homologous tissue. It would seem, however, that enzymes would not be capable of such fine discrimination as reported in the above HSA-BSA system.

Overall, in invertebrate immunity, there has been very little research into cell-hemolymph interaction, most workers having concentrated on one aspect or the other. The discovery of what on preliminary evidence appear to be similar opsonic substances in an Arthropod and a Mollusc would seem to indicate that such studies might be profitable. Of course not all opsonins need to possess other kinds of antibody-like activity; indeed an opsonin in octopus serum for uptake of erythrocyte by octopus hemocytes did not show hemagglutinating activity (STUART, 1968).

Another approach to the recognition problem has been to attempt the destruction of tolerance. Amebocytes from both male and female sipunculid worms failed to encapsulate, and therefore recognise as foreign, homologous normal eggs injected into their coelom; the eggs of another genus of sipunculid were rapidly encapsulated. Treatments of normal homologous eggs that led to their being encapsulated were eosin staining, repeated washing, heating and sonication (CUSHING, 1967). All these treatments killed the eggs. Frozen eggs, on the other hand, while reacting to vital dye as though they were dead, still retained much of their immunity. This suggested that the safety signal was not associated with active metabolism (HAND, 1966).

Some/

Some interesting studies on the problem of 'self' and 'not self' have been in the field of host-parasite relationships in insects. Eggs of the parasitic wasp Nemeritis, whether dead or alive, remain unencapsulated in the hemocoele of its normal caterpillar host Ephestia. Alterations to the surface of the egg, chemically with octane and physically by abrasion with iron filings resulted in complete encapsulation of the living eggs (SALT, 1965). The first instar larva hatches from the egg into the caterpillar hemocoele challenging the host defence reactions with a different surface, its chitinous cuticle. Again, no hemocyte reaction is evoked and experiments similar to those on the eggs again showed that its resistance was attributable to the surface of the larval cuticle (SALT, 1966). The origins of the immunity of both eggs and larvae were further investigated. In brief, the eggs were found to be coated with a protective substance (responsible for avoidance of encapsulation) as they passed through the calyx of the genital tract of the parent wasp (SALT, 1965). Eggs collected from below this point were observed to have a layer of transluscent material outside the chorion which served as substrate for the attachment of numerous small particles produced by the calyx wall (ROTHERAM, 1967). It appears that the larva acquires its protective surface within the egg between about 64 and 66 hours of age. Electron microscope studies have shown the presence of minute particles on the larval surface similar to those found on the egg chorion (SALT, 1968). Such protective substances/

substances are interpreted as mimicking the host's internal surfaces as they confer no protection when eggs and larvae are implanted in foreign hosts (SALT, 1960).

The invertebrate studied in this thesis, the cockroach, was selected because of some previous success in obtaining hemocytes from these insects, and maintaining them *in vitro*. The broad aim of the work was to increase the existing, relatively slender, store of information concerning invertebrate immunity, but, more particularly, it was hoped to learn something about the mechanism whereby the phagocytic hemocytes are able to distinguish between 'self' and 'non-self' material.

PART II

STUDIES ON THE AMERICAN COCKROACH (*P. americana* L.)

The animals used in this study were adult American cockroaches. These were trapped, as adults, in the Reptile House of the Royal Zoological Gardens, Edinburgh, and maintained in a glass tank at room temperature, being provided with rat cake and water *ad libitum*.

Cockroaches are amongst the most primitive of winged insects. Their origin dates back 250 million years to the Carboniferous period and fossil records show not only that they were extremely abundant at that time, but also that the present-day species have changed very little with the passage of time. The American cockroach is an extremely hardy animal with an adult life span of a year or more and has been the subject of extensive research by insect physiologists. For general data on the cockroach the reader is referred to CORNWELL (1968).

CHAPTER I HEMOLYMPH STUDIES

The hemolymph of insects is the fluid contained in the general body cavity where it freely bathes the various internal organs and also enters the appendages and tubular cavities of the wing veins. It consists of liquid plasma and numerous hemocytes.

A. HEMOLYMPH EXTRACTION.

Whole plasma. Hemolymph from adult cockroaches was extracted by a centrifugation technique based on that of SIAKOTOS (1960). Cockroaches from stock cultures were put into an ice bath for 30 minutes to inhibit blood coagulation; this process also served to anaesthetise the insects. A number were then removed from the bath and blotted with tissue. The head and foregut were carefully withdrawn with forceps, the last few segments of the abdomen, along with the head and midgut were similarly removed. Three such insects were placed thorax first into a 15 ml. conical centrifuge tube. The tube was then centrifuged for 45 seconds at 80-100Xg. Under these conditions, no fat body or other tissues contaminated the plasma. Samples thus obtained were centrifuged to remove cells and stored in aliquots at -20°C. The volume obtained from three insects in this manner was usually about 0.05-0.1 ml.

Saline extract. Cockroaches were anaesthetised with ether and 0.2 ml./

0.2 ml. of 0.85% sterile saline injected into the hemocoele via the intersegmental membrane between the abdominal sternites, using a 31 g. needle. No leak-back occurred and the animals were left for at least a minute to allow mixing of body fluid and saline. A metathoracic leg was then cut off at its base and the emerging fluid quickly taken up with a Pasteur pipette. Fluid thus obtained was centrifuged to remove cells, filter sterilised (Millipore HA 0.22μ) and stored in aliquots at -20°C. By this technique 0.1-0.2 ml. per cockroach was easily obtained

Whole plasma was a clear yellow fluid, often with a noticeable lipid layer. In saline extracts the lipid was not visible, otherwise the appearance was similar although slightly paler in colour. Samples of whole plasma left at room temperature for about 4 hours showed a slight brown colouration and, if left overnight at room temperature, were found to be dark brown; precipitation also occurred under these conditions. These changes did not occur when whole plasma was kept overnight in the refrigerator. In the frozen state whole plasma was completely stable. Saline extracts were completely stable for several days at room temperature. Melanisation* of the hemolymph of many

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* Melanisation is a darkening of the hemolymph due to the deposition of melanin pigments. It involves the oxidation of tyrosine by tyrosinase in the presence of oxygen and a chromogen (WIGGLESWORTH, 1965).

insects is known to occur on contact with air. If what was occurring here with whole plasma was melanisation, then presumably the saline extraction technique prevents this process in some way. Another possible explanation for the deterioration of whole plasma samples is the fact that the extraction technique involves breaking the gut inside the animal and this must have involved at least a slight release of gut contents or enzymes.

B. HEMAGGLUTINATING ACTIVITY OF HEMOLYMPH.

Hemagglutinating activity was found in cockroach hemolymph and the following experiments were designed to study this activity and its chemical basis. Hemagglutinating activity has been reported in the body fluids of many invertebrates (see Part I) and in the design of the experiments, particular reference was paid to the known characteristics of these. In fact, remarkably little was known about the nature of invertebrate hemagglutinins at the time of these experiments, the fullest and most recent study being that on the oyster hemagglutinin (TRIPP, 1966; McDADE & TRIPP, 1967a).

Hemagglutinin titration. Sheep red cells in Alsever's solution and defibrinated horse blood were purchased from Burroughs Wellcome Ltd. Chickens were bled from the brachial vein into a syringe containing heparin. Human red cells, in dextrose citrate were kindly provided by the Blood Transfusion Unit, Edinburgh Royal Infirmary/

Infirmary. Lampreys were bled into citrated 0.6% saline as described by PAPERMASTER et al. (1964).

Red blood cells were washed thrice in 0.85% saline (0.6% for the lamprey) and resuspended in saline at a concentration of 1% (v/v). Microtitrations were performed in V-shaped plastic wells (Linbro) by serially diluting hemolymph with 0.85% saline (0.6% for the lamprey). To 0.1 ml. diluted hemolymph was added 0.1 ml. 1% red cell suspension and all tests were incubated for 1 hour at room temperature and then in the refrigerator overnight. The results were read macroscopically and the titres recorded as the reciprocal of the last dilution of hemolymph that agglutinated red cells. Control red cells in saline were always negative.

Range and specificity of agglutinin. All the vertebrate erythrocytes tested were agglutinated by both whole plasma and saline extracts. Titres for whole plasma are shown in Table I, where it can be seen that they were quite considerable. Bacterial activity was ruled out by repetition with filter sterilised saline extracts. The agglutination patterns were identical to those obtained with a mammalian hemagglutinating antibody.

TABLE I: Agglutination of various vertebrate erythrocytes by cockroach hemolymph.

Red cell type:	Sheep	Chicken	Horse	Human	Lamprey
Hemaggn. titre:	256	256	64	32	128

Preliminary/

Preliminary experiments with sheep erythrocytes showed that the agglutinating activity was completely absorbed out from a volume of hemolymph mixed with an equal volume of packed sheep cells for 30 minutes at room temperature, this procedure being repeated once. Reciprocal absorption tests were set up to test for any specificity with respect to erythrocyte type. The results are set out in Table II. Unfortunately, no lamprey cells were available for this series of experiments.

TABLE II: Specificity of the hemagglutinins in cockroach hemolymph.

	Red cell used in titration		
Absorbing red cell	Sheep	Chicken	Human
Sheep	0*	64	128
Chicken	2	0	16
Human	4	32	8
None	32	64	128

* Figures in column are the reciprocal of the last dilution showing macroscopic agglutination.

Absorption with human cells in the manner described above failed to remove all the activity against human cells. Complete removal of the anti-sheep activity, the weakest, caused no reduction in either the chicken or human titres. Reduction of the strongest activity, anti-human, failed to cause a reduction of similar magnitude in chicken titre.

The/

The affinity for a wide range of vertebrate erythrocytes would appear to indicate that the red cell receptor involved is widely distributed and common to many vertebrate erythrocytes. A similar affinity has been reported for the hemagglutinins in the snail (GILBERTSON & ETGES, 1967), oyster (TRIPP, 1966; McDADE & TRIPP, 1967a) and the clam, mussel and crayfish (McKAY et al., 1969). However, from the absorption studies, it seems that there are present in cockroach hemolymph some hemagglutinins with a degree of specificity for certain vertebrate species, although marked cross reactivities are evident. Similar results are reported for the clam, mussel and crayfish (McKAY et al., 1969).

The presence of blood group reactive substances in invertebrate fluids has been reviewed in some detail in Part I and the cockroach agglutinin was next tested for any specificity with respect to the human A.B.O. blood group antigens. None was found (Table III).

TABLE III: Antigenic type of human erythrocyte titrated against cockroach hemolymph.

Human cell type:	A	B	O
Hemaggn. titre:	64	64	64

In vivo confirmation of agglutinating activity. Sheep red cells in saline were injected into the hemocoele of cockroaches which were then immediately killed with ether. At autopsy the red cells were clearly visible as agglutinated clumps, both free in the hemocoele/

hemocoele and loosely associated with the internal tissue surfaces.

Occurrence of agglutinin. COHEN (1967) reported that a hemagglutinin in the coconut crab (Birgus latro) was not found in the serum of relatively young individuals, which suggested a possible correlation of appearance with life cycle of the species. Whole plasma was extracted from eight cockroach nymphs (2.5-3.0 cm. in length), pooled and tested for agglutinating activity against sheep cells. Agglutinating activity was present at a titre of 512.

Heat treatment. 0.1 ml. aliquots of saline-extracted hemolymph were heated in a water bath at 56°C for 30 minutes, allowed to cool to room temperature and then assayed for hemagglutinating activity against sheep cells. Control hemolymph was maintained at room temperature. Agglutinating activity was completely abolished by heat treatment; the experimental and control titre being 0 and 64 respectively. Slide agglutination tests (microscopic) confirmed heat inactivation of the chicken cell agglutinin.

Dialysis. 0.1 ml. aliquots of saline-extracted hemolymph were dialysed against 2 litres of cockroach ringer (see Appendix I), phosphate buffered saline (P.B.S.) pH 7.4 or distilled water at 4°C for 24 hours. Control hemolymph was maintained at 4°C. Sheep cells were used in all assays and the results are set out in Table IV. Agglutinating activity was not dialysable against ringer or phosphate buffered saline. Dialysis against distilled water/

water caused precipitation and complete loss of activity. This was probably due to protein precipitation (see below).

TABLE IV: Dialysis of cockroach hemolymph.

Dialysed against:	Ringer	P.B.S.	Dist. H_2O
Hemaggn. titre:	64	64	0

Trichloro-acetic acid (TCA) precipitation. 0.15 ml. of 20% TCA was added to an equal volume of whole plasma at 0°C. Precipitation occurred for 5 minutes and the precipitate was then separated by centrifugation at 2°C. The supernatant was dialysed against 2 litres of phosphate buffered saline for 24 hours to remove TCA and then assayed for agglutinating activity against sheep cells. Control hemolymph mixed with an equal volume of buffered saline was similarly treated. TCA treatment completely removed agglutinating activity, the experimental and control titres being 0 and 64 respectively. This indicates the agglutinin to be protein or protein-bound; its insolubility in water referred to above suggests that the protein is euglobulin.

'Immunisation' with sheep red cells. The following experiments were to test whether the naturally occurring hemagglutinating activity was inducible i.e. whether the hemagglutinating titres were increased following injection of sheep red cells.

Nine cockroaches were each injected via the intersegmental membrane/

membrane between the abdominal sternites with 0.05 ml. of 5% washed sheep cells in 0.85% saline. At 12, 24 and 48 hours after injection, groups of three cockroaches were 'bled' using the saline extraction technique, and the pooled hemolymph samples were assayed for hemagglutinating activity against sheep cells. Control cockroaches received either 0.05 ml. saline or were uninjected. Aliquots of the pooled hemolymph samples were diluted 2% in saline and read at 280mμ in a spectrophotometer. In no case did the difference in protein concentration (O.D. units) exceed one doubling dilution. The results are set out in Table V. No increase in hemagglutinating activity after injection of red cells was found.

Both TRIPP (1966) and ROWLEY (1967) reported that they too were unable to increase the titres of the naturally occurring hemagglutinins in the oyster and crayfish respectively. The acquired anti-bacterial principles described in insects by GINGRICH (1964) and STEPHENS (1962) both showed peak activity around 24 hours.

TABLE V: Changes in titre of the naturally occurring sheep red cell agglutinin in cockroach hemolymph following injection of red cells.

	Inoculum		
Time after injection	Sheep cells	Saline	Non-injected
12 hrs.	32*(0.50)+	32(0.56)	32(0.42)
24 hrs.	32 (0.55)	32(0.37)	32(0.28)
48 hrs.	32 (0.38)	32(0.46)	64(0.55)

* Hemagglutination titre.

\+ Figures in brackets refer to protein concentration in O.D. units.

Complement fixation. Agglutinations of sheep cells by whole cockroach plasma and rabbit anti-sheep-cell serum (see page 88) were set up in parallel and allowed to settle out in the refrigerator overnight. The red cells were then resuspended and to each dilution was added 0.1 ml. of complement (normal guinea pig serum diluted 1/4 in saline). Complete lysis of red cells occurred at all dilutions of rabbit serum (titre 512), whereas no lysis was visible at any hemolymph dilution after 1 hour at room temperature. This lack of complement fixing activity is in keeping with the reported absence of such activity amongst insects (CHADWICK, 1967) and invertebrates in general. Complement activity is considered to have evolved among the lower vertebrates, weak activity being detectable among the lower elasmobranchs (GEWURZ et al., 1966). A naturally occurring hemolytic system has been described recently in the lobster, but it differs from the classical guinea pig complement system, and may be attributable to enzyme action (WEINHEIMER et al., 1969).

Hemagglutination inhibition. McDADE & TRIPP (1967a) in their studies on the oyster hemagglutinin, tested a range of saccharides for their ability to inhibit agglutination. Activity against human cells was completely inhibited by n-acetyl glucosamine and n-acetyl-D-galactosamine suggesting attachment to the ABO haptens since these saccharides are known to be part of the 'backbone' molecule common to the ABO antigens. Similarly, JOHNSON (1964) found that both these saccharides inhibited the anti-human A_1 activity/

activity which occurred naturally in the butter clam (Saxidomus giganteus).

The following saccharides; n-acetyl-D-glucosamine, n-acetyl-D-galactosamine, D-mannose and D-xylose (Sigma) were tested for any inhibitory action with the cockroach hemagglutinin. 5 mg. of saccharide in 0.05 ml. of 0.85% saline were added to 0.1 ml. dilutions of saline extracted hemolymph. These mixtures were incubated for 30 minutes at room temperature and 0.05 ml. of a 2% suspension of human red cells (Blood group A) was added. After 1 hour at room temperature and overnight in the refrigerator, titres were read as before. The results are set out in Table VI. Both D-mannose and D-xylose, which had no effect on the oyster or clam hemagglutinating activity, caused a possible slight reduction in titre. There was no reduction in the presence of either n-acetyl-D-glucosamine or n-acetyl-D-galactosamine.

TABLE VI: Inhibition of hemagglutinating activity.

Treatment	Human A cells Titre
None	128
D-Xylose	64
D-Mannose	64
n-acetyl-D-galactosamine	128
n-acetyl-D-glucosamine	128

Sperm/

Sperm agglutination. The heteroagglutinin described in lobster serum was capable of agglutinating both vertebrate sperms and erythrocytes (TYLER & METZ, 1945). Cockroach hemolymph was therefore tested for its effect on vertebrate sperm.

Spermatozoa from random bred Q strain mice were stripped from the vas deferens into buffered saline. The suspension was then lightly centrifuged to remove epithelial cells and clumped sperm. 0.05 ml. of sperm suspension was added to 0.05 ml. of serially diluted hemolymph (saline extract) and examined microscopically for agglutination after 20-30 minutes at room temperature. A negative control consisted of sperm in saline and a positive control of antiserum to soluble antigens of mouse sperm was kindly provided by Dr. E.B. Bell.

With hemolymph of hemagglutinating titre 32, mouse sperms were observed to be agglutinated to the same titre. The agglutination pattern (Fig. I) was similar to that observed in the positive control and also to that of pictures of iso-antibody agglutination of mouse sperm (BELL, 1968). It appeared to be predominantly tail to tail agglutination, many sperms having their tails enmeshed and their heads beating vigorously. In the negative controls sperms remained free, although occasional head to head agglutination was observed.

To determine whether the hemagglutinin was identical with the sperm agglutinin, the experiment was repeated with hemolymph that had been absorbed with sheep cells and shown to contain no residual/

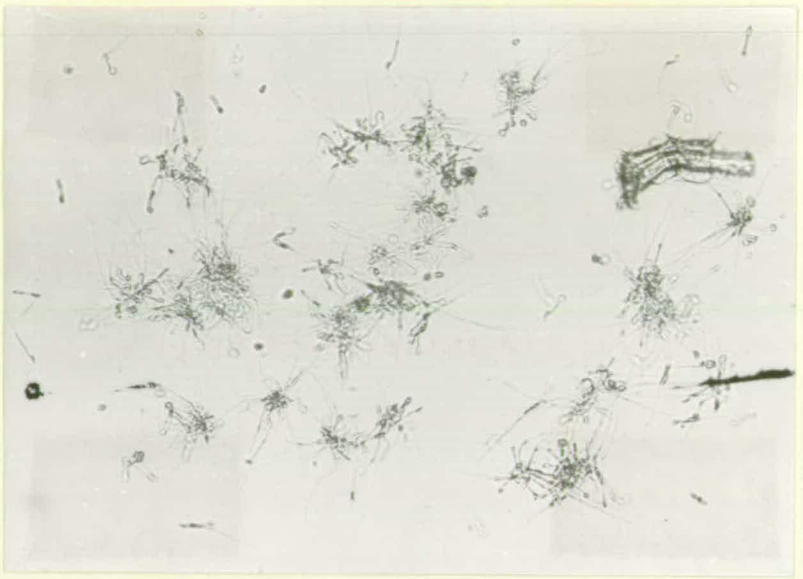
residual hemagglutinating activity. Again the saline control showed free sperms with occasional head to head agglutination. Non-absorbed hemolymph gave a typical 'immune type' agglutination pattern (titre 32), but with absorbed hemolymph no such agglutination occurred. Instead, at low dilutions of hemolymph, there was marked head to head agglutination with tails thrashing freely. At the same time many free sperms were visible (Fig. 2). This head to head agglutination fell off below the 'immune' agglutination titre (8 as compared to 32) and may correspond to the non-specific agglutination which is known to occur with mammalian sperms in serum (CHANG, 1947). Alternatively, another type of agglutinin may have been unmasked, since complete removal of sheep cell activity did not remove all the activity against other erythrocytes in previous experiments. In any case, the agglutination patterns with absorbed and non-absorbed hemolymph were so completely different (see Figs. 1 and 2) that it was concluded that absorption with sheep cells had completely removed immune type sperm agglutinating activity, and that the hemagglutinin and sperm agglutinin were therefore identical.

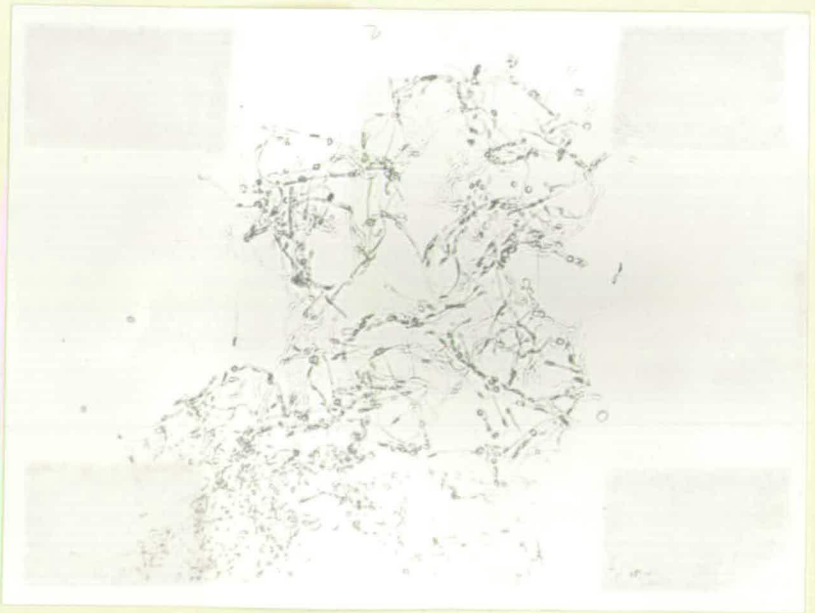
Cockroach hemolymph was next tested for activity against homologous cockroach sperm. Cockroach sperms were obtained by dissecting out the mushroom glands from two adult male cockroaches and placing them in a watch glass containing buffered saline. Some lobes of the glands were then snipped off with scissors and the sperms escaped into the surrounding saline. After about

5/

Fig. 1 : Agglutination of mouse sperms by cockroach hemolymph - predominantly tail to tail agglutination.

Fig. 2 : Head to head agglutination of mouse sperms by cockroach hemolymph absorbed with sheep red cells.





5 minutes, the surrounding saline was pipetted up and lightly centrifuged to remove contaminating cell debris and clumped sperms. Hemolymph was assayed for agglutinating activity against the free sperms in the supernatant as with mammalian sperms. No agglutinating activity was found. The sperms remained motile in buffered saline for many hours.

Bacterial and yeast agglutination. In an effort to throw light on a possible functional reason for the existence of hemagglutinating activity in the cockroach, hemolymph was tested to see whether the activity cross reacted with any activity directed against micro-organisms. Four species of yeast and ten species of bacteria covering a wide range of types were used. Dr. I. Campbell (Microbiology Department, Heriot Watt University, Edinburgh) kindly advised, as well as preparing and providing the cultures. The bacteria included two known potent insect pathogens, P. aeruginosa and B. thuringiensis. Slide agglutination tests were carried out in saline and the results are set out in Table VI.

The only positive result was against the capsulated bacterium Aerobacter aerogenes. To establish whether it was in fact the hemagglutinin that was acting, heat inactivated hemolymph, with confirmed negative hemagglutinating activity, was tested with no reduction in intensity of bacterial agglutination. It was concluded that separate agglutinins were involved.

TABLE VII/

TABLE VII: Agglutination of various micro-organisms by cockroach hemolymph.

	Micro-organisms	Agglutination
Yeasts:	Hanseniaspora valbyensis	-
	Schizosaccharomyces pombe	-
	Saccharomyces cerevisiae	-
	Rhodotorula	-
Bacteria:	Pseudomonas aeruginosa	-
	Micrococcus sp.	-
	Staphylococcus albus	-
	Bacillus macerans	-
	Escherichia coli	-
	Salmonella typhimurium	-
	Bacillus thuringiensis	-
	Staphylococcus aureus	-
	Aerobacter aerogenes	+
	Serrata marcescens	-

\+ present - absent

Comment. Natural hemagglutinating activity has been reported before in insects; in lepidopterous larvae (BERNHEIMER, 1952; BERNHEIMER et al., 1952) and the large milkweed bug (FIER & WALZ, 1964). In these cases, hemagglutinating activity was characterised by its extreme lability; activity being lost on standing at room temperature and on freezing. The activity described here for the American cockroach appears to be different; one sample of whole plasma showed unimpaired agglutinating activity in a slide test/

test after 2 years storage at -20°C. Bacterial studies revealed no cross reactivity with the organisms screened, and hemagglutinating activity in no way resembled the highly heat-stable, non-protein, acquired anti-bacterial principles described in some insects (HINK & BRIGGS, 1968; GINGRICH, 1964; STEPHENS, 1962). From the protein nature and heat lability of the hemagglutinating activity, it more closely resembles the acquired anti-tetrahymena principle described in the male American cockroach by SEAMAN & ROBERT (1968). However, hemagglutinating activity occurs naturally and is also present in female cockroaches.

From the previous experiments, the cockroach hemagglutinin can be characterised as being, or being bound to, a heat labile, non-dialysable protein which behaves as a euglobulin. Table VIII compares some of the characteristics of the cockroach hemagglutinin with those reported to date for other invertebrate hemagglutinins. The cockroach is the only terrestial invertebrate in the group, but a wide range of invertebrates is represented; fresh water gastropods, marine molluscs and both fresh water and marine crustaceans. Regarding the characteristics listed, none of the other studies has been as comprehensive as those on the oyster on which the cockroach studies were based. The cockroach agglutinin so far closely resembles that in the oyster. The lack of inhibition of human red cell agglutination by acetyl-D-glucosamine and acetyl-D-galactosamine would indicate that the cockroach hemagglutinin combines with a site on the cell other than the blood group/

REFERENCES FOR TABLE VIII.

(1) This Thesis.

(2) TRIPP (1966), McDADE & TRIPP (1967a).

(3) TYLER & METZ (1945), TYLER & SCHEER (1945).

(4) GILBERTSON & ETGES (1967).

(5) JOHNSON (1964).

(6) MARCHALONIS & EDELMAN (1968).

(7) McKAY *et al*. (1969).

(8) McKAY *et al*. (1969).

		Protein	Non-dialysable	Heat labile	Precicipitation in dist. H_2O	Agglutinates wide range of vert. erythrocytes	A.B.O. Specificity	Inhibited by some saccharides	Inducible	Agglutinates sperm
Cockroach (P. americana)	(1)	+	+	56°C	+	+	-	-	-	+
Oyster (C. virginica)	(2)	+	+	65°C	+	+	-	+	-	
Lobster (P. vulgaris)	(3)	+?	+	56°C		+				+
Planorbid snails	(4)			56°C	+	+				
Butter clam (S. giganteus)	(5)		+	70°C			A_1	+		
Horseshoe crab (L. polyphemus)	(6)	+								
Crayfish (P. bicarinatus)	(7)	+	+	57°C		+	-		-	
Mussel (V. ambiguus)	(8)									

Blank spaces = not tested

TABLE VIII: Some properties of some invertebrate hemagglutinins.

group hapten.

Generalising and extrapolating from the relatively scant data presented in the Table, the various hemagglutinins show a marked degree of similarity in that they all might be heat labile, non-dialysable, euglobulin-type proteins capable of agglutinating a wide range of vertebrate erythrocytes. Initially then, the cockroach hemagglutinin bears no resemblance to hemagglutinins previously described in insects, but resembles more the activities described in a variety of non-insect invertebrates. This aspect of the cockroach agglutinin will be considered further in Chapter IV, in the light of further investigations. With regard to the heat lability of these substances, it should be mentioned here that preliminary reports on purified mussel hemagglutinin show it to be protein in nature and heat stable at 57°C, whereas hemagglutinating activity of whole hemolymph is destroyed at this temperature. This would suggest that the loss of hemagglutinating activity might be due to absorption onto other denatured protein (McKAY et al., 1969).

C. ELECTROPHORETIC ANALYSIS OF HEMOLYMPH.

MATERIALS AND METHODS.

Both simple and immuno-electrophoresis were carried out with L.K.B. microelectrophoresis apparatus. 1% agarose (L'Industrie Biologique, Francaise S.A.) in veronal buffer pH 8.2 was used throughout, except for the simple electrophoretic comparison of mouse serum and hemolymph, when 1.5% Special Agar Noble (Difco) was/

was substituted.

Simple electrophoresis. With a 1 mm. diameter well, undiluted whole plasma was used. With a 1 mm. x 15 mm. well the plasma was mixed 2:1 with 46°C agarose. Migration in both cases was for 1 hour at 250 V. Slides were fixed in 2% acetic acid for 30-40 minutes, rinsed in distilled water for 15 minutes and air-dried at 37°C prior to staining.

Rabbit anti-whole plasma serum. An adult rabbit was injected with 0.5 ml. pooled whole plasma from adult cockroaches, mixed with 0.5 ml. pertussis vaccine (Burroughs Wellcome Ltd.) and emulsified in 1 ml. Freund's Complete Adjuvant (Difco). Half the dose was injected subcutaneously into the top of each thigh. The dose was repeated at 4 and 5 weeks, the animal being bled 2 weeks after the last injection. Serum was prepared and stored in aliquots at -20°C.

Immunoelectrophoresis. Undiluted whole plasma from male cockroaches and rabbit anti-whole plasma serum were used. Separation was for 1 hour at 250 V. Precipitation occurred over 24 hours and unprecipitated protein was removed with 3 washes of 1.5% NaCl over 3 days. Slides were then rinsed in distilled water for 5 minutes, air-dried at 37°C under filter paper and stained.

Staining techniques (see Appendix II for procedures).
Protein - Napthalene Black, Ponceau Red or Lissamine Green.
Glycoprotein/

Glycoprotein - phenylhydrazine-HCl.

Lipid/lipoprotein - Sudan Black.

Esterase - β napthyl acetate.

RESULTS.

Comparison of mouse serum and hemolymph. A comparative simple electrophoresis of normal mouse (C57BL) serum and whole plasma was run in agar (Fig. 3). No cockroach protein was found in the gamma globulin region, all hemolymph components migrating towards the anode at pH 8.2. The bulk of hemolymph proteins migrated in the fast alpha and slow beta regions. This lack of proteins migrating in the gamma region is in keeping with data for other invertebrates (ENGLE et al., 1958)

Proteins. Simple electrophoresis revealed a fast (A) and a slow (B) moving protein component Fig. 4. Immunoelectrophoresis revealed six distinct protein arcs labelled in Fig. 8.

Glycoproteins. Both the A and B components gave a positive reaction for glycoprotein, their identity being confirmed by counterstaining with Lissamine Green. All immunoelectrophoretic arcs, except IV, gave a positive reaction, their identity being confirmed by counterstaining with Napthalene Black (Figs. 5, 9).

Lipid/lipoprotein activity. A thin, slow moving band of lipid-positive material appeared in simple electrophoresis. Ponceau Red/

Fig. 3 : Simple electrophoresis of normal mouse (C57BL) serum and cockroach hemolymph.

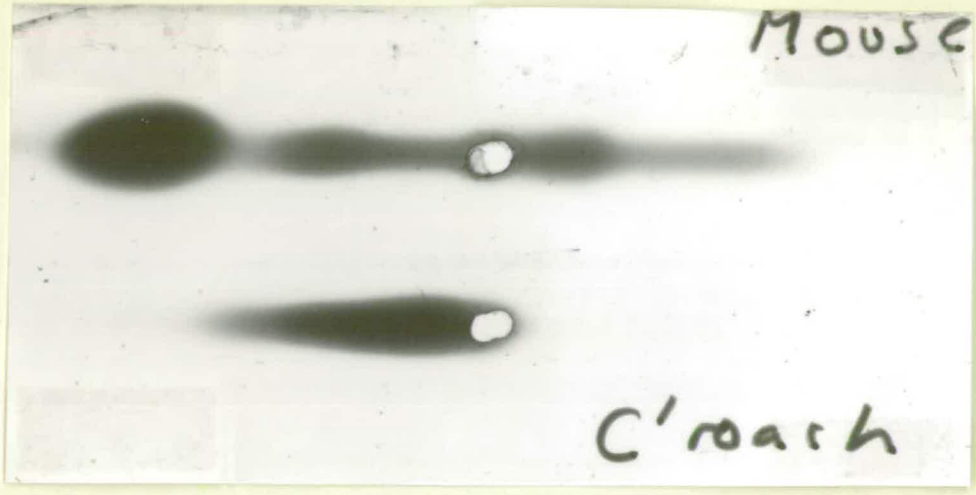
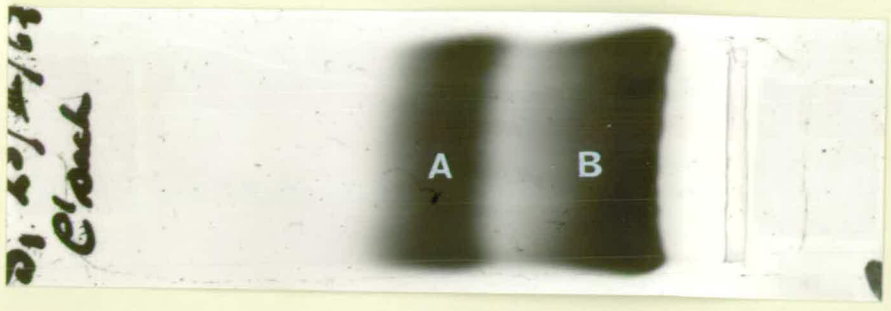

Mouse
C'roach

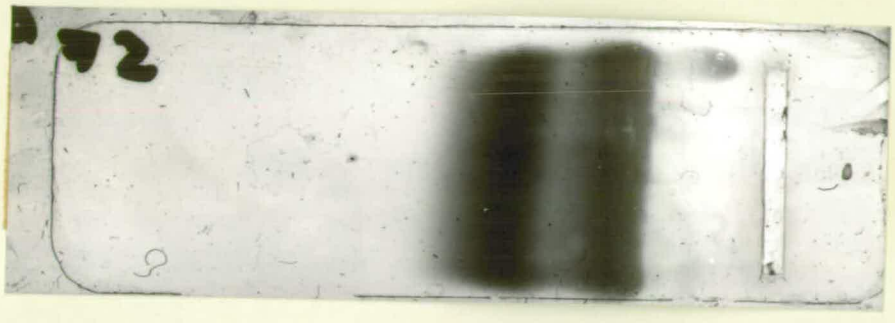
Fig. 4 : Simple electrophoresis of cockroach hemolymph stained for protein with napthalene black.

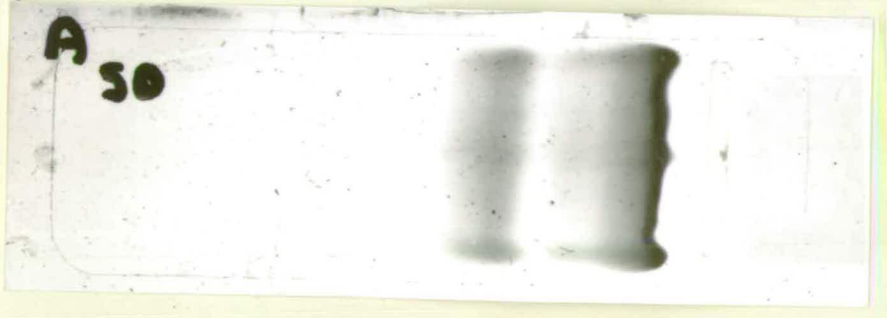
Fig. 5 : Cockroach hemolymph stained for saccharide and counterstained for protein with lissamine green.

Fig. 6 : Cockroach hemolymph stained for lipid and counterstained for protein with napthalene black.

Fig. 7. Cockroach hemolymph stained for esterase activity and counterstained for protein with napthalene black.


A
B





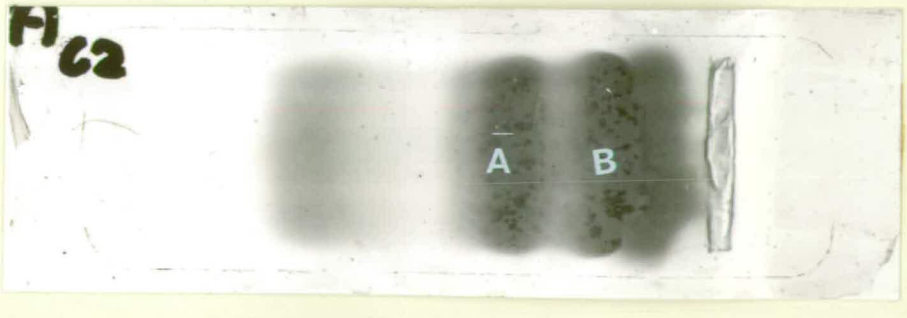
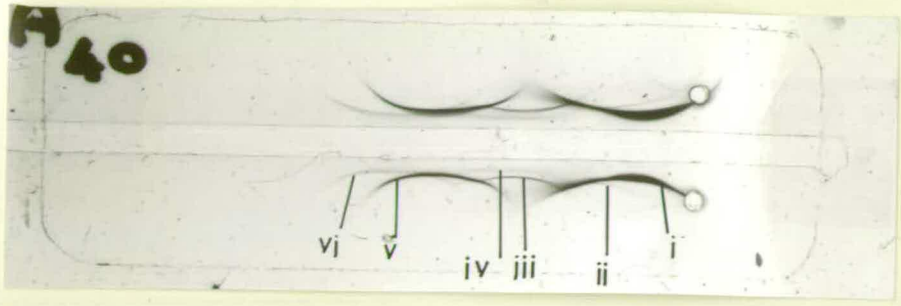

A
B

Fig. 8 : Immunoelectrophoresis of cockroach hemolymph stained for protein with napthalene black.

Fig. 9 : Cockroach hemolymph stained for saccharide.

Fig. 10 : Cockroach hemolymph stained for lipid.

Fig. 11 : Cockroach hemolymph stained for esterase activity.


A40
vi
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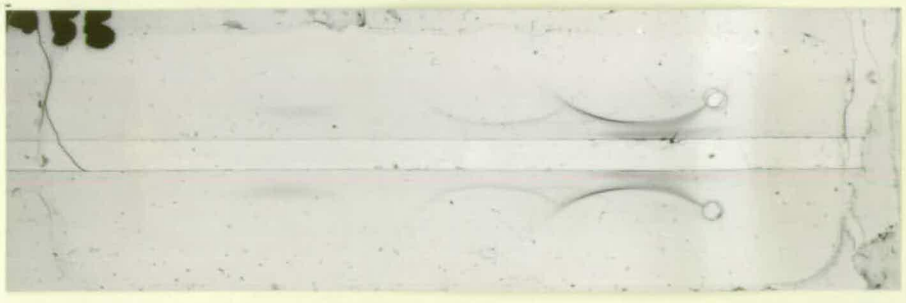
A66


A46


A55

Red counterstaining showed it to be the trailing edge of component B. Immunoelectrophoretically, lipid was confined to arc I (Figs. 6, 10).

Esterase activity. A slow and a very fast moving band stained for esterase activity. Counterstained with Napthalene Black, the slow moving band was distinct from component B and the fast band considerably faster than A. The fast moving band appeared as a new arc in immunoelectrophoresis. The slow band was identical with arc I and another arc appeared on the trough side of arc I (Figs. 7,11).

D. ANTIGENIC IDENTITY OF AGGLUTINATING ACTIVITY.

Absorption. The first attempts to characterise hemagglutinating activity antigenically were by absorbing out the activity and looking for the disappearance of any of the six antigen arcs.

Preliminary experiments established that the activity was indeed either itself antigenic or associated with an antigenic protein. Rabbit anti-whole plasma serum was absorbed with an equal volume of packed sheep cells for 1 hour at room temperature, and again with fresh packed cells for 1 hour at $0^{o}C$ to remove natural anti-sheep-cell activity. To 0.1 ml. saline-extracted hemolymph was added 0.1 ml. absorbed anti-hemolymph serum and precipitation occurred for 30 minutes at room temperature. The precipitate was then spun down and the supernatant tested for agglutinating/

agglutinating activity against sheep cells. Control hemolymph was similarly treated with absorbed normal rabbit serum and no precipitation was observed. Treatment with anti-whole plasma serum completely abolished agglutinating activity, the experimental and control titres being 0 and 16 respectively.

Saline-extracted hemolymph, hemagglutinating titre 64, was used. Immunoelectrophoretic comparison of this with whole plasma showed arcs I, III, IV, V and VI to be present. 0.1 ml. hemolymph was absorbed twice with packed sheep cells during 1 hour at room temperature. Control hemolymph was maintained at room temperature during this period. Before immunoelectrophoresis, slide agglutination tests confirmed the presence and absence of agglutinating activity in experimental and control hemolymphs respectively. The immunoelectrophoretic results are shown in Fig. 12 , there being no apparent difference between absorbed and non-absorbed hemolymph. It would appear then that agglutinating activity does not represent the whole of any of the six distinct antigens present in whole hemolymph, but only constitutes a part of one or more of them. McDADE & TRIPP (1967a) were likewise unable to characterise the oyster hemagglutinin antigenically by absorption followed by Ouchterlony double diffusion tests.

Ethanol fractionation. Crude ethanol fractionations were carried out on whole plasma using Analar Ethanol at $0^{o}C$. Preliminary experiments determined that no precipitation of hemolymph occurred with/

with 10% ethanol, whereupon fractionation was carried out according to the scheme shown in Table IX. After dialysis against buffered saline, samples were assayed for agglutinating activity against sheep cells. Agglutination was read microscopically and graded semi-quantitatively from xxxx to 0. The results are shown in Table X.

Nearly all hemagglutinating activity was precipitated consistently with 25% ethanol. Activity was also precipitated with 15% ethanol and is therefore among the first proteins to be precipitated with increasing ethanol concentration.

The 25%, 50% and 80% fractions were examined immunoelectrophoretically. The second wells contained whole plasma for comparison and identification of arcs and the results are shown in Figs. 13, 14, 15.

The 25% fraction consisted of a strong and well defined arc I, with a diffuse trace of what appeared to be arc III. The 50% fraction consisted of arc V. The 80% fraction consisted of arc VI.

From this somewhat crude analysis, agglutinating activity is associated with the electrophoretically slow moving fraction of the cockroach hemolymph proteins. The slight trace of arc III in the 25% fractions should not be disregarded but, from the strength of the activity in these fractions, it seems likely that the activity might be associated with the slowest moving antigenic arc I. This has been demonstrated to contain lipid and/

TABLE IX: Ethanol (EtOH) fractionation of cockroach hemolymph.

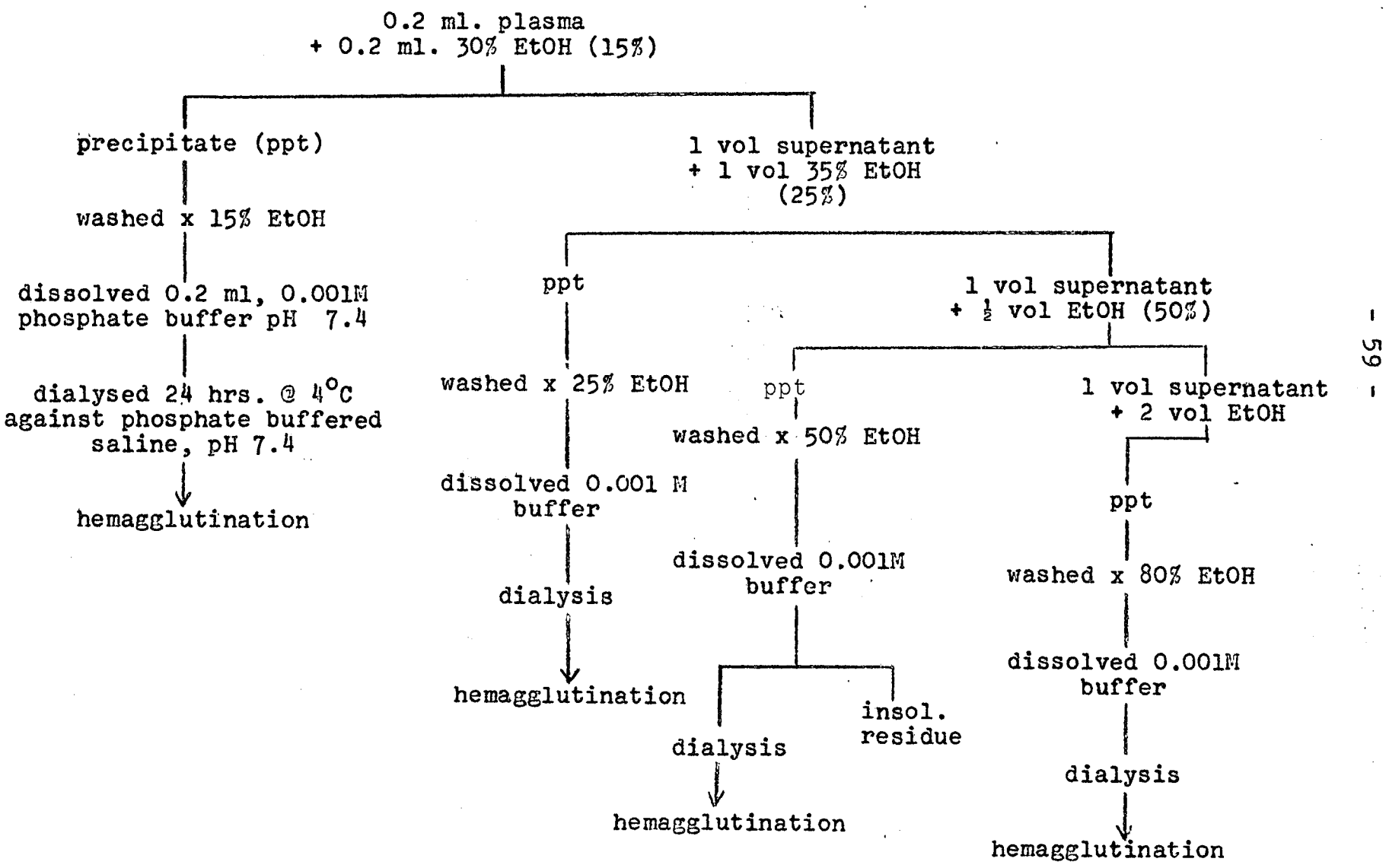


TABLE X: Hemagglutinating activity of ethanol fractions of cockroach hemolymph.

Hemolymph	% Ethanol	Hemolymph dilution 1/2	1/4	1/8	1/16	1/32	1/64	1/128	Control
Lot 1	10	No precipitation							
	15	++++	++++	+++	++	±	-	-	-
	25	++++	++++	++++	+	±	-	-	-
Lot 1	25	++++	++++	++++	++++	+++	+	-	-
	50	+	-	-	-	-	-	-	-
	80	-	-	-	-	-	-	-	-
Lot 2	15	++++	++++	++	+	-	-	-	-
	25	++++	++++	+++	+	-	-	-	-
	50	++++	+	-	-	-	-	-	-
	80	-	-	-	-	-	-	-	-
Lot 3	15	++++	++++	++	±	±	-	-	-
	25	++++	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-
	80	-	-	-	-	-	-	-	-

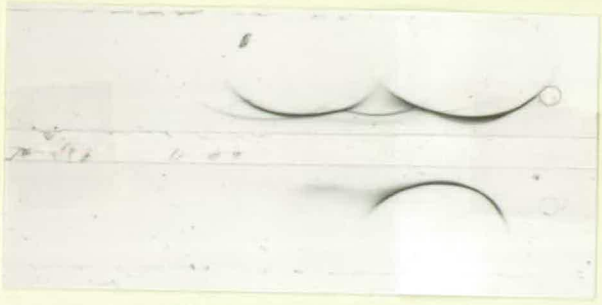
Fig. 12 : Immunoelectrophoretic comparison of normal cockroach hemolymph (C) and hemolymph absorbed with sheep red cells (A).

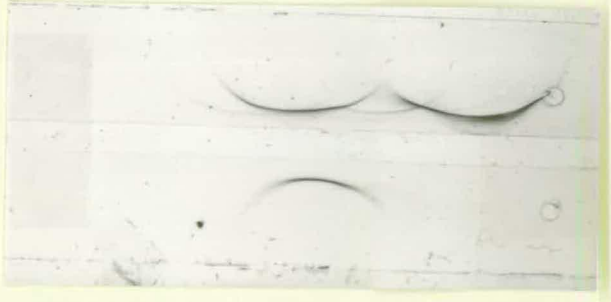
Fig. 13 : Hemolymph fraction precipitated with 25% ethanol, compared with normal hemolymph.

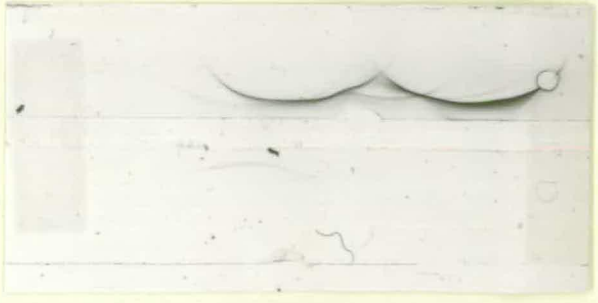
Fig. 14 : Hemolymph fraction precipitated with 50% ethanol, compared with normal hemolymph.

Fig. 15 : Hemolymph fraction precipitated with 80% ethanol, compared with normal hemolymph.


A
C







and saccharide, and to possess esterase activity.

McDADE & TRIPP (1967a) reported failure to fractionate the oyster hemagglutinin with Ammonium sulphate. Zone electrophoresis on starch (pH 8.0) of whole crab hemolymph has shown that hemagglutinating activity is associated with the electrophoretically slow moving fraction of the hemolymph proteins (MARCHALONIS & EDELMAN, 1968).

CHAPTER II HEMOCYTE STUDIES

A. COCKROACH HEMOCYTES.

Current concepts concerning insect hemocytes have been comprehensively reviewed by WIGGLESWORTH (1959) and JONES (1962). The hemocytes are those cells which either circulate in the hemolymph and/or are attached loosely to the various tissue surfaces within the hemocoele. The extent to which hemocytes are freely circulating varies among different insects. JONES (1953) demonstrated that, in fixed specimens of adult P. americana, there are usually abundant free hemocytes in both the heart lumen and the hemocoele. The origin and proposed functions of hemocytes, which include roles in connective tissue formation, intermediary metabolism and hemolymph coagulation, are discussed in the above reviews. The function that we are concerned with here has been clearly established by a wealth of observation and experiment; this is the hemocyte's phagocytic role and the part it plays in immunity and protection from bacterial infection. Phagocytosis of a whole range of target particles by the hemocytes of various insects has been recorded by JONES (1962), and more particularly, BETTINI et al. (1951) describe the uptake of vertebrate erythrocytes by P. americana hemocytes.

Hemocyte extraction and monolayer formation. Hemocytes were obtained by lavage of the cockroach hemocoele. A cockroach was lightly anaesthetised with ether and placed on a clean glass slide. The/

The meso- and meta-thoracic legs were cut off close to the body. 0.1-0.2 ml. Parker's tissue culture medium 199 (Glaxo) were then injected into the hemocoele by means of a 27g needle inserted through the intersegmental membrane between the abdominal sternites. The hollow centres of the legs are continuous with the hemocoele and fluid emerges from the leg stumps, whence it is readily taken up with a Pasteur pipette. This procedure was repeated two to three times. Care was taken not to puncture and inject into the gut; if this happened fluid emerged from the mouth and anus and such animals were discarded. Pooled cells from several cockroaches were washed by centrifugation and resuspension in medium 199, the final concentration being such that cells from six cockroaches were resuspended in 0.1 ml. medium 199.

Culture wells, similar to those used in mammalian cytophilic antibody studies (BERKEN & BENACERRAF, 1966), were constructed using lucite rings, 2-3 mm. high with inner diameter 10 mm. Silicone grease was applied to the flat surfaces and the rings stuck onto clean 22 mm^2 coverslips making watertight 'reaction wells'. 0.1 ml. aliquots of hemocyte cell suspension were added to the wells and left at room temperature for 40 minutes to allow the hemocytes to settle and adhere to the bottoms of the wells. Hemocyte monolayers were prepared for examination by tilting the well, pipetting off the overlying fluid, and filling the well with medium 199 containing 3% of a neutral red solution/

solution (100 mg. neutral red dissolved in 100 mls. 0.85% saline). A clear glass slide was placed atop each well and the well inverted and examined under the light microscope (Fig. 16).

Cockroach hemocytes. Insect hemocyte populations are made up of many cell types. The generally accepted classification and terminology is that of JONES (1962) who describes nine morphologically distinct types of hemocyte. There is agreement that usually only a few of these types are found per species, with one cell type predominating. The cell type is most commonly dominant is the 'plasmatocyte', which is also generally the most active in phagocytosis (JONES, 1964; WIGGLESWORTH, 1965).

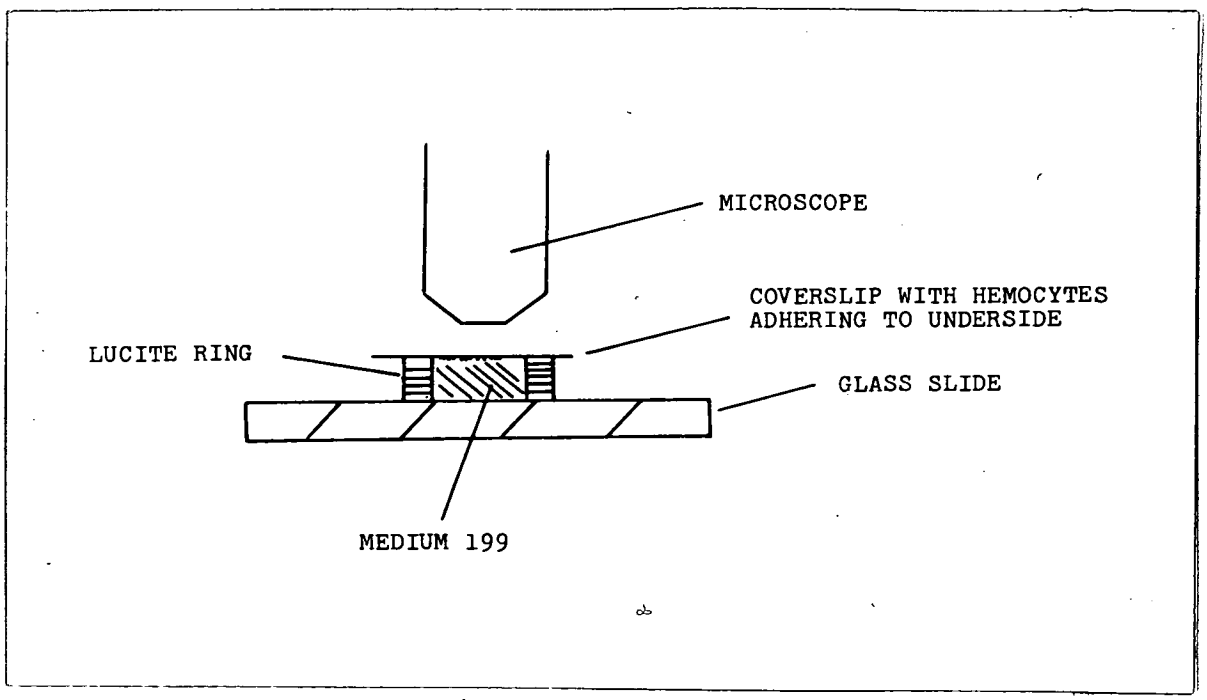
Both WHEELER (1963) and BRADY (1967) experienced difficulty in diluting adult American cockroach blood *in vitro* without the cells clumping. Clumping, in both these instances, was prevented with 0.2% versene. In my own experiments, where dilution of the blood was effectively carried out *in vivo*, the presence of clumped cells in 'wash outs' was only occasional and then slight.

Monolayers of cockroach hemocytes, prepared as described, consisted of essentially homogeneous populations of cells which readily adhered to glass and showed marked uptake of the vital dye neutral red within minutes. Incidentally, these two characteristics are shared by mammalian macrophages (GESNER & HOWARD, 1967). Once on glass, the hemocytes spread out into various shapes and were often seen to extend numerous filamentous pseudopodia/

pseudopodia. On spreading, the large, usually centrally located nucleus became visible (Figs.17,18). Methanol fixed monolayers stained with May Grünwald-Giemsa showed the nucleus and basophilic, granular, vacuolated cytoplasm (Fig. 19). Occasional cells contained bacteria. The cells conformed to the description of plasmatocytes as in JONES (1964) - ' highly pleomorphic cells usually with a large centrally located nucleus in an abundant, basophilic, finely granular cytoplasmic envelope'. The stained preparations were similar to stained monolayers of Cutworm plasmatocytes shown by MARTIGNONI & SCALLION (1961), who also noted adherence to glass. SMITH (1938) studied the blood cells of the American cockroach and was impressed by their constancy and uniformity of structure. From his photographs they are identifiable as plasmatocytes. JONES (1957) making differential counts of hemocytes from adult American cockroaches reported 60-95% plasmatocytes.

Culture medium. Medium 199 is of course a mammalian tissue culture medium, but prolonged survival of insect tissue in this medium has been reported by BECKEL (1956), and certainly the cells used in these studies remained healthy throughout the experiments as judged by appearance, uptake of the vital dye neutral red, exclusion of trypan blue and extent of spreading.

Cell counts. The number of hemocytes obtainable from an insect is, by vertebrate standards, very small. This is due not only to the /

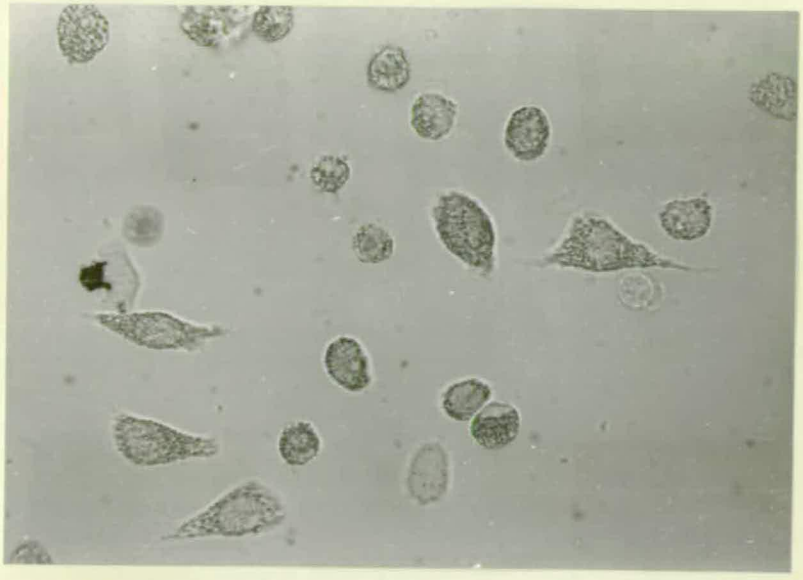

MICROSCOPE
COVERSLIP WITH HEMOCYTES
ADHERING TO UNDERSIDE
LUCITE RING
GLASS SLIDE
MEDIUM 199

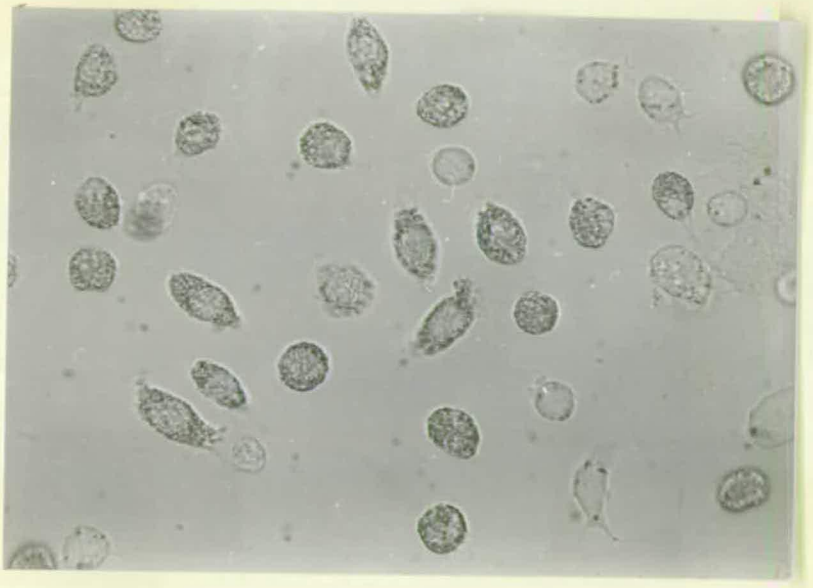
Figs. 17 & 18 : Monolayers of hemocytes with neutral red.

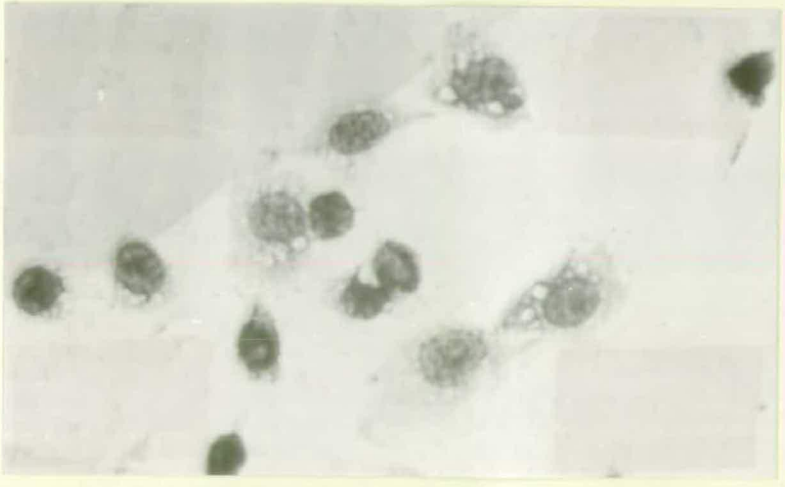
Fig. 19 : Hemocyte monolayer, methanol fixed and stained with May Grünwald-Giemsa.

Figs. 17 & 18 : Monolayers of hemocytes with neutral red.

Fig. 19 : Hemocyte monolayer, methanol fixed and stained with May Grünwald-Giemsa.







the small size of the organism, but also the low cell densities. Insect hemocytes are usually found at concentrations of less than 1 x 10^4/µl (TAUBER & YEAGER, 1935, 1936). Values for the adult American cockroach have been recently calculated as 3.6 x 10^4/µl (BRADY, 1967) and 1.1 x 10^4/µl (WHEELER, 1963) with hematocrit values of 2.6% and 5.9% respectively. SMITH (1938) and WHEELER (1963) considered hemocyte counts along with hemolymph volume and calculated 1.65 x 10^7 and 1.8 x 10^7 as rough estimates of the total hemocytes of the average American cockroach and total number of circulating hemocytes in old, randomly selected, adult American cockroaches respectively.

In this study, cell counts of the total number of hemocytes obtained from individual cockroaches, flushed out with a constant volume of medium 199, were carried out in an improved Neubauer Hemocytometer with Crystal violet in 2% glacial acetic acid diluent. The results are shown in Table XI.

TABLE XI: Total cell counts (X10^3) - individual adult cockroaches.

2, 25, 6, 2, 4, 30, 40, 30, 7, 10, 10, 4.5, 36, 61, X10^3

Variations in individual hemocyte counts have been referred to in Part I. With regard to the previously quoted figures for the cockroach, the wash out technique used here would appear to be somewhat inefficient. However, the amount of any internal hemolymph/

hemolymph coagulation is not known, and also the method only collects hemocytes from the abdominal and thoracic region, bypassing the hemocoelic region of the legs, wings and antennae.

B. STUDIES WITH SHEEP AND CHICKEN RED BLOOD CELLS.

Uptake of sheep and chicken cells. Sheep and chicken red cells were washed thrice in 0.85% saline and resuspended at a concentration of 1% in medium 199. At no time were cells older than one week used.

Monolayers of hemocytes were formed and the overlying fluid was pipetted off and replaced with 0.1 ml. aliquots of either chicken or sheep erythrocyte suspensions. The red cells were allowed to settle for 1 hour at room temperature. Any red cells not attached to hemocytes were then pipetted off and the wells were filled with medium 199 containing neutral red and inverted as before. The wells were left for 5-10 minutes to allow any remaining cells not attached to hemocytes to fall to the bottom before examination.

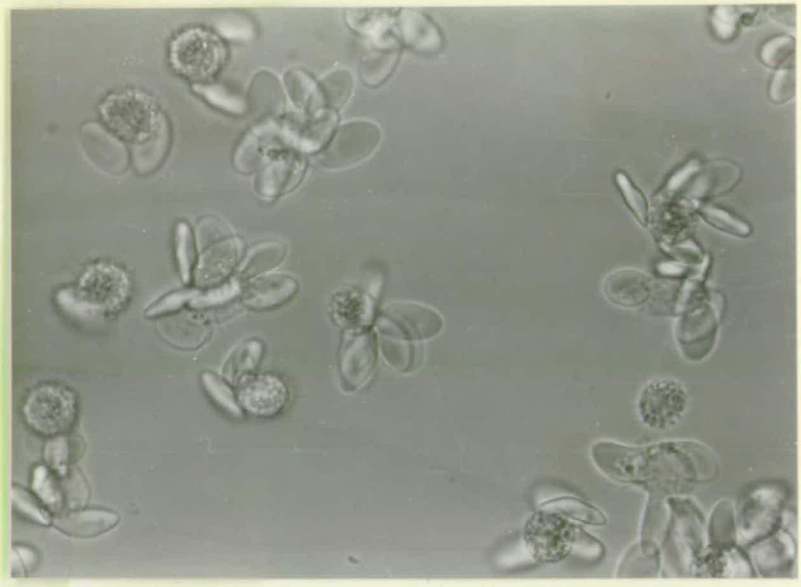
On examination, red cells were seen adhering to hemocytes. The result was rosettes similar to those formed with mammalian macrophages in the presence of cytophilic antibody (BOYDEN, 1964; BERKEN & BENACERRAF, 1966) See Figs. 20,21. Actual phagocytosis of the red cells was only occasionally observed (Fig.22), even when observations were continued up to 5 hours. The association between hemocyte and red cell was, however, extremely intimate. This/

This was especially clear in the case of the chicken cells where the erythrocytes appeared to become deformed (Fig.23). Surface adhesion of a target particle to a mammalian phagocyte precedes the actual transport of the particle across the phagocytic cell membrane. These two stages of phagocytosis can be separated, for example, reduction of temperature arrests the uptake of particles at the surface adherence stage (JANSCO, 1955). BERKEN & BENACERRAF (1966), were able to demonstrate in vitro intake by macrophages of temperature-arrested adhering red cells upon reheating to 37°C. Similar results have been obtained with insect hemocytes. Injection of spores of Bacillus popilliae into larvae of Bombyx mori, maintained at 4°C, resulted in marked adherence of spores to the hemocyte surface with little phagocytosis. The opposite effect was obtained with larvae maintained at 24°C (VAGO & VASILJEVIC, 1965). Adherence as a prerequisite for phagocytosis would appear to be general throughout the animal kingdom. It has been shown to precede phagocytosis of bacteria by American oyster hemocytes (BANG, 1961) and also uptake of erythrocytes by the ameba Entameba histolytica (SEMENOFF, 1938).

In the present experiments, lack of actual phagocytosis was possibly due to a combination of sub-optimal culture conditions and the large size of the target particles. In experiments using considerably smaller target particles - Berlin blue (0.1% in medium 199), the blue particles were clearly seen within the cytoplasm/

Fig. 20 : Hemocytes with adhering sheep red cells.

Fig. 21 : Hemocytes with adhering chicken red cells.



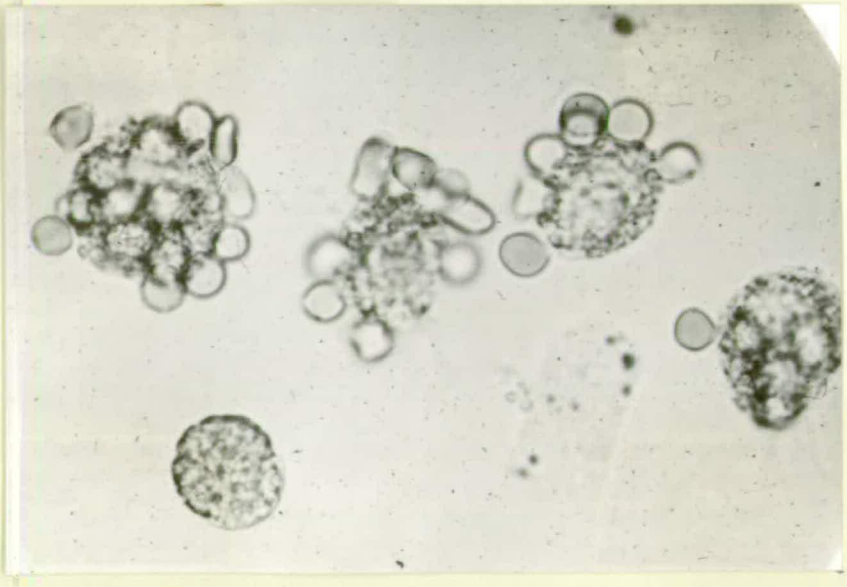
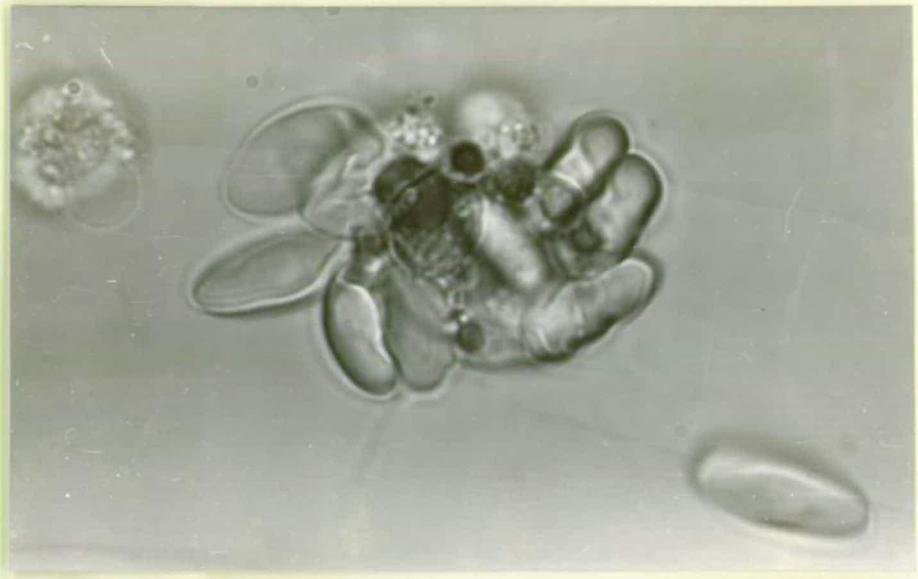
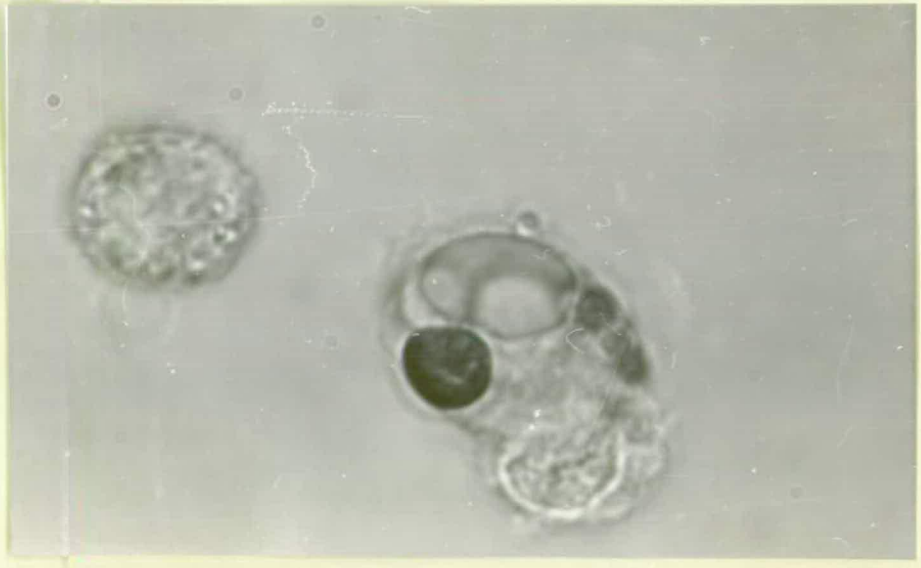


Fig. 22 : Hemocyte containing neutral red vacuole and phagocytosed chicken red cell.

Fig. 23 : Association between chicken red cells and a neutral red containing hemocyte.





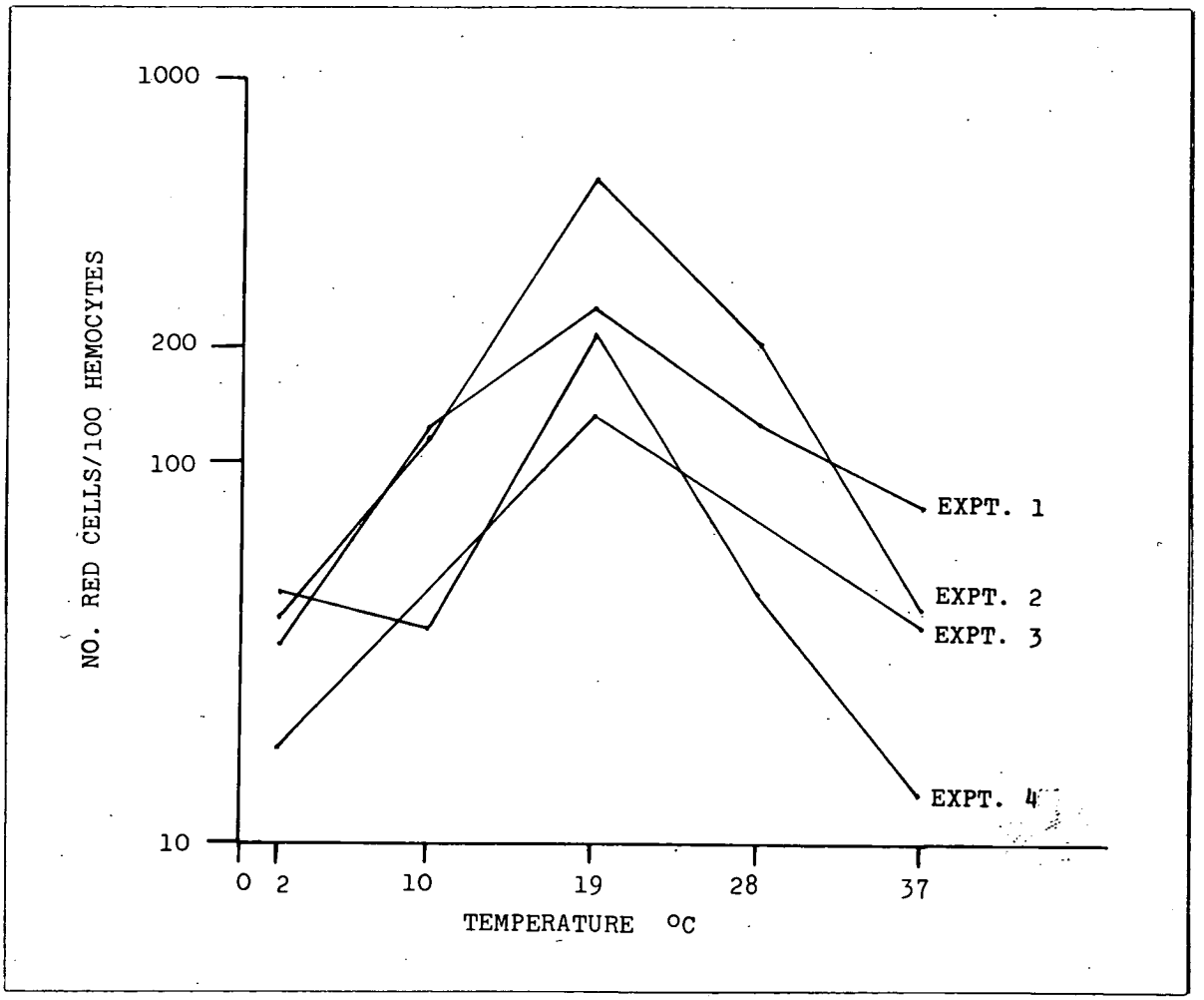
cytoplasm, especially when the hemocytes were 'counterstained' with neutral red. It is of interest here to note the recent work of McKAY et al. (1969). They demonstrated that in vitro cultures of crayfish phagocytic hemocytes would take up erythrocytes that had been presensitised with crayfish hemagglutinin, but they were only able to demonstrate adherence of erythrocytes, never phagocytosis. It was suggested that the effect might be due to the EDTA which was present in their system.

In vivo phagocytosis of red cells. 2 µl of 10% washed sheep cells in 0.85% saline were injected into the hemocoele of each of seven cockroaches using a micrometer activated syringe and 31 g needle. After 1 hour the hemocytes were flushed out, allowed to settle out on coverslips and examined with an inversion microscope. Most of the injected red cells were agglutinated due to the agglutinating activity of the hemolymph and were visible as clumps on the internal tissue surfaces at autopsy. This clearly did not leave many free red cells for phagocytosis by individual hemocytes. However, on examining the hemocytes some were seen to contain an individual red cell and sometimes agglutinated clumps of 2-3 red cells. An occasional free red cell was present.

Effect of temperature on red cell adherence. Sheep cell suspensions, at the requisite temperature, were added to hemocyte monolayers/

monolayers and incubated at that temperature for 1 hour. Non-adhering red cells were then removed and the monolayers examined for red cell uptake. Cell counts of at least 250 hemocytes and the number of red cells adhering to these, were made. Counts were restricted to the central area of the wells as some non-specific sticking of red cells to the coverslip often occurred around the edges due to the silicone grease. The results, expressed as the number of red cells adhering to 100 hemocytes are shown in Fig. 24. As can be seen, adherence was markedly decreased at 2°C suggesting that it is indeed an active process requiring expenditure of metabolic energy and not merely passive sticking. From the temperatures tested, uptake would appear to reach a peak at temperatures that might be construed as optimal for the cockroach. The reason for the fall off in uptake at higher temperatures is not understood. Hemocytes maintained at 37°C were completely normal with respect to appearance and amount of neutral red uptake. RABINOVITCH (1967), using glutaraldehyde treated horse erythrocytes, was able to dissociate the attachment and ingestion phases of their phagocytosis by mouse macrophages. Such erythrocytes adhered to the macrophages but were not ingested in a serum free system, whereas phagocytosis occurred in the presence of serum. The effects of temperature on adherence were studied with results which were similar to my own except that adherence fell away around 43-48°C in the mammalian system. RABINOVITCH suggested that this might be/

Fig. 24 : Effect of temperature on the uptake of sheep red cells by cockroach hemocytes.


1000
200
100
10
NO. RED CELLS/100 HEMOCYTES
0
2
10
19
28
37
TEMPERATURE °C
EXPT. 1
EXPT. 2
EXPT. 3
EXPT. 4

be due to the lack of the protective effect of serum, as phagocytosis in the presence of serum increased at these temperatures. It seems that increased motion at both the hemocyte and macrophage surface might account for this loss of adherence with increased temperature, the macrophage surface being stabilised by the addition of serum (30%).

Effect of calcium and magnesium ions on red cell uptake. The uptake of sheep cells, washed and resuspended in medium 199 containing 0.2% diamino-tetra-acetic-acid (disodium salt), was compared with those prepared in normal medium 199. The presence of EDTA had no adverse effect on the hemocytes and red cell uptake was comparable in both cases:-

TABLE XII: Effect of EDTA on the uptake of sheep red cells by cockroach hemocytes.

Treatment	
EDTA-199	199
251*	236*

* red cells/100 hemocytes

The presence of calcium and magnesium ions was not therefore necessary for red cell uptake by hemocytes.

Enzyme and chemical treatment of hemocytes. On the assumption that a 'receptor site' on the hemocyte surface for sheep red cells might exist, hemocytes were treated with various enzymes and/

and reactive chemicals in attempts to elucidate the nature of the site.

The following enzymes were used (see Appendix III); trypsin, lecithinase-c and lipase. All enzymes were dissolved in medium 199 and monolayers of hemocytes were exposed to the various enzyme preparations at the concentrations indicated for 30 minutes at 37°C. The cells were then washed three times by filling and emptying the wells with fresh cold medium 199. After the final wash, 0.1 ml. of 1% sheep red cell suspension in medium 199 was added, and after 1 hour at room temperature the hemocytes were assessed for red cell adherence. Trypsinised hemocytes adhered less readily to glass and tended to be lost during the washings. Therefore, in two of the trypsin treatments reported, hemocytes were trypsinised in a tube, washed by centrifugation and resuspension, and then transferred to a well and allowed to settle out and form a monolayer. Sheep cells were then added as above. The results are set out in Table XIII.

Pretreatment with a proteolytic enzyme, trypsin, almost completely abolished uptake of sheep cells. At the same time there was no apparent reduction in neutral red uptake by the trypsin-treated hemocytes. There was no reduction but rather enhancement of red cell uptake following lipase treatment. Again there was no decrease in neutral red uptake. This enhancement of uptake is not understood, but, since hemolymph is known to have a high lipid/

lipid content, and the fat body is a prominent tissue, it might be that a masking layer of lipid was being removed. With lecithinase-c there was an obvious reduction in neutral red uptake and a concomitant reduction of sheep cell uptake. This effect may be attributed to the impairment of the integrity of the hemocyte membrane.

TABLE XIII: Enzyme treatments of cockroach hemocytes - effect on the uptake of sheep red cells.

Enzyme	Control	Treated	% Control
Trypsin (0.2%)	225.0*	9.3	4.1
	129.8	2.9	2.2
	117.4	2.0	1.7
	174.4	0.5	0.3
Lipase (0.2%)	89.9	228.8	254.5
	187.5	311.9	116.3
	309.3	404.8	130.9
	162.1	154.0	95.0
Lecithinase-c (0.2%)	124.0	57.6	46.5
	309.3	117.3	37.9
	187.5	10.7	5.7
	89.0	1.4	1.6

* Figures in columns refer to red cells/100 hemocytes.

Chemical treatment. The following chemicals were used; iodoacetamide, sodium nitrate and 2-mercaptoethanol. They were all dissolved in medium 199 and hemocyte monolayers exposed to them at the concentrations indicated for 1 hour at 37°C. Monolayers were then washed and sheep cells added as before. The results are set out in Table XIV.

TABLE XIV/

TABLE XIV: Chemical treatment of cockroach hemocytes - effect on uptake of sheep red cells.

2-Mercaptoethanol

Control	50 mM	200 mM
132.1*	52.4	2.7
74.8	63.4	1.0
Normal+	Normal	Normal or Slightly Reduced

Sodium Nitrite

Control	5 mM	10 mM	50 mM
215.1	228.9	212.3	n.d.
368.5	n.d.	441.2	334.6
Normal	Normal	Normal or Slightly Reduced	Reduced

Iodoacetamide

Control	0.01 mM	0.05 mM	0.5 mM
137.6	168.4	97.2	n.d.
186.2	189.2	197.1	n.d.
175.4	n.d.	n.d.	4.4
Normal	Normal or Slightly Reduced	Reduced	Marked Reduction

* Figures in columns are red cells/100 hemocytes.

\+ Comments refer to neutral red uptake by the hemocytes.

n.d. - not done.

With the reducing agent, 2-mercaptoethanol at 50 mM, there appeared to be a slight reduction in red cell uptake with no corresponding/

corresponding decrease in neutral red uptake. At 200 mM, when neutral red was normal or only slightly reduced, red cell uptake was abolished. Thus the site was sensitive to mercaptoethanol. With 50 mM sodium nitrite, neutral red was reduced with no concomitant decrease in red cell uptake. The receptor site was therefore deemed insensitive to this oxidising agent.

With 0.01 mM iodoacetamide, where neutral red uptake was normal or only slightly reduced with respect to the control, there was no reduction in red cell uptake. Neutral red was reduced at 0.05 mM when there was a slight decrease in red cell uptake. At 0.5 mM, neutral red was markedly reduced and red cell uptake virtually abolished. It was concluded then that iodoacetamide, in its capacity to react with sulphydryl groups, did not affect the receptor site itself, but rather reduced red cell uptake by reason of some antimetabolic effect.

In summary, the reactivity of the putative red cell receptor site on the hemocyte surface was inhibited by a reducing agent (mercaptoethanol) but was insensitive to an oxidising agent (sodium nitrite). Free sulphydryl groups appeared to play no part in its activity. Proteolytic enzyme treatment almost completely abolished activity and it therefore appeared to be at least partly protein in nature. The term 'receptor site' is of course vague, and it is perhaps better to say merely that a protein on the hemocyte surface appears to be involved in the uptake/

uptake of red cells by the hemocyte.

C. HEMOLYMPH FACTORS INVOLVED IN RED CELL UPTAKE.

We have so far considered the uptake of red cells in an *in vitro* serum free system. The possibility still remained that it might be mediated by humoral factors adsorbed onto the hemocyte surface and not removed during the washing. Indeed the likelihood of this was increased by the finding that red cell adherence was abolished by trypsin treatment. VAUGHAN (1965b) found that the uptake of guinea pig effete red cells by homologous macrophages was abolished by trypsin treatment. Activity was restored to the trypsin treated macrophages by incubation in normal guinea pig serum, which suggested that a normal serum protein component adsorbed onto the macrophage surface was involved in red cell uptake.

Similar experiments were carried out with cockroach hemocytes and hemolymph. Hemocyte monolayers were trypsin-treated as before for 30 minutes at 37°C. After washing, they were incubated in undiluted whole plasma for 1 hour at room temperature and washed 4 times; sheep cells were then added. Red cell uptake was assessed as before and the results are shown in Table XV.

The hemocytes remained completely normal throughout the experiments but the ability to take up red cells was not restored on incubation with hemolymph. This negative result does not of course/

course rule out the possibility of the trypsin having irreversibly damaged a hemocyte receptor for any humoral component.

TABLE XV: Incubation of trypsin treated hemocytes with hemolymph - effect on uptake of sheep red cells.

Treatment		
Control-199	Trypsin	Trypsin & Hemolymph
175*	0.5	0
n.d.	1.4	1.9

* Red cells/100 hemocytes.

n.d. - not done.

TRIPP (1966) and McKAY et al. (1969) showed that the naturally occurring hemagglutinins in oyster and crayfish hemolymph were opsonic for the uptake of vertebrate erythrocytes by oyster and crayfish hemocytes respectively. Phagocytosis was enhanced when oyster hemocytes were incubated with red cells in oyster hemolymph, compared to incubation in a balanced salt solution. No adherence of red cells to crayfish hemocytes occurred unless the red cells had been previously sensitised with crayfish hemagglutinin.

Hemolymph in medium 199 was prepared in the same way as the saline extracts. 0.1 ml. doubling dilutions of this were set up in tubes with equal volumes of 1% sheep cells and left for

3/

3 hours at 18°C. A sample from each tube was then examined microscopically and the dilution where trace agglutination had occurred was recorded. (Control hemolymph, maintained at 18°C for 3 hours and then assayed for hemagglutinating activity, showed no loss of agglutinating activity during the incubation period). The contents of the tubes showing trace agglutination, and also one dilution above and below, were added to three prepared hemocyte monolayers. A control monolayer received red cells that had been incubated in medium 199 alone. The red cells were allowed to settle out for an hour at room temperature and then uptake was assayed as before. The results are set out in Table XVI.

TABLE XVI: Uptake, by cockroach hemocytes, of sheep red cells sensitised with cockroach hemagglutinin.

Hemagglutinin concentration			
None	$\frac{\text{Trace}}{2}$	Trace	Trace x 2
38.4*	25.5	40.0	31.0
71.6	68.6	48.7	48.0
103.7	88.0	59.7	54.1

* Red cells/100 hemocytes.

There was no increase in red cell uptake following sensitisation with hemagglutinin, the trend, if any, being towards a reduction. The red cells at the dilutions of hemagglutinin used were never grossly clumped, and there was always a large excess/

excess of free red cells.

The technique was modified to that of TRIPP (1966) so that higher concentrations of hemolymph could be used. Here the red cell - hemolymph mixture was added immediately to the monolayer without prior incubation. At all times there were many free red cells but again no increase in uptake was observed (see Table XVII). Also, even with hemolymph in the system, actual phagocytosis of red cells was not observed.

TABLE XVII: Uptake, by cockroach hemocytes, of sheep red cells in cockroach hemolymph.

Hemolymph concentration			
None	1/128	1/64	1/16
263.9*	211.0	173.9	248.5

* Red cells/100 hemocytes.

Erythrocyte uptake by cockroach hemocytes appears then to be independent of the presence of hemolymph factors; the naturally occurring hemagglutinin shows no opsonic activity as reported for the oyster and crayfish. TRIPP (1966) did not conclusively demonstrate the oyster hemagglutinin itself to be the opsonic factor, since all the other hemolymph components were present in the system. Indeed, it was not surprising to find that oyster hemocytes functioned more efficiently in a medium approaching their in vivo environment than in a balanced salt solution. However/

However in the case of the crayfish hemocytes (McKAY et al., 1969), increased red cell adherence was demonstrated with erythrocytes that had been sensitised with hemagglutinin and then thoroughly washed before incubation with hemocytes.

D. UPTAKE OF FUNGAL SPORES.

Preparation of fungal spores. Puff Ball Spores (3.21μ diam.), as used for calibration of electronic cell counters, were obtained from Coulter Electronics Ltd., Dunstable, Bedfordshire, England. They were chosen as a target particle since they did not adhere to glass and readily lent themselves to the assay system used for red cells. The spores were wetted in 0.85% saline containing 0.01% of an ion free detergent (Triton X), by sonication at an amplitude of microns for 30 seconds with an MSE ultrasonic disintegrator. The spores were then centrifuged, washed in medium 199, again centrifuged and finally resuspended in medium 199 at a concentration of 0.5% by sonication. These suspensions showed a marked tendency to clump, but spores which did aggregate came out of suspension and collected at the surface of the liquid.

Uptake of fungal spores. 0.1 ml. aliquots of spore suspensions were added to hemocyte monolayers and left at room temperature for 1 hour. Excess and non-adhering spores were removed and the monolayers were assessed for spore uptake in the usual way. On examination, the spores were found adhering to hemocytes. Nonspecific/

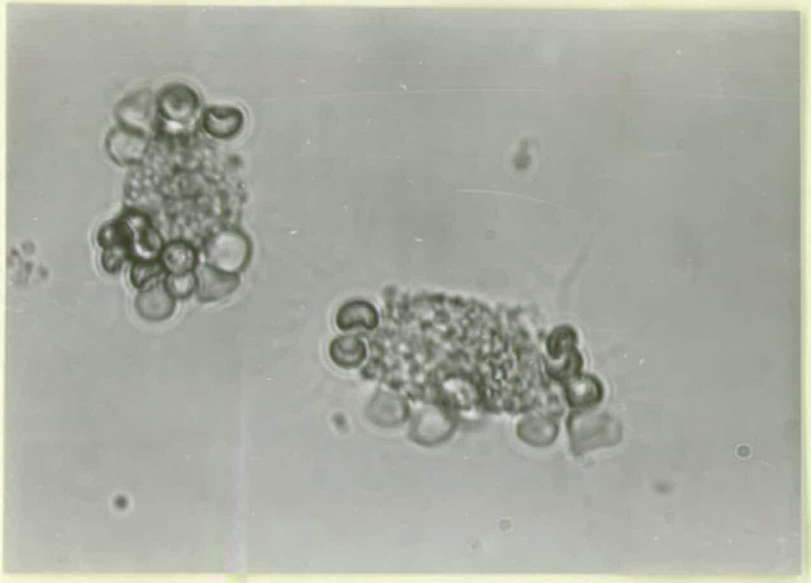
Non-specific sticking to the coverslip occurred only at the edges of the wells. Adherence was invariably maximal i.e. full rosettes on almost every hemocyte. Once again little or no actual phagocytosis had occurred.

Effect of temperature on uptake of fungal spores. Uptake of spores by hemocytes maintained at 2-3°C, and at room temperature, were compared using the technique described for erythrocytes. Uptake was obviously reduced at the lower temperature, nearly every hemocyte showing only partial rosetting. Full rosetting occurred at room temperature and thus uptake of spores appeared to be an active process dependent on metabolic energy

Uptake of spores and erythrocytes. In the following experiments a mixture of puff ball spores and erythrocytes was added to monolayers of hemocytes to see whether individual hemocytes were capable of taking up two different particles simultaneously.

0.1 ml. aliquots of a mixed suspension (1:1) of spores and erythrocytes was added to monolayers for 1 hour at room temperature. On examination, the spores and erythrocytes were easily differentiated by size and shape, and particularly by the different light refracting properties of the two particles. Mixed rosettes i.e. spores and erythrocytes adhering to the same hemocyte (Fig. 25) were present in all monolayers. A preliminary count on one monolayer showed 73% of rosettes to be mixed. In these monolayers there was always a predominance of/

Fig. 25 : 'Mixed rosettes' - the uptake of Puff ball spores and sheep red cells by the same hemocyte. The spores are the darker, kidney shaped particles.



of spores taken up by the hemocytes. Previous work had shown that with monolayers presented with either spores or erythrocytes, spore uptake was invariably greater. In the experiments with mixed suspensions, the puff ball spore is a considerably denser particle than the erythrocyte and it might have been that the spores settled out more quickly than the erythrocytes, thus physically precluding a completely free choice situation.

A detailed count on 100 hemocytes in one monolayer revealed that 11 hemocytes had taken up neither spores nor erythrocytes, 1 had only erythrocytes, 43 had only spores and 45 had both spores and erythrocytes. Since uptake of an erythrocyte was a relatively rare event, a statistical analysis on the number of erythrocytes taken up by spore-containing hemocytes was carried out to see if the frequency distribution of erythrocytes assumed a Poisson distribution. The actual frequency distribution of the erythrocytes, compared with the theoretical Poisson distribution for the same mean, is shown in Table XVIII.

A χ^2 test for goodness of fit showed the two distributions to be significantly different (probability - 0.005). This was also true even if only the data of from 0-8 red cells per hemocyte was considered. From this apparent non-random distribution of erythrocytes (Table XVIII), it seems that a heterogeneity might exist among the spore-containing hemocyte population e.g. some hemocytes having a stronger affinity for erythrocytes than/

than others, or even some hemocytes rejecting erythrocytes. The statistics are based on a minimal amount of data but suggest that further work with such a system might be interesting. One further statistical test on the data was a Mann Whitney U test to determine whether the number of spores taken up by hemocytes not containing erythrocytes was significantly different from the number taken up by those containing both particles. The result was insignificant (probability 0.4).

TABLE XVIII: Comparison of experimental and theoretical distribution of red cells taken up by spore containing hemocytes.

No. red cells/hemocyte	No. hemocytes (experimental)	Theoretical Poisson distribution (mean = 1.7)
0	43	16.4
1	13	27.9
2	9	23.8
3	8	13.5
4	7	5.7
5	0	1.9
6	2	0.5
7	1	0.1
8	1	0.03
9	1	0.005
10	2	0.0009
11	0	0.0000
12	0	0.0000
13	0	0.000
14	1	0.0000

BURNET (1968), considering invertebrate phagocytic recognition mechanisms (see Part 1) proposed that invertebrate hemocyte populations were made up of monospecific hemocytes of differing specificities. The specificity was conferred upon the hemocyte by a 'globulin like' protein, produced by the hemocyte and associated with its surface. A search was advocated to see whether hemocytes were divided into specific sub-populations e.g. by their power to differentially adsorb specific particles. With regard to Burnet's proposal, from the above *in vitro* experiments, using considerably different target particles, it would seem that any such differentiation of the hemocyte population certainly does not exist entirely at the level of monospecific hemocytes.

The experiments were repeated *in vivo*. 0.05 ml. of a suspension, made up of equal volumes of 10% sheep erythrocytes and 10% puff ball spores in saline, was injected into the hemocoele of each of six cockroaches. The first cockroach was etherised after 1 hour, a hemolymph sample taken from the leg stump, and the animal dissected to examine the hemocoele. The remaining cockroaches were similarly treated at hourly intervals.

When the hemocoele was opened, the inoculum was visible as a series of reddish brown clumps, loosely associated with the internal tissue surfaces, or free in the hemocoele. The clumps were removed, squashed, and examined. One hour after injection hemocytes were already seen to be associated with the clumps/

clumps, and capsule formation (see Part 1 and Chap. III) progressed with time. At 3 hours all clumps were completely surrounded by hemocytes and at 6 hours they were grossly encapsulated. Every capsule examined contained both spores and erythrocytes. The red cells had been agglutinated and it was presumed that the clumping of the spores was due to the known instability of such suspensions. However, due to this agglutination and clumping, very few red cells and spores remained free for phagocytosis. Examination of the hemolymph samples showed that only 1-2% of hemocytes in each animal had phagocytosed a particle. Although one hemocyte was seen to contain nine spores, all other hemocytes showing uptake contained only either a single red cell or spore. No hemocyte that contained both a spore and a red cell was found, but, because of the small number of hemocytes showing phagocytosis, this could not be considered significant. The fact that the hemocyte capsules contained both target particles was inconclusive. Although encapsulation is generally oonsidered to be the phagocytic response to a target particle too large to be phagocytosed by a single hemocyte, it is quite possible that the hemocytes were acting specifically i.e. the hemocytes actually in contact with the sheep cells and spores were acting specifically while adherence in the process of capsule thickening (hemocyte - hemocyte adherence) was probably nonspecific.

Further attempts were made to see if a single hemocyte could/

could be demonstrated to have phagocytosed two different particles simultaneously. Using yeast (S. pombe) and Berlin Blue, injection in saline always resulted in clumping rather than free dissemination throughout the hemocoele. A few free hemocytes contained either yeast or Berlin Blue, but again, the small amount of free material made the chance of a hemocyte contacting both highly remote. The cause of this clumping of materials introduced into the hemocoele is not known. It might be due to an initial immiscibility between the suspending saline and the highly lipid hemolymph, or even to localised hemolymph coagulation caused by the saline.

E. RECOGNITION OF FOREIGNESS BY HEMOCYTES IN VITRO.

This section briefly summarises some unsuccessful attempts to demonstrate discrimination between 'self' and 'non-self' material by cockroach hemocytes in vitro.

Uptake of dead hemocytes. Throughout the various preparation procedures, healthy hemocytes had rarely shown any signs of association with one another. The following experiments were designed to investigate whether or not dead hemocytes would be recognised as foreign by healthy hemocytes. It was established that hemocytes were killed by heating at 56°C for 30 minutes. Such treatment did not disrupt the hemocytes, but they failed to take up neutral red/

red and no longer adhered to glass. Monolayers of living hemocytes were completely covered with heat killed cells and left for 1 hour at room temperature. Non-adhering dead cells were removed in the same manner as red cells and the monolayers were examined. Live and dead hemocytes were distinguished by the presence and absence respectively, of neutral red. Two such monolayers were prepared and, in both, only occasional dead cells were seen to adhere to live cells. Possibly heat treatment had not sufficiently altered the hemocytes to render them 'foreign'. The self immunity of parasitic eggs and larvae in insects is known not to necessitate active metabolism (SALT, 1965, 1966). The investigations were not continued since to provide enough dead cells to cover one monolayer of living hemocytes necessitated the killing of over fifty cockroaches.

Uptake of red cells coated with homologous protein. The second approach to an *in vitro* demonstration of recognition was to coat erythrocytes with hemolymph protein and see whether they were recognised as 'self', as judged by reduced uptake compared to non-treated erythrocytes.

To facilitate uptake of protein, sheep cells were formalinised and tanned as described in Appendix IV . Hemolymph (saline extract) was absorbed twice with packed, formalinised cells for 1 hour at room temperature to remove agglutinating activity. 0.5 ml. absorbed hemolymph was incubated with 0.5 ml. tanned cell suspension (5% in phosphate buffered saline pH 7.0) for 30 minutes/

minutes at room temperature. (Preliminary experiments with I^{131} labelled hemolymph had shown an association of label with similarly treated cells - Appendix V). Control tanned cells were incubated with buffered saline. Both experimental and control cells were washed once and resuspended in medium 199 at 1%. 0.1 ml. aliquots of these suspensions were added to prepared hemocyte monolayers for 1 hour at room temperature and uptake was assessed in the usual manner. The tanned erythrocytes were obviously 'sticky' and adherence was maximal. Every hemocyte was completely surrounded with attached red cells, resulting in the formation of 'mulberries', and there was considerable non-specific adherence of red cells to the coverslip. With the large numbers of adhering erythrocytes counting was impossible, but experimental and control wells appeared identical with respect to red cell adherence to hemocytes and sticking to glass. The fact that adherence to the coverslip was similar with both coated and uncoated red cells implies that the coating may have been incomplete.

F. UPTAKE OF MAMMALIAN SERA.

Glycerinated rabbit anti-sheep red cell sera (Batch nos. K7927, K8175) were obtained from Burroughs Wellcome, Beckenham, Kent, England. These preparations had haemolytic titres 1:2000 - 1:3000. Before use the sera were dialysed against Phosphate Buffered Saline pH 7.2 for 24 hours to remove glycerine.

After/

After dialysis both sera showed agglutination titres of 512.

0.1 ml. aliquots of serum were added to monolayers of hemocytes at the dilutions indicated. All serum dilutions were made in medium 199. Hemocytes and serum were incubated together for 1 hour at room temperature, the serum then pipetted off and the monolayers washed 3-5 times with fresh cold medium 199. 0.1 ml. aliquots of 1% sheep red cell suspension in medium 199 were added to the wells, left for 1 hour at room temperature and then removed before the cells were examined for sheep cell uptake. Control wells were incubated with medium 199. Results are shown in Table XIX.

TABLE XIX: Uptake of mammalian serum by cockroach hemocytes.

	Serum Dilution				
Serum	1/2	1/4	1/8	1/16	199 Control
K7927 -	433.9*	263.6	255.8	117	86.0
K8175 -	271.0	198.0	168.9	98.8	28.0

* Red cells/100 hemocytes.

Serum treatment of normal hemocytes resulted in increased uptake of sheep cells compared with controls. Full rosetting invariably occurred at serum dilution 1/2 and sheep cells were often attached not only to the periphery of hemocytes but over the lower surface as well. This made accurate counting of red cells difficult and for subsequent experiments a semi-quantitative assessment of sheep cell uptake was resorted to.

Monolayers/

Monolayers were assessed from ++++ to 0 using the same scoring system as BERKEN & BENACERRAF (1966).

++++ - rosettes on almost every hemocyte

+++ - red cells on most hemocytes, rosettes on about half the cells

++ - red cells on about half the hemocytes, frequent rosettes

+ - red cells on some hemocytes in most fields, occasional rosettes

The serum employed was originally glycerinated and to confirm that the effect was not due to any glycerine remaining after dialysis, crude γ globulin extracts of dialysed serum were prepared by ammonium sulphate precipitation (see Appendix VI). Treatment of monolayers with these fractions again resulted in increased sheep cell uptake (Table XX).

TABLE XX: Uptake of gamma globulin by cockroach hemocytes.

	Control-199	γ Globulin
Serum K7927	+	++++

Uptake of serum following treatment with a metabolic inhibitor - iodoacetamide. To determine whether the uptake of serum was due to active uptake or merely to passive sticking, hemocyte monolayers were treated with 0.1 ml. aliquots of 0.5 mM iodoacetamide in medium 199 for 1 hour at 37°C. They were then washed three times and incubated with serum (dilution 1/2) for/

for 1 hour. After washing to remove serum red cell uptake was assayed as before and the results are shown in Table XXI.

TABLE XXI: Effect of iodoacetamide treatment on uptake of serum by cockroach hemocytes.

Treatment	Expt.I	Expt.2	Neutral Red
199-199	++	+++	Normal
Iodoacetamide-199	-	-	Marked Reduction
199-Serum (1/2)	++++	++++	Normal
Idoacetamide-Serum (1/2)	++	++	Marked Reduction

There was a decrease in red cell uptake by serum-treated hemocytes following prior treatment with iodocetamide. Whether or not this represented a decrease in the uptake of serum by iodoacetamide-treated hemocytes was unclear, since, with control monolayers that had not been treated with serum, iodoacetamide treatment abolished the background red cell uptake (i.e. not mediated by serum). It seemed possible that the decreased uptake after iodoacetamide treatment might merely be reflecting the abolition of this background and not represent a reduction in the amount of serum taken up. In that case, red cells taken up by serum-treated hemocytes would have comprised two groups of those where adherence was serum-mediated and those where it was not.

To investigate this, the action of complement on rosettes formed/

formed by serum-treated hemocytes was examined. BERKEN & BENACERRAF (1966) demonstrated complement lysis of red cells bound to macrophages by cytophilic antibody, whilst control cells not bound by antibody remained intact. Two sources of complement were used in the hemocyte experiments; Burroughs Wellcome preserved guinea pig serum and Flow Laboratories lyophilised guinea pig serum. Before use, complement was absorbed with an equal volume of packed sheep red cells at $0^{o}C$. Sheep cells were allowed to adhere to serum-treated and control monolayers in the normal way. After pipetting off the excess and non-adhering red cells, the wells were filled with 0.1 ml. complement diluted 1 in 10 with neutral red containing medium 199. Wells were then sealed, inverted and examined at room temperature.

Hemocytes showed good neutral red uptake and the amount of rosetting was similar to previously observed ratios between serum-treated and control wells. The wells were then placed in a $37^{o}C$ incubator and re-examined after 15 minutes. After this incubation period both experimental and control hemocytes showed signs of swelling and some loss of neutral red. This was presumably due to complement-mediated cytotoxic activity present in normal guinea pig serum. Ideally the guinea pig serum should have been absorbed with hemocytes prior to use, but shortage of animals made this impracticable. However, despite the damage to hemocytes, the red cells remained adherent. In the control wells, they were not lysed and were visible as rosettes/

rosettes. No adherent red cells were immediately apparent in the serum-treated wells but, on closer examination, red cell ghosts could be made out adhering to the dead or damaged hemocytes. They were easily seen under phase contrast illumination. Non-adherent red cells which had fallen away from the monolayers were intact in both serum-treated and control wells. Control wells were scanned under phase contrast for the presence of lysed red cells, but none were found. Since all the red cells adhering to serum-treated hemocytes were lysed, and following the reasoning outlined above, it would appear that they were all bound by antibody and that the effect of iodoacetamide treatment had been to reduce the amount of serum taken up.

Uptake of serum by trypsin-treated hemocytes. It had been established that trypsin treatment of hemocytes completely abolished the uptake of sheep red cells. The effect of trypsin treatment on the uptake of serum was therefore examined. Hemocyte monolayers were trypsinised using the modified technique described previously. Serum was then added for 1 hour at room temperature and, after washing, red cells were added and their uptake assayed as before. The results are shown in Table XXII.

Trypsin-treated hemocytes were still capable of taking up serum. Whether or not serum uptake by trypsin-treated hemocytes was less than by non-treated, was not investigated.

TABLE XXII/

TABLE XXII: Uptake of serum by trypsin-treated hemocytes.

Treatment	
Trypsin-199	Trypsin-serum
0.8*	292.2
3.3	164.6

* Red cells/100 hemocytes.

HOWARD & BENACERRAF (1966), while investigating the nature of the macrophage cytophilic antibody site, found that it too was resistant to trypsin treatment. This provoked interest as to whether the uptake of antiserum by hemocytes might depend on a receptor site specific for immunoglobulin molecules. The existence of such a site, on an invertebrate cell surface, before the evolution of antibody, would of course have interesting implications.

Pretreatment of hemocytes with Rabbit Serum Albumin (RSA). If serum uptake was non-specific in the sense that all rabbit serum components were taken up indifferently, then one would expect pretreatment with rabbit serum albumin to block the receptor sites and reduce the amount of the γ globulin component taken up by the hemocytes. Rabbit serum albumin (RSA), Cohn fraction V (Batch no. 38812) was obtained from Koch Light Laboratories, Colnbrook, Buckinghamshire, England. The concentration used was 40 mg/ml. in medium 199. Hemocyte monolayers were treated with/

with RSA at room temperature for 30 minutes, and washed thrice prior to serum and red cell treatment. The results are set out in Table XXIII. No reduction in uptake was apparent.

TABLE XXIII: Effect of pretreatment with Rabbit Serum Albumin (RSA) on the uptake of rabbit antiserum by cockroach hemocytes.

Treatment	Expt.1	Expt.2
199-199	++±	++
RSA-199	++	++
199-Serum	+++±	++++
RSA-Serum	++++	++++

It thus appeared possible that there might indeed be a specific uptake of the gamma globulin component of rabbit serum and the experiments described in the following paragraphs were designed to investigate this further.

A gamma globulin receptor site on the hemocyte surface? The rabbit anti-sheep cell serum used in these experiments was tested for cytophilic activity with guinea pig alveolar macrophages using the passive direct technique described by BERKEN & BENACERRAF (1966). Cytophilic activity was found, the cytophilic titre being 1/512.

One of the features of cytophilic antibody described by BERKEN & BENACERRAF (1966) was that if sheep cells were treated with antiserum before being added to the macrophage monolayers (indirect technique), cytophilic activity could be detected at

20-30 times lower serum dilutions than when macrophages were passively coated with antiserum and then washed before adding normal red cells (direct technique). This effect was considered to be due to elimination of other cytophilic antibodies not specific for sheep cells (BERKEN & BENACERRAF, 1966).

Cockroach hemocytes have been described to take up antiserum, known to be cytophilic, using the direct technique, and they were therefore tested for uptake using the indirect technique: sheep cells were incubated for 1 hour at 37°C in dilutions of rabbit antiserum which showed only trace agglutinating activity. The red cells were kept in suspension by flicking the tubes every 15 minutes. Control sheep cells were incubated in medium 199. After incubation, the red cells, still in the suspending medium, were added to monolayers of hemocytes for 1 hour at room temperature. At the serum dilutions used nearly all the red cells were free, with only occasional clumps. The results of this experiment are shown in Table XXIV.

TABLE XXIV: Uptake of 'sensitised' red cells by cockroach hemocytes.

Red cell treatment		Uptake (red cells/100 hemocytes)
Control-199	-	115.9
Sensitised* 1/512	-	160.7
Sensitised* 1/256	-	83.8

* Serum K7927 aggn. titre 1/512.

In/

In a second experiment, a positive control for sensitisation of red cells was included. This was a monolayer of mouse peritoneal macrophages obtained by peritoneal lavage with medium 199. The macrophages were washed in medium 199 and allowed to form a monolayer in the same manner as hemocytes. The results are shown in Table XXV.

TABLE XXV: Uptake of 'sensitised' red cells by cockroach hemocytes and mouse peritoneal macrophages.

Red cell treatment	Hemocytes	Macrophages
Control-199	20.0*	71.2+
Serum 1/250	15.5	>1000

* red cells/100 hemocytes. + red cells/100 macrophages.

In neither case was there a detectable increase in the uptake of sensitised, as compared to normal red cells by the cockroach hemocytes. The increased uptake of sensitised red cells by mouse macrophages confirmed the sensitised state of the red cells. From this it is concluded that cytophilic antibody is not being preferentially taken up by the hemocyte surface, and therefore no receptor site similar to that on the mammalian macrophage surface is present on the hemocyte surface.

With the indirect technique, the red cells are sensitised with whole serum and not just the cytophilic fraction. Therefore all serum components directed against the red cell (mainly gamma globulin/

globulin) will go onto the red cell surface. These factors, which are opsonic for uptake of sheep cells by mouse macrophages, are not opsonic for uptake of sheep cells by cockroach hemocytes. This suggests that there is no receptor site for gamma globulin on the hemocyte surface, and that the uptake of rabbit antiserum described is merely a general uptake of whole serum. STUART (1968) has since reported his failure to demonstrate opsonisation of red cells for octopus hemocytes with rabbit anti-red cell serum. He likewise concluded that no gamma globulin receptor site existed on the octopus hemocyte surface.

CHAPTER III ENCAPSULATION STUDIES

Encapsulation in insects has been described briefly in Part I. It is one of the defence reactions to metazoan parasites and implants too large to be phagocytosed by a single hemocyte. The description of the development and structure of the capsule in Part I was based on the light microscope studies of SALT (1960). GRIMSTONE et al. (1967) have studied capsule formation in flour moth caterpillars (Ephestia) using the electron microscope. Their studies have distinguished three layers, as defined by cell shape, in the 72 hour capsule. Such capsules are typically 50-60 cells thick.

The cells of the inner layer are closely applied to the implant and appear to be necrotic or in the process of becoming so. Most of their cytoplasm is occupied by strikingly prominent cytolysomes, which often displace the nucleus. The main feature of the middle layer is the extreme flattening of the cells into a compact, many-layered tissue, there being no evidence of necrosis or cytolysomes. The outer layer consists of hemocytes which have recently joined the capsule. The cells of the extreme outer layer resemble free blood cells, whilst the others represent various stages of flattening.

Encapsulation then is obviously more complex than a situation in which hemocytes, attempting to ingest a large surface, merely adhere to and completely surround it. Encapsulation does/

does not stop once the implant has been completely enveloped, but continues to develop until a relatively structured capsule of defined size has been formed. It has been reported to occur in fourteen orders of insects (SALT, 1963à) and more specifically in response to methylcholanthrene or talc pellets implanted in the hemocoel of Periplaneta americana (SCHLUMBERGER, 1952).

A. SOME OBSERVATIONS WITH NYLON IMPLANTS.

It has been established that implants of very inert substances such as glass, polythene and polyfluorocarbon are encapsulated as readily as parasites or foreign tissue (SALT, 1965).

Preparation and implantation of nylon. Nylon monofilament (I.C.I. 'Luron 2') diameter 0.009 inches was guillotined into 1-2 mm. lengths. These were placed on a filter paper in an open petri dish and autoclaved at 15 lbs./sq. inch for 20 minutes. Using fine forceps, a single length was placed in the bevel of a 19 g disposable needle (Gillete Scimitar) primed with sterile saline and attached to a syringe loaded with saline. The needle was carefully introduced into the hemocoel of an etherised cockroach via the intersegmental membrane between the abdominal sternites. The syringe was then rotated through 180° and approximately 0.05 ml. saline expelled along with the nylon. Care was taken not to penetrate the/

the gut, and little or no leaking occurred from the wound.

Recovery of nylon implants. At the times specified after implantation, cockroaches were killed with ether and dissected from the dorsal side. The wings were removed and the body cavity opened. The exposed organs were bathed in saline and the internal tissues carefully searched with fine forceps until the implants were located. Pieces of nylon were relatively easily found because of their green colour and were carefully removed and placed in saline. Any contaminating fat body was carefully dissected away before examination.

Encapsulation of nylon. Cockroaches were each injected with a single nylon monofilament and these were recovered at varying intervals of time after injection. They were found either free in the hemocoel or loosely associated with the internal tissue surfaces. After removal they were placed in saline and gross observations were made with both a dissection and an inversion microscope.

The onset of encapsulation was extremely rapid; nylon recovered after only 40 minutes had received a patchy coating of hemocytes although usually only one cell thick. Capsules thickened irregularly until, after 4 hours, all nylon recovered was grossly encapsulated with capsules generally 60-100μ thick but with highly irregular outlines. Some hemocytes were extremely loosely associated with the capsule surface and often came/

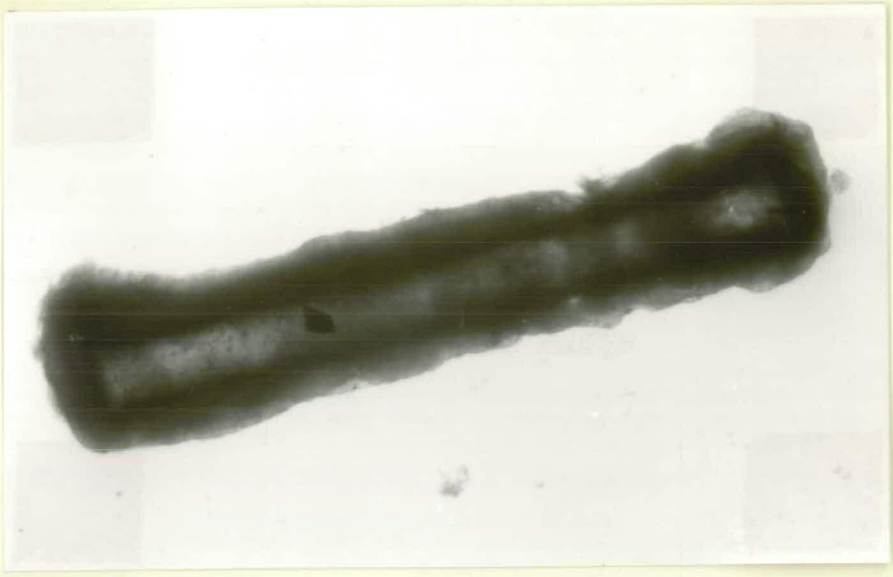
came free in the saline. 24 hour capsules consisted of densely packed hemocytes, again typically 60-100μ thick, but with capsule outlines considerably more regular than at 4 hours. A typical 24 hour capsule is shown in Fig. 26 . GRIMSTONE et al. (1967) reported that differentiation into the three concentric layers was only just beginning after 24 hours, being most apparent in the flattening of the middle cells. The inner cells showed no pathological changes and overall, except for shape, 24 hour capsule cells resembled free blood cells (GRIMSTONE et al.,1967).

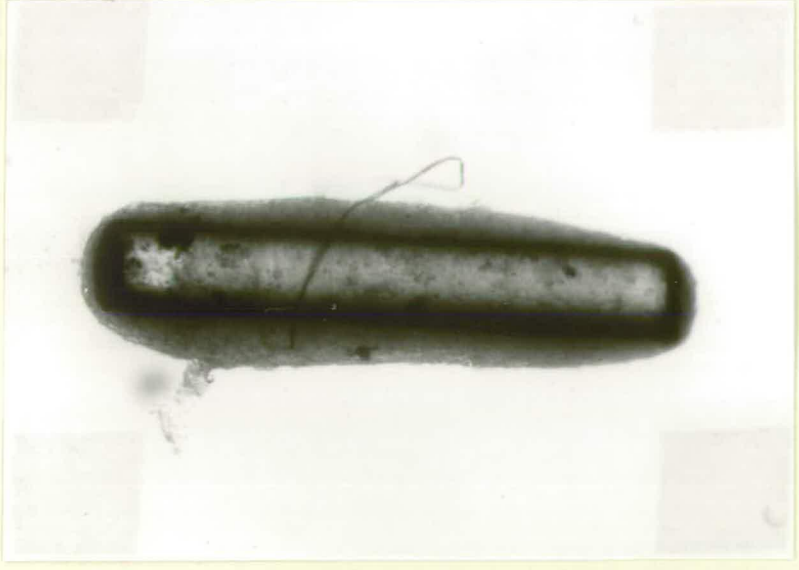
The contained nylon could easily be removed through a tear in the 24 hour capsule leaving the rest of the capsule intact and demonstrating that hemocyte-hemocyte adherence was considerably stronger than that of hemocyte to nylon. The remaining capsule was elastic in nature and, although it could be easily torn into parts, this did not result in the liberation of individual cells. This elastic nature of the capsule has been described by SALT (1960) in circumstances where the elasticity seems to have been necessary to accommodate the contortions of a recently encapsulated parasitic larva.

Capsules recovered after 15 days in the cockroach were extremely smooth and regular in outline, see Fig. 27 . Patches of reddish brown also appeared to have been deposited on the surface of the contained nylon. This was presumably melanin which is known to be deposited in capsules in some insects (SALT, 1963b)/

Fig. 26 : 24 hour capsule of hemocytes around a nylon monofilament.

Fig. 27 : 15 day capsule of hemocytes around a nylon monofilament.





1963b). GRIMSTONE et al. (1967) showed that 20 day capsules were still cellular but no longer differentiated into layers, all cells being flattened and a large proportion of them often showing necrosis and containing cytolyzomes. Pieces of nylon recovered from 15 day cockroach capsules were discovered to be squashy, the slightest pressure permanently deforming them. Nylon recovered after 24 hours was in pristine condition and was extremely resistant to deformation. The reason for this squashiness is not understood, and is of special interest in view of the generally recognised resistant nature of nylon. It could be due to prolonged exposure to something liberated by the capsule cells (possibly from the cytolyzomes), but as yet little is known about the mechanisms of degrading contained material. According to RIZKI (1968) bacterial cells encapsulated by Drosophila hemocytes were destroyed by lysis within the capsular confines, there being no evidence of phagocytosis or intracellular digestion. He suggested that the capsule hemocytes secreted lytic factors.

One cannot, of course, comment on the fine structure, but from these gross observations the American cockroach gave a typical insect encapsulation response (SALT, 1960) to implanted inert material with respect to time of onset of encapsulation and the nature of the capsule. It has been demonstrated that there is no difference in the electron microscope appearance of capsules elicited by inert material and foreign tissue (GRIMSTONE et/

et al. 1967)

Effect of temperature on encapsulation. Three cockroaches were anesthetised by cooling to 3°C and each injected with a single nylon monofilament. They were then maintained at 3°C for 24 hours, during which time they remained completely immobile. (Cockroaches are not killed by this treatment and fully recover within about 10 minutes of their return to room temperature). They were allowed to recover at room temperature just long enough to ascertain that they were alive and were then immediately dissected, the implanted nylon being recovered. Control cockroaches were maintained at room temperature for 24 hours. The results are shown photographically in Fig. 28 . As shown, there was a gross reduction in capsule formation at the low temperature.

Encapsulation of vertebrate cells implanted in the cockroach Leucophaea maderae had been reported to be abolished at 4°C and this was assumed to be due to decreased hemocyte mobility (DAWE et al., 1967). My previous experiments showing reduced adherence of target particles to hemocytes at low temperatures suggest that the decreased response might be due not only to a reduced number of contacts with the implant because of lowered hemocyte mobility, but also due to decreased adherence on contact. In either case, the decreased encapsulation response is in keeping with the obviously lowered metabolism of the whole animal.

Enzyme/

24 hour capsules recovered from cockroaches maintained at room temperature.

Fig. 28 : EFFECT OF TEMPERATURE ON ENCAPSULATION.

24 hour capsules recovered from cockroaches maintained at 3°C.

KODAK PANATOMIC-X FILM
KODAK SAFETY

1
1A
2
2A
3
3A
4
4A

Enzyme treatment of capsules. 24 hour capsules were treated with various enzymes in attempts to reduce them to individual cells amenable to counting and morphological study. The cell concerned was thought to be the plasmatocyte because a) capsules immersed in neutral red containing saline readily took up the vital dye, and b) the cells loosely associated with the early capsule surfaces appeared to be plasmatocytes. GRIMSTONE et al. (1967) noted that many of the capsule-forming cells in flour moth caterpillars contained phagocytosed bacteria.

Another reason for performing the enzyme treatments was the hope that something might be learned about the contact relationships of the hemocytes comprising the capsule. Electron microscope studies (GRIMSTONE et al., 1967) showed that the membranes of adjacent cells were often in direct contact, but that in many areas they were separated by a gap almost invariably filled with dense extracellular material. This substance was possibly acting as a cement, consolidating the hemocytes into a compact tissue.

The following enzymes (see Appendix III) were dissolved in medium 199 at the concentrations shown in brackets: trypsin (0.3%), collagenase (0.1%), hyaluronidase (0.3%), lecithinase-c (0.3%). 24 hour capsules were removed from cockroaches and placed immediately in tubes containing the enzyme solutions. The tubes were then stoppered and vigorously shaken for 30 minutes at 37°C in a shaking water bath. On removal, each tube was held against/

against a high speed vibrator (Rotamixer, Hook & Tucker Ltd.) for 30 seconds. Both the capsules and the suspending medium were then examined microscopically.

The hemocytes were destroyed by trypsin treatment under these conditions, however the cell debris was still associated with the nylon in the form of the original capsule. Some of the innermost cells were still intact and occasional cell debris was visible in the suspending medium. With the three remaining enzymes, all the capsules remained completely intact and no hemocytes were visible in the surrounding medium. In this they resembled medium 199-treated controls. Nothing positive came from the series of experiments then, save for the absence of effect of the various enzymes on the capsule. GRIMSTONE et al. (1967) reported failure in their attempts to characterise the intercellular material cytochemically, but stated that sections of capsules stained weakly and apparently uniformly with the Periodic Acid-Schiff (PAS) technique for polysaccharide. WIGGLESWORTH (1956) earlier reported that Rhodnius hemocytes, collected around injected Indian ink, contained great quantities of PAS-positive material which they appeared to discharge into the intercellular spaces. However in the above enzyme treatments, hyaluronidase did not cause the capsules to break up.

Encapsulation response to large doses of nylon. It was obvious, merely/

merely from observation, that a very large number of hemocytes must go into the formation of a single capsule, and it seemed possible that a massive dose of nylon might be capable of overwhelming the hemocytes i.e. exhausting the reactive cells so that some nylon might be only thinly coated or might escape encapsulation altogether. This would be the equivalent of 'superparasitism' in nature and any such failure of the encapsulation system could result in the insect succumbing to the parasites.

The experimental scheme was to inject the 'blocking' nylon dose (18 mm.) into the hemocoele followed immediately by a test strip (2 mm.). The test strip was then assayed for encapsulation after 24 hours. The 18 mm. nylon were cut into 3 x 6 mm. strips. An injection of this amount of material in the usual manner would have involved a large volume of saline entering the insect, so a small trochar was constructed with a 19 g needle and a wire plunger. This unit was sterilised, and the three nylon strips were placed inside the needle and inserted into the hemocoele. The 2 mm. strip was then injected, in saline, at a different site in the hemocoele. Control insects merely received the test strip along with a trochar wound as wounding is known to affect the hemocyte count (SHAPIRO, 1968). After 24 hours the test strips were removed and, for the purpose of comparison, were photographed in saline by means of a Nikon Stereo Zoom dissection microscope with photo attachment and/

and constant illumination and magnification. Contact prints of the results are shown in Fig.29. where it is obvious that any differences were slight. Enlargements of these pictures were made and given to three independent assessors to whom the experiment had been briefly explained. These people were asked to rank the pictures in order of thickness of cell coating and the results are shown in Table XXVI. The probability (calculated by the Mann-Whitney U test) that the controls were indeed thicker than the experimentals was significant at the 0.05% level in two cases, and not significant in the third.

TABLE XXVI: Encapsulation response to a large dose of nylon. Ranking of experimental and control nylon strips according to capsule thickness.

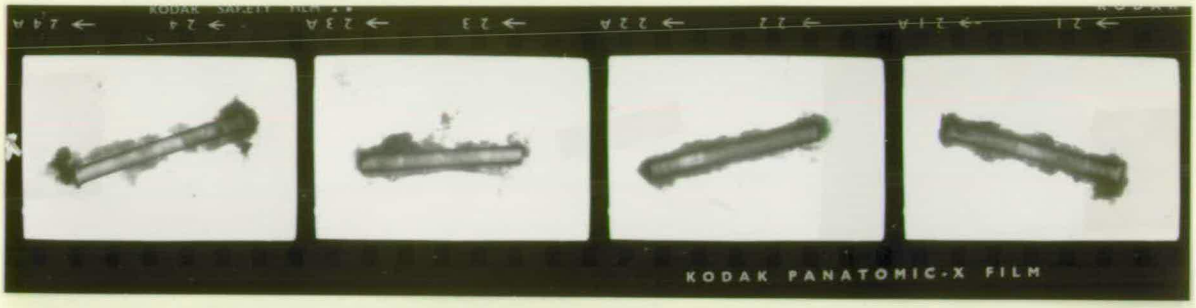
Order	1st Assessor	2nd Assessor	3rd Assessor
1 (thickest)	X	C	X
2	C	X	X
3	C	C	C
4	C	C	C
5	C	C	C
6	C	C	C
7	X	X	X
8	X	C	C
9	C	C	C
10	C	X	C
11	X	X	X
12	X	X	X
13	X	X	X
14	X	X	X
15 (thinnest)	X	X	X
Probability	0.027	0.014	0.116

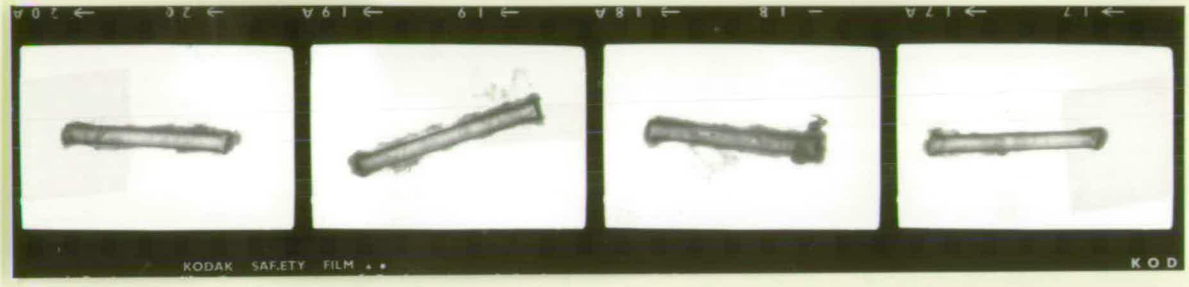
X - Experimental. C - Control.

Control capsules (see text).

Fig. 29 : ENCAPSULATION RESPONSE TO A LARGE DOSE OF NYLON.

Experimental capsules (see text).


KODAK PANATOMIC-X FILM


KODAK SAFETY FILM
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KODAK SAFETY FILM


KODAK PANATOMIC-X FILM
KODAK SAFET

In many cases the 3 x 6 mm. nylon strips were examined too, and always they were completely encapsulated, sometimes separately, sometimes two or three together. What can be stated then, is that although the massive dose of nylon had possibly reduced capsule size, the cockroach was capable of dealing effectively with what must have constituted a considerable challenge.

SALT (1960) reported that when caterpillars were superparasitised with five parasitic eggs, all the eggs were encapsulated. Although no actual data was quoted, he reported that after 24 hours the eggs were more thinly encapsulated than usual, the capsule being normal at 48 hours, presumably due to formation of new cells. Whether the original complement of cockroach hemocytes was able to deal with the challenge or whether new cells had been formed is of course not known. The time 24 hours was chosen to minimise the latter effect, but SHAPIRO (1968) has since reported a significant increase in the number of hemocytes in mitosis 24 hours post wounding in the wax moth caterpillar. Whatever the origin of the cells, the encapsulation response must be considered extremely effective.

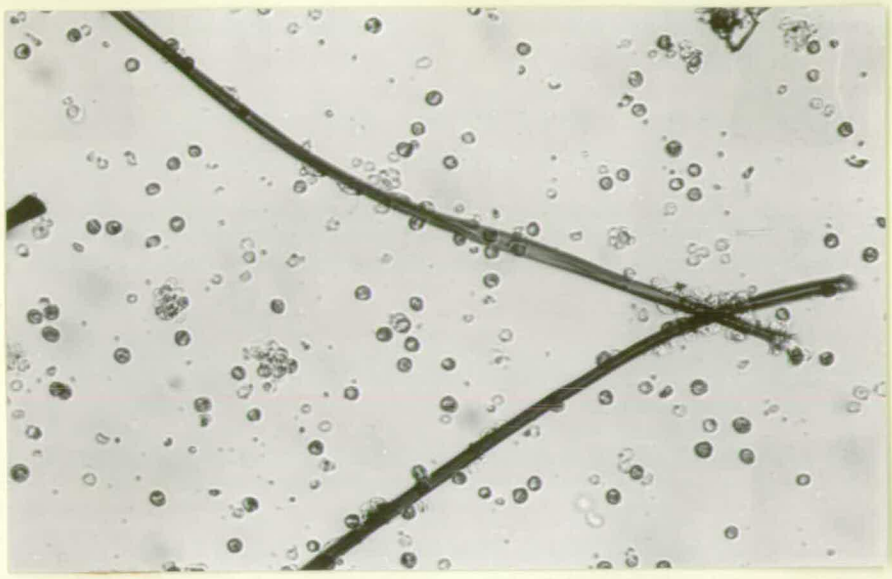
In vitro encapsulation. Attempts were made to form capsules in vitro as follows. Individual fibres of black cotton thread (1 mm. in length and diameter 0.01 mm.) were soaked in ether to remove/

remove grease, and then placed inside one end of a 100µl disposable micropipette (Drummond Scientific Company, Broomall, Pennsylvania, U.S.A.). Pooled hemocytes from twenty-five adult cockroaches were washed and resuspended in 0.05 ml. medium 199. The end of the micropipette containing the threads was dipped into the cell suspension and both hemocytes and threads were drawn in by capillary action. With a microburner, the other end of the pipette was sealed and allowed to cool. The pipette was then centrifuged to deposit the cotton threads, packed with hemocytes, at the bottom of the tube. After 3 hours at room temperature, the hemocytes and threads were resuspended by gently tapping the tube. The sealed end of the tube was then sawn off and the contents blown out into a culture well, containing medium 199 and neutral red, for microscopic examination. The hemocytes appeared normal and took up neutral red, but there was no evidence of encapsulation. Most of the hemocytes had remained free although a few clumps were noted. Occasional hemocytes had adhered to the cotton thread (Fig. 30). A control cockroach received an injection of cotton thread which was recovered after 3 hours completely encapsulated (Fig. 31).

The possibility remained that hemolymph was necessary for the encapsulation process. From their electron microscope studies, GRIMSTONE et al. (1967) reported that the spaces between the hemocytes most recently added to capsules in Ephestia, sometimes contained precipitated hemolymph. The experiments were/

Fig. 30 : Failure of *in vitro* encapsulation of cotton thread.

Fig. 31 : *In vivo* control for above - encapsulation of cotton thread after 3 hours.



were therefore repeated incubating the hemocytes and cotton threads in saline extracted hemolymph. The results were the same as in the hemolymph-free system. Occasionally a piece of cotton was associated with a clump of hemocytes but there was no definite evidence of encapsulation.

Relatively little is known about the actual mechanism of capsule formation in insects and, in this respect, my own experiments were unsuccessful. Although encapsulation is usually described as the quasi-phagocytic response to a target particle too large to be phagocytosed by a single hemocyte, from our considerations of the phenomenon the process cannot be accounted for merely in terms of a phagocytic response. Simple phagocytosis could readily account for the first stages of capsule formation i.e. the initial laying of hemocytes around the target particle. This might be interpreted as the hemocyte attempting to phagocytose the object, the result being that it merely spreads out on the object's surface. However, once the implant has been completely covered with hemocytes, all further adherence is between the hemocytes themselves. In the insect, hemocytes do not usually adhere to one another and here we have an apparent reversal of this situation. Since the cells involved are phagocytic, it is as though the cells already forming the capsule are recognised as 'foreign' by the hemocytes joining the capsule. GRIMSTONE et al. (1967), pointed out that the dense extracellular material, found in/

in some of the spaces between the capsule cells, might possibly be of significance here. If the adhesion of hemocytes to foreign bodies or to themselves stimulated them to produce this material, and if this was identified as foreign, then continued growth of the capsule could be accounted for. The actual cells adhering to implants were not markedly different in fine structure from free hemocytes save for some degree of flattening, (GRIMSTONE et al., 1967).

The problem also remains as to how and why capsules tend to assume a relatively definite size. Does the stimulus for further hemocyte adherence stop, or is the size attained that compatible with continued existence in the hemolymph without interfering with organ movements, hemolymph circulation etc, any further hemocytes being mechanically removed? Certainly, the phenomenon of encapsulation constitutes one of the most fascinating problems in the field of invertebrate defence mechanisms.

B. TISSUE IMPLANTATION.

What constitutes foreigness to an invertebrate has been discussed in Part I and the theme of the following experiments was to inject implants of considered degrees of foreigness into the hemocoele of the American cockroach and observe any discrimination on the part of the hemocytes.

Animals. Besides American cockroaches, the following animals were/

were used. Lobster cockroaches (Nauphoeta cinerea) were from a culture maintained in the Zoology Department, University of Edinburgh. Calliphora larvae (maggots) were purchased from A.Bryant & Co. Ltd., Erling Works, Jerusalem Farm, Thornton, Bradford, Yorkshire, England. The mice used were an outbred strain .

Preparation of tissue implants.

Mouse tendon. Strips of tendon dissected from the brachial region were washed in a petri dish containing saline and, still under saline, were cut into 1-2 mm. lengths. A microscopic check confirmed them to be undamaged, save for the cut end, and they were immediately implanted in the same way as nylon.

Cockroach nerve cords. Etherised cockroaches were dissected from the dorsal side. Throughout the operation the internal organs were bathed in saline. The gut was removed revealing the ventral nerve cord lying in the floor of the body cavity. The cord, in the abdominal and thoracic region, was carefully cleared of any adhering tissue, cut anteriorly and posteriorly, and transferred to a dish of saline. Any remaining tissue was then removed and the nerves cut off as close to the nerve cord as possible. Sections of nerve cord, about 4 mm. long and each containing a ganglion, were cut. These were washed in saline, checked for damage and implanted. Throughout the whole/

whole procedure, the utmost care was taken to prevent damage to the nerve cord. It was found that the sections were best handled using fine forceps and one of the cut nerve ends.

Calliphora 'brain'. Calliphora larvae were similarly dissected. In the larva, the nerve cord is condensed into a fused ganglionic mass ('brain') situated at the anterior end of the body and from which individual nerves radiate to all parts of the body. This was carefully removed, cleared of surrounding tissue and checked for damage as with cockroach nerve cords. Whole brains, usually about 4 mm. in length were implanted in the usual manner.

Implantation experiments.

Homologous nerve cord implants - P. americana. These involved the removal of nerve cord sections from adult American cockroaches and their implantation into other adult American cockroaches. One section of nerve cord was implanted in each recipient. After 24 hours, nerve cords were successfully recovered from twenty out of twenty-four recipient animals. The sexual combinations of donor and recipient in these twenty cases were as below.

Donor		Recipient	No.
Male	-	Female	6
Male	-	Male	6
Female	-	Male	4
Female	-	Female	4

At/

At autopsy, the nerve cords were found free in the hemocoele or loosely associated with the tissue surfaces. Examination of the cords in situ with a high powered dissection microscope placed in a drop of saline on a clean glass slide, and subjected to a critical microscopic examination using both a standard and an inversion microscope.

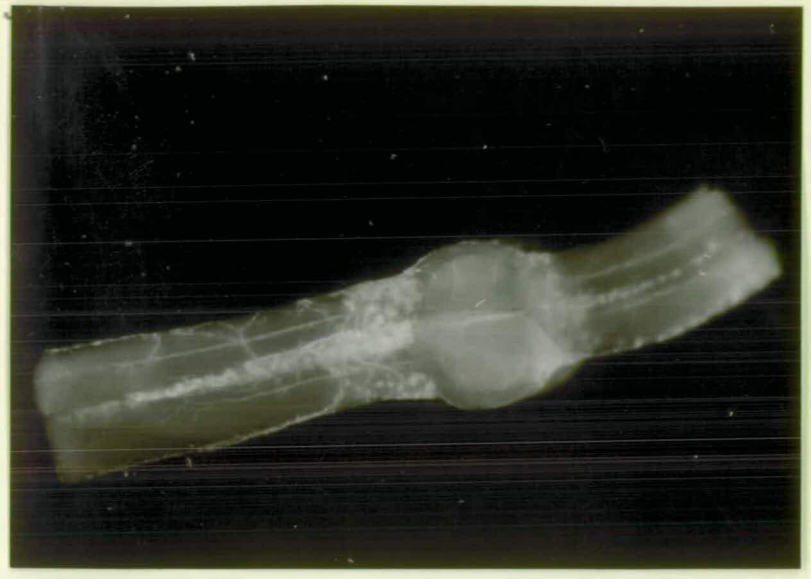
None of the cords showed any signs of deterioration. 17 cords showed a slight aggregation of hemocytes at one or both cut ends and occasional similar slight aggregations around some of the cut nerve endings. The remainder of the cord was completely free except for occasional individual hemocytes. The remaining 3 cords were completely free from hemocyte adherence - Figs. 32, 33. The sexual combinations of these 3 were male ⟶ male, male ⟶ female, female ⟶ male.

Heterologous cockroach nerve cord implants. Difficulty was experienced in removing nerve cords from N. cineria. In this cockroach, the bulk of the fat body is extremely intimately associated with the actual nerve cord and it proved impossible to obtain nerve cord that was free from torn fragments of fat body. This presence of damaged tissue really defeated the purpose of the experiments, however three implants of Nauphoeta nerve cord into Periplaneta were carried out. All three cords were successfully recovered 24 hours later. Two of them had been completely encapsulated (Fig.34,35), the remaining one showed partial encapsulation.

The/

Fig. 32 : P. americana nerve cord prior to implantation.

Fig. 33 : The same nerve cord as shown in Fig. 32 recovered after 24 hours in P. americana. Completely free from hemocytes.



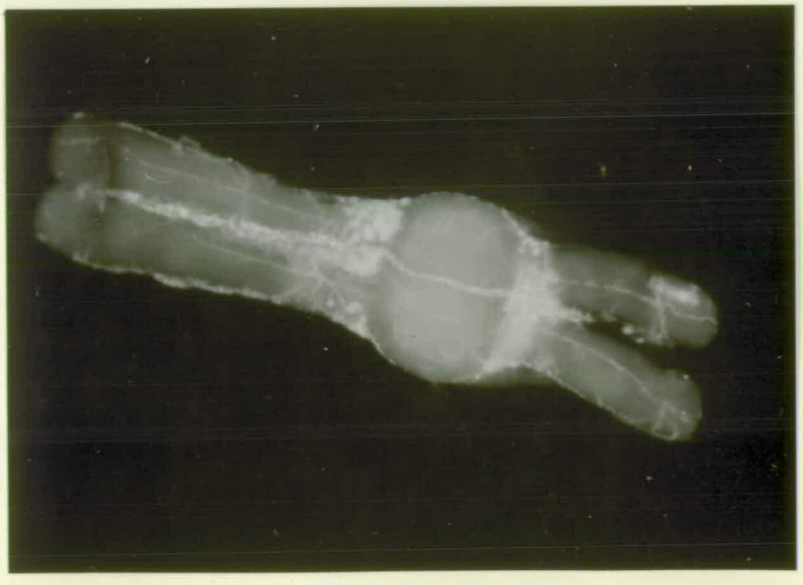
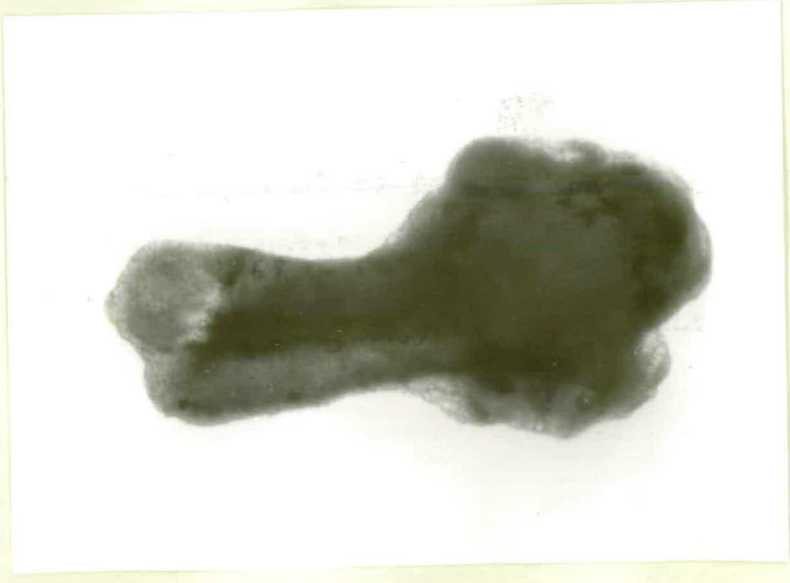


Fig. 34 : N. cineria nerve cord prior to implantation. The black associated with the nerve cord is fat body.

Fig. 35 : Same nerve cord as in Fig. 34, recovered completely encapsulated after 24 hours in P. americana.



The reciprocal transplant (Periplaneta ⟶ Nauphoeta) provided no problems and nine such operations were successfully carried out. Of these nine, one was completely clear of hemocytes, two were partially encapsulated and the remaining six were completely encapsulated. The capsules had a characteristic 'wispy' appearance (Fig. 36). Nauphoeta capsules around nylon after 24 hours also showed this same 'wispy' appearance. (Fig. 37).

Implants of Calliphora 'brain'. A Calliphora brain was implanted into each of three Periplaneta and recovered successfully after 24 hours. All had been grossly encapsulated (Figs. 38, 39). The cut nerves were just visible through the capsule but in each case the capsule was removed for conformation.

Implants of mouse tendon. A piece of tendon was successfully recovered from each of three Periplaneta after 24 hours. As with Calliphora nerve, all had been grossly encapsulated. (Figs. 40, 41). The striations of the tendon were faintly visible through the capsule, but all three capsules were removed to reveal the implanted tendon.

Homologous nerve cord implants were not recognised as foreign by recipient cockroaches, as judged by encapsulation. Heterologous nerve cord from another species of cockroach was recognised as foreign and reacted against. Nerve cord from an

Fig. 36 : *P. americana* nerve cord recovered encapsulated after 24 hours in *N. cineria*.

Fig. 37 : Nylon monofilament recovered encapsulated after 24 hours in *N. cineria*.

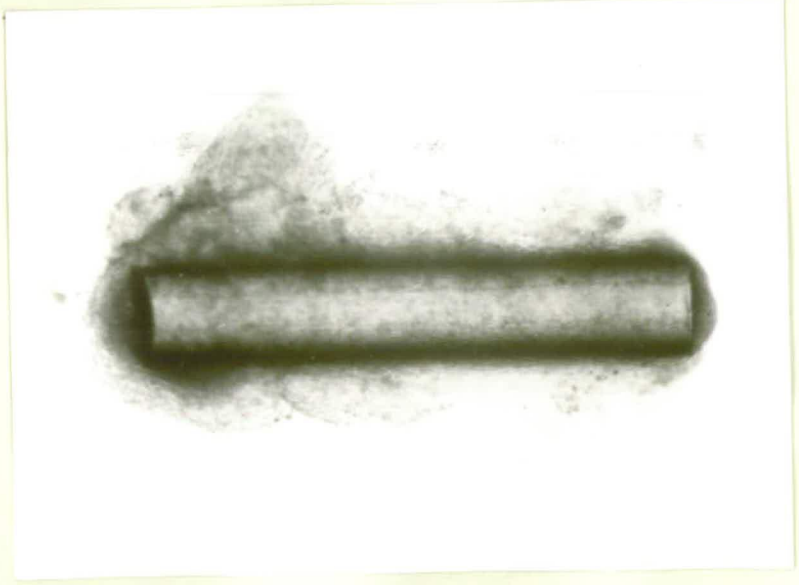


Fig. 38 : 'Brain' from Calliphora prior to implantation.

Fig. 39 : 'Brain' from Calliphora recovered encapsulated after 24 hours in P. americana. Nerves are visible through the capsule.

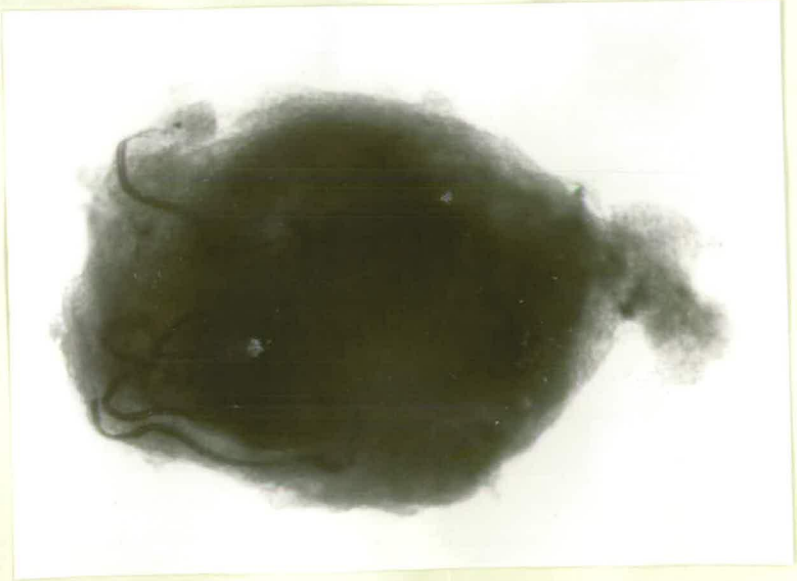
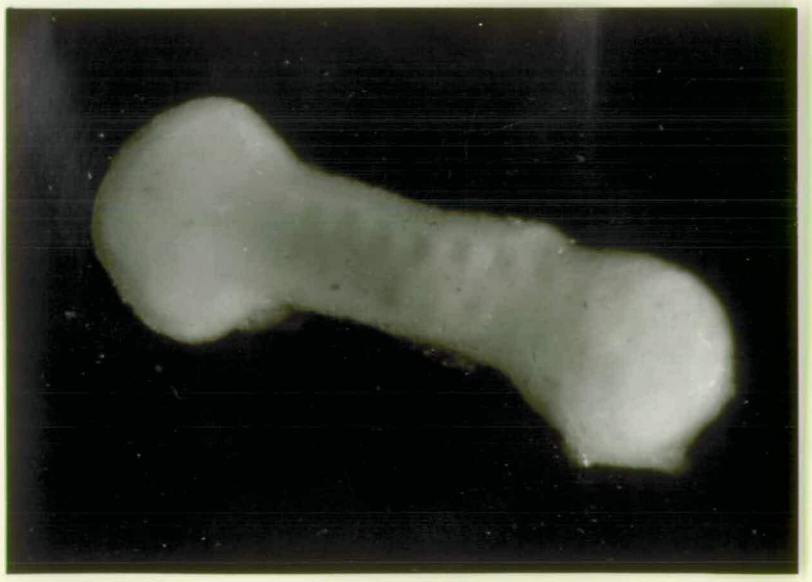


Fig. 40 : Mouse tendon prior to implantation.

Fig. 41 : Mouse tendon recovered encapsulated after 24 hours in P. americana. The striations are visible through the capsule.



an unrelated Dipteran evoked a response, as did vertebrate tissue. The cockroach N. cineria was selected because of its close phylogenetic relationship with P. americana. CORNWELL (1968) described the two cockroaches as different super families of the same sub order Blatteria. To determine the serological relationship between them, Nauphoeta whole plasma (prepared as described for Periplaneta) was analysed immunoelectrophoretically using rabbit anti-Periplaneta hemolymph. The result was a diffuse line (Fig xxxx) indicating almost no cross reactivity between the two hemolymphs. This could be relatively meaningless with respect to the 'degree of foreignness' of the implants, as it is not known how much the hemolymph constituents reflect those of the surfaces they bathe.

The homologous nerve cord implants carried out here are the equivalent of vertebrate homografts. The failure of the American cockroach to show a homograft reaction agrees with the results of SALT (1960) who, also using the encapsulation response, was unable to demonstrate the reaction in caterpillars. From the assessment of other insect transplant data by SALT (1961), it seems that the absence of a homograft reaction might be frequent among the insects.

C. IMPLANTATION OF ENCAPSULATED NYLON.

This technique involved the implantation on nylon, that had already been fully encapsulated with hemocytes, into another insect to see whether or not further encapsulation occurred i.e. were/

were the hemocytes being treated as foreign? SALT (1960) reported that nylon encapsulated for 72 hours in Diatarixia larvae and injected into another member of the same species, was not significantly further encapsulated except at points of damage or where the nylon had been exposed. Similar capsules from Tenebrio larvae implanted in Diatarixia doubled their thickness and multiplied in volume.

An experiment with Periplaneta and Nauphoeta is reported in detail. To ensure that hemocyte recruitment to the capsule had stopped, nylon that had been encapsulated for 7 days was used. Pieces of nylon were injected into adult Periplaneta and Nauphoeta and removed after 7 days. Great care was necessary to avoid damaging the capsules. They were then photographed in saline and carefully injected into adult Periplaneta. After 24 hours they were removed and re-photographed. The results are shown in contact print form in Fig. 42 . It must be remembered that the outlines of the capsules might not be exactly the same due to the pressure of the viscera of the second host. Also, the capsules are not symmetrical and the second photograph might show a different view.

Five of the six control capsules (Periplaneta capsules into Periplaneta), despite an obvious addition of hemocytes, had not been grossly re-encapsulated. A fifth showed a relatively gross accumulation of hemocytes at both ends, but the original capsule was extremely thin and possibly incomplete. A sixth showed/

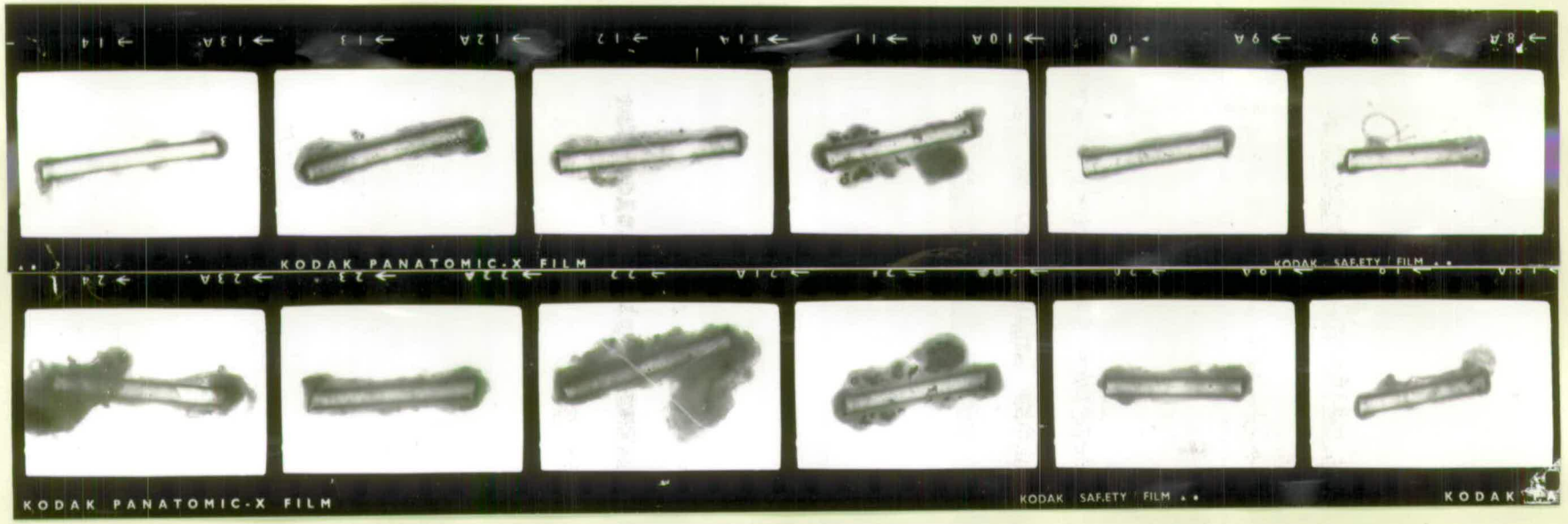
Fig. 42 : Implantation of encapsulated nylon.

A. Nylon encapsulated after 7 days in *P. americana*.

B. The same capsules (adjacent pictures) recovered after implantation for a further 24 hours in other *P. americana*.

C. Nylon encapsulated after 7 days in *N. cineria*.

D. The same capsules (adjacent pictures) recovered after implantation for 24 hours in *P. americana*.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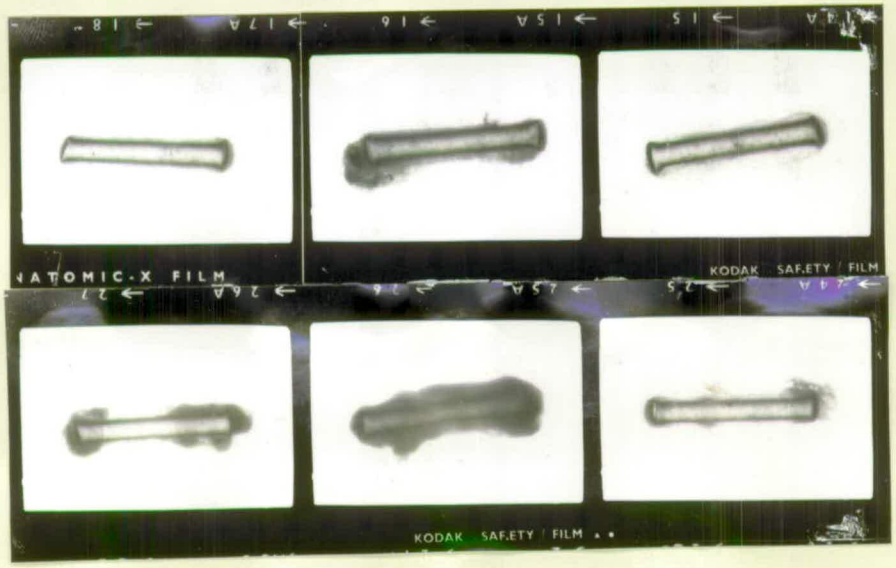
A

B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C

D

showed massive re-encapsulation for no apparent reason.

Of the six Nauphoeta that had been injected with nylon, three died within the 7 day encapsulation period. Of the remaining three capsules injected into Periplaneta, one case of gross re-encapsulation with an increase in both size and density, was noted. A second capsule had been grossly encapsulated except for the mid-part of the original capsule. A third capsule showed no signs of gross encapsulation, being similar to the majority of the control series above.

Further experiments using this technique gave inconsistent results with re-encapsulation of control capsules and neglect of hetero- capsules for no apparent reasons. Not even trends could confidently be described. Judging whether or not a capsule had been damaged or whether further encapsulation had occurred, was very difficult. Staining the original capsule with neutral red and methylene blue was unsuccessful as no colour was visible on recovery from a second host. (CHIRADELLA, 1965 had reported that staining homotransplants with methylene blue did not result in their being recognised as foreign by the starfish host).

The difficulties experienced in handling capsules without damaging them, and the problem of assessing re-encapsulation, along with the possibility that the capsule itself is more dynamic than assumed, most probably frustrated what, in principle, would seem to be a useful technique in studies of this kind/

kind.

D. ENZYME TREATMENT OF NERVE CORDS.

It has been established that the hemocytes of the American cockroach do not react against its own healthy tissue or implanted homologous nerve cords. The aim of the following enzyme treatments was to try to destroy the immunity of implanted homologous nerve cords and possibly learn something about the 'self signal' involved.

METHOD.

The following enzymes; collagenase, hyaluronidase, lecithinase-c, trypsin, lipase and neuraminidase (see Appendix III) were dissolved in saline at a concentration of 3% except for neuraminidase which was used at 2%. Sections of Periplaneta nerve cord were incubated in enzyme for 30 minutes at 37°C. They were then removed, washed in 15 ml. saline and implanted in adult Periplaneta. On recovery, 24 hours later, they were examined for signs of encapsulation. Control nerve cords were incubated in saline before implantation.

RESULTS.

Controls - Nine implants. All nine were unencapsulated. Two of these were completely free of hemocytes and the remaining seven showed slight aggregations of hemocytes at the cut ends. Two of these seven also had occasional single hemocytes along the edges.

Lipase/

Lipase - Six implants. All six were unencapsulated. Five were completely free save for slight associations of hemocytes at the cut ends. The remaining one showed slight associations of hemocytes at points other than the cut ends.

Hyaluronidase - Six implants. Five were unencapsulated save for association at the cut ends and occasional single hemocytes along the edges./ (Fig. 43). The remaining one had a dense aggregation of hemocytes at one end, attributable to damage which had been noted at the time of implantation.

Neuraminidase - Five implants. Three were completely clear of hemocytes. One of the remainder showed slight association at the cut ends, and the other had a dense aggregation of hemocytes at one cut end.

Collagenase - Eight implants. Nerve cords treated with collagenase were recovered intact, but had a slightly 'chewed' appearance. Only one was deemed to be unencapsulated and this had hemocytes at both cut ends and occasional single hemocytes sticking to the surface. None of the rest could be termed grossly encapsulated, in fact, in terms of nylon and hetero-transplant capsules, all were only slightly encapsulated. However, in all cases, there was a clear association of hemocytes with almost the whole surface of the nerve cords. As far as could be judged, the hemocyte layers were 1-2 cells thick, but in/

in one case the cord was recovered, thickly, and completely, encapsulated.

Lecithinase-c - Eleven implants. Only one was judged to be unencapsulated. Of the remainder, six were thickly encapsulated (Fig. 44) and the final four showed a clear association of hemocytes, 2-3 layers thick, almost over their entire surface. When this latter group were placed in a drop of saline on a glass slide, left for a minute or two and then removed, some free hemocytes and some associated to form a sheet, remained adhering to the glass (Fig. 45). These cells had obviously been only loosely associated with the cord, possibly because encapsulation was only in its early stages. It did, however, make possible examination of the cells. These took up neutral red and resembled the plasmatocyte type of the previous studies.

Trypsin - Six implants. Trypsin treated nerve cords again appeared slightly 'chewed'. Compared to the results obtained with other enzymes, where at least trends had been established, the results with trypsin were somewhat inconsistent. Two nerve cords were recovered completely free of hemocytes. One was grossly encapsulated and the capsule was removed and the nerve cord recovered. The remaining three had hemocytes, 1-2 layers thick, associated with their surfaces; one was completely covered, a second partially and the third at one end only.

Comment/

Fig. 43 : Hyaluronidase treated P. americana nerve cord recovered unencapsulated after 24 hours in P. americana.

Fig. 44 : Lecithinase-c treated P. americana nerve cord recovered encapsulated after 24 hours in P. americana.

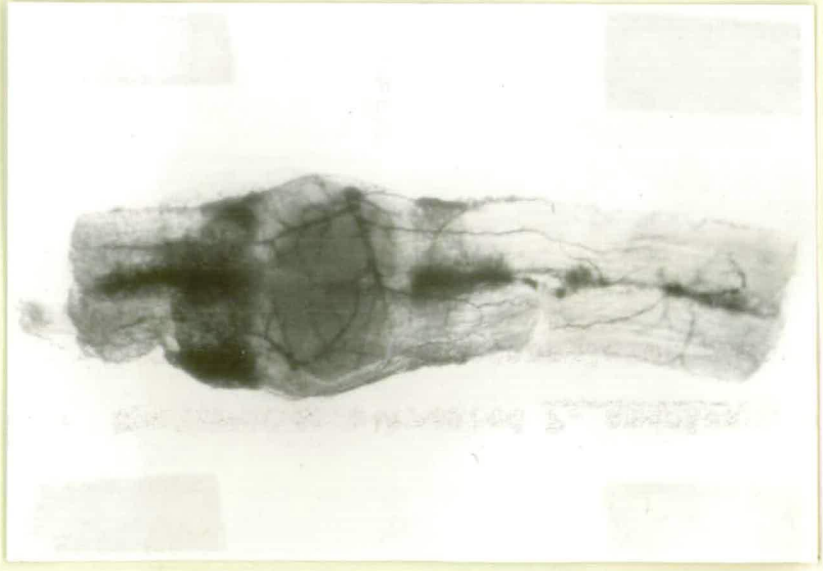
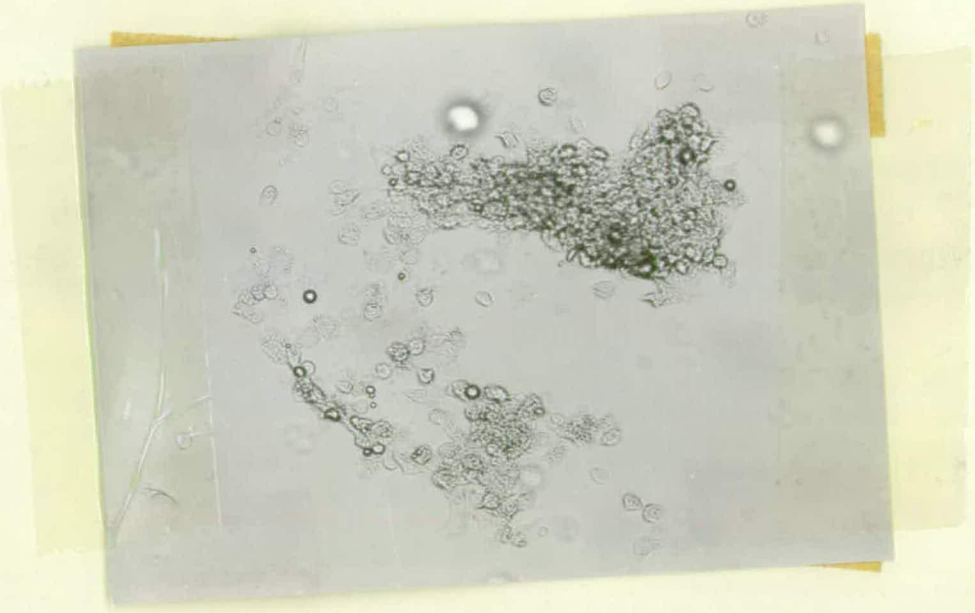


Fig. 45 : Hemocytes which have come away from the capsule surrounding an implanted lecithinase-c treated nerve cord.



Comment. The enzymes, lipase, hyaluronidase and neuraminidase, were without effect. Both trypsin and collagenase treated nerve cord showed signs of deterioration following enzyme treatment, but of these two, only collagenase treatment caused consistent hemocyte adherence. This result is interesting as other studies on Periplaneta americana suggested that the outermost structural layer of the nerve cord - the neural lamella - consisted of a collagen type protein with neutral mucopolysaccharide (ASHURST, 1961). In the same study she also noted that hyaluronidase appeared to have no effect on the neural lamella.

The enzyme which had the most prominent effect in these encapsulation studies was lecithinase-c. After such treatment the nerve cords showed no signs of deterioration. The result immediately suggested that the membrane (neural lamella) around the nerve cord was being disrupted, but ASHURST (1961) reported the neural lamella to have no lipid content detectable histochemically. However, RICHARDS & SCHNEIDER (1958) reported optical evidence to suggest the presence of lipid, histochemical tests again being negative.

The results of the enzyme treatments would seem to indicate the immunity of the implanted nerve cords is dependent on an intact neural lamella.

CHAPTER IV DISCUSSION

Throughout the reporting of the experimental work on the cockroach, it has been compared and contrasted where possible, with data from other insects and invertebrates. As stated at the outset, and evidenced in Chapter I and successive chapters, it is still not clear whether we are justified in generalising about immune mechanisms in a group as diverse as the invertebrates, except perhaps with respect to cellular responses. However, with our present lack of data, for some of the more general and speculative aspects of this discussion it will be necessary, and more informative, to do just this and assume a general invertebrate.

From the literature it seems that *in vitro* studies of invertebrate hemocytes, other than those concerned only with morphology, have been rare. Certainly previous *in vitro* studies of insect hemocytes have been mainly morphological, and nearly all demonstrations of their phagocytic capacity have been *in vivo*. Thus far, the experiments described in this thesis appear to constitute the first experimental study of insect hemocytes in an *in vitro*, hemolymph-free system. Washed cockroach hemocytes under these conditions were demonstrated to take up erythrocytes and fungal spores actively onto their surfaces. For the reasons outlined previously, this adherence was judged to be preliminary to phagocytosis of the particles. Uptake in the absence of hemolymph was considerable and experiments with hemocytes and hemolymph indicated/

indicated an apparent lack of any humoral factors associated with the process. These two findings contrast markedly with the generally serum-dependent phagocytic process in mammals.

No similar data are available for insects other than the cockroach, but JONES (1956) was unable to demonstrate in vitro any signs of phagocytosis by Sarcophaga hemocytes. Later, reviewing phagocytosis by insect hemocytes, he concluded that in vitro phagocytosis could not be demonstrated using the methods available at that time (JONES, 1962). GILLIAM & SHIMANUKI (1967) have since demonstrated in vitro phagocytosis of Nosema apis spores by honey bee (Apis mellifera) hemocytes in undiluted hemolymph, and the outcome of future studies with insect hemocytes in hemolymph free conditions is eagerly awaited.

Very few data are available on the performance of non-insect invertebrate phagocytic hemocytes in the absence of serum. Oyster hemocytes were capable of taking up erythrocytes in the absence of serum, although oyster serum was demonstrated to be opsonic for the process (TRIPP, 1966). No such uptake occurred with crayfish hemocytes (McKAY et al., 1969), and similarly STUART (1968), was unable to demonstrate uptake of erythrocytes by octopus hemocytes in the absence of octopus serum.

In retrospect, the choice of an insect as an invertebrate on/

on which to study hemocyte-hemolymph interaction may have, in one respect, been unfortunate. In general, untreated hemolymphs are highly toxic to insect tissues in vitro. This is due to melanisation on exposure to air and the resulting deposition of toxic phenolic compounds. The general practice to prevent this involves inhibiting the phenol oxidase activity by heating the hemolymph to 60°C before use (JONES, 1964). The hemagglutinating activity described here for the cockroach would be destroyed by such treatment, and so probably would many other hemolymph components. Visible deterioration of undiluted cockroach hemolymph was not evident until after about 4 hours at room temperature, and did not appear to be toxic for hemocytes when used experimentally during this period. Most of the hemolymph-hemocyte work was carried out with saline-extracted hemolymph which was stable for days at room temperature. How melanisation was prevented in this manner and whether it would work for other insects is not known.

The cockroach can now be added to the list of various invertebrates which have been demonstrated to have naturally occurring hemagglutinating activity in their hemolymphs. From our earlier considerations it appeared that the different hemagglutinins might be essentially similar and therefore possibly due to a common molecule. In this respect it is interesting/

interesting to compare two recent electron microscope studies of invertebrate hemolymphs. FERNANDEZ MORAN et al. (1968) examined the hemagglutinating fraction of Horseshoe Crab (Limulus polyphemus) hemolymph and demonstrated the hemagglutinin molecules to be ring shaped structures (diam∿100A) with hexagonal outlines. Similar ring structures (diam∿120-150A) appear in the micrographs of some insect hemolymphs (KAY et al., 1969). It is not known whether the insect hemolymphs concerned possessed agglutinating activity, but, since activity has been shown in the cockroach, an electron microscope search for such structures in cockroach hemolymph might be rewarding.

The demonstration of hemagglutinins in invertebrate body fluids was naturally of interest to those people seeking 'invertebrate antibodies' - but what resemblance to antibodies do these invertebrate substances in fact have? Although they are proteins and show a degree of specificity, they are not gamma globulins, are not increased after injection of red cells and do not fix complement.

Detailed investigation of the hemagglutinin of the crab, Limulus polyphemus, by MARCHALONIS & EDELMAN (1968) showed that in many respects it differed structurally from vertebrate immunoglobulins. Only one class of subunit on the basis of molecular weight was found and the subunits were not joined covalently in the intact molecule. Furthermore, the subunits did not show the starch gel electrophoretic heterogeneity/

geneity characteristic of the polypeptides of antibody. Both the amino acid composition and peptide maps were different and although, until amino acid sequence comparisons have been made, one cannot completely exclude the possibility of some relationship, the conclusion was that the hemagglutinin and antibody molecules were unrelated evolutionary developments.

If structurally they bear little resemblance to immunoglobulins, do they then serve any immunological purpose in the invertebrate? Or does their existence mean no more than that any soluble protein may carry a sequence of amino acids which confer physical properties of similar quality to those of an antibody? The degree of specificity could be accounted for by simple polymorphism, any affinity other than for self components having no selective value. Cockroach hemagglutinating activity could be ascribed no immune function, and indeed so far, none of the hemagglutinating activities described have been demonstrated to cross react with microorganisms likely to be encountered in nature. An immunological role has recently been ascribed to the hemagglutinins in the oyster and cray fish, where they appear to be opsonic for the uptake of red cells by the respective hemocytes (TRIPP, 1966; McKAY et al., 1969). This is directly analogous to the activity of some mammalian antibodies and the widespread occurrence of, possibly similar, opsonins in a variety of invertebrates would be very interesting. No such activity was demonstrable/

demonstrable with cockroach hemocytes and hemolymph, and the outcome of similar experiments with other insects and non-insect invertebrates is awaited.

Before attempting to reconcile my findings in the cockroach with current ideas about the recognition mechanisms of invertebrate phagocytes, the question "What constitutes foreignness to an invertebrate?" raised in Chapter I, deserves final comment.

First, a note on technique. Much of the invertebrate transplant data has come from grafting experiments. Tissue grafting in invertebrates is still in its infancy, and exactly how much is actual immunological rejection and what part is played by non-specific inflammation and wound healing processes, is not understood. The insect capsulation studies, on the other hand, involve the implantation of tissue with intact surfaces which circulates freely in the hemolymph. No host damage is involved, and what is more, the encapsulation response is known to be an immune defence mechanism against invading organisms.

The failure of some invertebrates to discriminate between auto and homografts has been described in the Introduction. So too has the rejection of second set homografts by earthworms (DUPRAT, 1964). However, generalising from the available data, it would seem that invertebrates are not capable of such a fine degree of discrimination as the vertebrates. An alternative to this could be that it is not a failing on the part/

part of the invertebrate recognition system, but that the individual differences between invertebrates within a species are not as clearly defined as in vertebrates - nothing is yet known about invertebrate histocompatibility antigens. It may be surmised that some populations of land invertebrates, with low mobility and high reproductive capacity, might be subject to considerably more inbreeding than their vertebrate counterparts. In this respect it is interesting to note that DUPRAT (1964) found that homografts between earthworms from different geographical regions of France were treated as heterografts. The cockroaches used in this study, although trapped as adults, were from the same building. Although this reasoning might apply to the cockroach and the earthworm it would be difficult to reconcile it with many other invertebrates e.g. marine invertebrates where the gametes are seaborne.

BURNET (1968, 1969) has recently speculated as to how the vertebrate immune mechanism might have developed during the course of evolution, and in doing so touches on a possible origin of histocompatibility antigens. Vertebrates are known to be prone to cancer and malignant diseases, and Burnet adopts the suggestion of THOMAS (1959), that the vertebrate adaptive immune system was not initially concerned with defence against infections, but arose from the need for dealing with 'inconsistencies' in the cellular integrity of the body, i.e. foreign antigens appearing as a result of somatic mutation or equivalent chromosomal/

chromosomal process. In its primitive form, the mechanism for the recognition and destruction of these foreign cells is envisaged as purely cellular (see below) and the development of antigenic heterogeneity (histocompatibility antigens) is seen as being complementary to such a system. For example - if, in a vertebrate population, all individuals were alike, then a tumour might rapidly spread through the population by contagious transfer. It would affect not only the old, but also those who had not yet attained reproductive age. However, if there were strong histocompatibility differences between individuals, with an immune system designed to seek and destroy 'nonconformist' cells such a transfer would not occur. BURNET (1969) also points out that histocompatibility differences are genetically based and presumably arose by mutation. This being the case, he suggests that there might even be selection towards a 'non-lethal' hypermutability of the histocompatibility locus.

What then about the invertebrates? Are we to assume that they are not prone to such malignancies? HARKER (1958) describes the histology of tumours in American cockroaches following implantation of sub-oesophaleal ganglia from cockroaches conditioned to a reversed light-dark cycle. The tumours showed metastasis and, when transplanted into normal cockroaches, although encapsulated they induced tumours in these secondary hosts. Other examples of invertebrate tumours can be found in a review by HARSHBARGER (1967) of invertebrate responses to various/

various vertebrate carcinogens. In brief, some invertebrates showed no detectable cellular responses to various agents, whilst others did. In many cases the responses induced manifested themselves quite differently from those in vertebrates. Thus, although comparatively little is known about invertebrate neoplasms, they do appear to exist. It is of course true that with a large, long-lived animal whose maintenance is dependent on a large turnover of cells, somatic mutation might be a very real source of potential danger, whereas this would be considerably reduced in small, short-lived invertebrates. What is not true is that all invertebrates are small and short-lived. The comparison might well be between a mouse and a giant squid or octopus.

It might be that invertebrate tumours are strong'antigens' easily recognised and dealt with by the hemocyte response; the hereditary melanotic tumours of Drosophila melanogaster (RIZKI, 1957; HARSHBARGER, 1967) are melanotic masses of hemocytes associated with a tissue. The formation and appearance of these is similar to the hemocyte response to foreign and wounded tissue, and it is presumed that the initiation is due to a change in the tissue rendering it foreign to the hemocytes (HARSHBARGER, 1967). The suggestion is of course mere conjecture, but certainly this is a field of invertebrate immunity, as yet unexplored, and potentially relevant to the evolution of the vertebrate immune response.

In/

In a serum-dependent phagocytic system, the lack of reactivity against self components can readily be explained by lack of serum components directed against such material. My findings with the cockroach cannot be reconciled with such a system and an alternative hypothesis is considered here.

A whole range of objects has been implanted in insects, and nearly all are reacted against and coated with hemocytes. In general the only surfaces that do not attract a hemocyte response are the unbroken internal tissue surfaces of the insect itself, undamaged homotransplants and normal parasites. SALT (1960) suggested that the reason these surfaces did not excite the hemocytes was because they were protected from them in some way, possibly by a substance laid on them by the hemocytes (such substances would be mimicked by successful parasites). Although the encapsulation response was primarily the subject of discussion, the first stages of this are considered to be a phagocytic response, and Salt's suggestion is interesting in the light of the apparent lack of any humoral intermediaries in phagocytosis by cockroach hemocytes. The implication is that phagocytosis by insect hemocytes is a non-serum-dependent process and that the hemocytes will react to any particle except where they are signalled not to do so by a 'self' substance. In comparison, the mammalian phagocyte is unreactive until activated by a serum component directed against the foreign substance. In the first case there would be no discrimination between different foreign particles. All differences/

differences in uptake would presumably be due to inherent differences in susceptibility to phagocytosis, related for example to surface charge.

A problem with such a hypothesis concerns the nature of the self signal. It is difficult to envisage what properties would cause two surfaces to repel one another. Electrostatic repulsion seems improbable under the circumstances and it may merely be that the cells' surfaces have been evolved to be dynamically stable to any other surface or soluble component that they encounter normally.

There is some evidence that the relationship between insect hemocytes and the internal tissue surfaces might be more than mere co-existence. Almost all insect tissues, including the epidermis, are separated from the hemocoele by a loose neutral polysaccharide layer - the basement membrane, and it is with this that the hemocytes normally come into contact. SALT's (1960) original suggestion that this was coated with a substance laid on it by the hemocytes was stimulated by the work of WIGGLESWORTH (1956) who reported that during the later stages of moulting in Rhodnius, the amebocytes (plasmatacytes) liberated the muccopolysaccharide material which formed, or contributed to, the connective tissue and basement membrane. Autoradiographic studies by SHRIVASTAVA & RICHARDS (1965) in Galleria mellonella showed that whilst hemocytes play some role in the destruction of the larval neural lamella (basement membrane) they are not involved/

involved in the formation of the connective tissue sheaf around the adult nervous system. Hemocytes gather at breaks in the internal tissue surface sealing off wounds in a manner similar to encapsulation. The finer details of wound repair in insects are not fully understood, but restoration of the integument is not complete without the renewal of the basement membrane. Once again there is controversy over whether the new membrane is secreted by the hemocytes (WIGGLESWORTH, 1937) or the epidermis (LAI FOOK, 1968).

Whether the above examples of possible, prior hemocyte - basement membrane interaction might in some way be related to the existing immunity of the membrane from hemocyte attack, is not known.

The experimental data on which the above reasoning has been based have all come from insects. However, such an approach to the recognition problem is interesting when considering invertebrates in general. To summarise, the concept represents the invertebrate phagocyte as something very like an ameba, independently and indiscriminately taking up all foreign material and ignoring only self. Such an approach naturally conflicts with BURNET's (1968) postulate of a humoral recognition factor as do my data with those which have subsequently seemed to favour Burnet's idea (see Part Chapter I). As yet, experimental support for either approach is such that both must be considered/

sidered highly speculative. It is difficult to reconcile Burnet's suggestion of monospecific hemocytes with some of the smaller, more primitive invertebrates. The number of specificities required to deal with potential foreign particles might well approach the total number of hemocytes. Also, Burnet postulates that cell division does not occur following hemocyte-particle contact. Even if the hemocytes were non-specific and did not produce the recognition factors themselves, but merely became passively coated with them, the same number of specificities would need to exist in the hemolymph. Not only does this seem to contradict the low specificity of invertebrate humoral substances reported to date, it is again difficult to reconcile this with the sheer simplicity of some of the more primitive animals.

In the sponge, which has no definite digestive apparatus, the wandering phagocytes of the mesoglea, in addition to phagocytosing offending material have also retained a nutritive function, taking up food particles. Such circumstances would seem to favour an indiscriminate uptake of all foreign material.

It seems that, in some sense, the invertebrate phagocytic hemocyte must be ancestral to the vertebrate phagocyte. If, as reasoned above, the primitive hemocyte is ameboid in nature, then the evolutionary trend might have been one of increased serum dependence. What may have started as a mere facilitation of the phagocytic process by opsonins (possibly non specific) culminated/

culminated in the almost completely serum dependent mammalian system with its highly specific opsonins. Such an evolutionary trend might be expected to be reflected within the invertebrate line and thus the two approaches to the invertebrate recognition problem should not be considered mutually exclusive. Indeed, as a guide to further research, both seem to be useful working hypotheses, which readily lend themselves to experimentation.

Most interest in invertebrate immunity has been, and, to a large extent, still is, from an evolutionary viewpoint. Are we able to detect amongst the invertebrate immune mechanisms, the raw material which evolution could have fashioned into the vertebrate adaptive immune response?

We must first generalise and consider what constitutes an invertebrate immune response. The primary defence mechanism is a cellular one. Foreign particulate matter is rapidly and efficiently removed from the hemolymph by phagocytosis or encapsulation, and it is assumed that soluble material is pinocytosed with equal efficiency. The hemocytes responsible have at least a limited capacity to recognise foreigness. Invertebrates do not possess gamma globulins, but some substances with antibody-like activity (hemagglutinins etc.) are found in some invertebrate hemolymphs. As yet it is uncertain whether such substances play an immune role. Both natural/

natural and acquired anti-bacterial principles so far recorded have been found to be of low specificity. There is no activity against many of the soluble proteins (e.g. HSA, BSA) known to be immunogenic in invertebrates. Indeed, so far, most of the immune substances described would be expected to have a direct selective value for the survival of the animal i.e. are directed against potential pathogens. No direct selective value can be attributed to the naturally occurring hemagglutinins until they have been shown to have activity against bacteria, or that their reported opsonic activity applies to the uptake of micro-organisms as well as erythrocytes.

Do any of the immune substances described in invertebrates qualify as the primitive building blocks of the immunoglobulins of vertebrates? BURNET (1968) favours the substances with 'pseudo-antibody' like activity as candidates for such 'protoglobulins', but structural analysis of a hemagglutinin has since shown it to have little or no affinity with immunoglobulins as we know them today. No equivalent information is available for the substances known to play an immune role and it seems that they may be diverse in nature throughout the invertebrates. In insects at least the antibacterial principle is known to be non-protein. There is of course no real necessity that any such 'protoglobulins' should have a function similar to their evolutionary products. Indeed, component parts of the immunoglobulin molecule may have existed/

existed separately until joined under the influence of an organised lymphoid system. A search for such building blocks, e.g. isolated L-chains or any small proteins with adsorptive properties, could possibly prove productive.

BURNET (1968, 1969) has also speculated as to how, at the cellular level, the vertebrate immune response may have arisen from the invertebrates. He reasons that, if the primary function of the mechanism which was later to develop into adaptive immunity was to recognise and remove body cells which had become recognisably foreign, then the earliest manifestations of this function would be expected to be wholly at the cellular level. The process of active antibody production is presented as having all the marks of a later addition to the more primitive cell-based systems concerned with delayed-type hypersensitivity and homograft rejection. The delayed hypersensitivity reaction is represented as a primitive surveillance mechanism, based solely on cell-to-cell associations and designed to seek and destroy cells with aberrant surfaces properties. The recognition mechanism of the primitive immunocyte is visualised as involving a modified globulin on the immunocyte surface, capable of recognising an unfamiliar pattern on the surface of another cell. Making the assumption that the evolutionary precursor of the vertebrate immunocyte is an invertebrate phagocytic hemocyte with a crude, but definite, capability/

capability of recognising foreigness based on the same principle, there is little difficulty in deriving the adaptive response from the essentially non-specific, non-adaptive invertebrate response. Only two major changes would be required at the cellular level to result in the essential features of an adaptive response; a) an increased flexibility in the part of the genome concerned with coding of the protoglobulins, to provide greater diversity, must be developed; and b) contact of foreign particles and recognition globulins should result in proliferation of the hemocyte concerned, with retention of its specificity throughout the descendant clone (BURNET, 1968).

We have clearly already moved too far, too rapidly into the realm of speculation, and further comment should await further data. With regard to future research, some approaches at both the humoral and cellular levels have already been outlined. Only a systematic study of all the invertebrate groups will reveal how general the various defence mechanisms are, but, for the present at least, the studies of those interested in the evolution of the invertebrate immune response, should be restricted to invertebrates of considered evolutionary significance. There is often a tendency for work on invertebrates to be compared with various aspects of the mammalian immune response, and it should be remembered that the latter is itself the result of evolution within the vertebrate line. Possibly/

Possibly the expansion of work on the primitive immune systems of early chordates, especially cellular immunity, might reveal some clues. In this respect Amphioxus seems to be a much neglected animal. It may yet, however, turn out that the search for the invertebrate origins of the vertebrate immune response may have been confounded by evolution itself, in that, in its present state, no invertebrate can be considered ancestral to the vertebrates.

SUMMARY

The literature concerning invertebrate immunity has been reviewed with particular emphasis on recent developments in a field which is currently being reconsidered in the light of current immunological thinking.

The animals used throughout the experimental work described in this thesis were adult American cockroaches (Periplaneta americana L.)

1. Hemolymph Studies.

Undiluted cockroach hemolymph, obtained using a centrifugation technique, underwent melanisation if left at room temperature for more than 4 hours. Using a saline extraction technique, hemolymph was obtained more easily, and such extracts were completely stable at room temperature for several days.

Simple electrophoretic comparison of hemolymph with mouse serum showed that no cockroach proteins migrated in the γ globulin region, the bulk of them migrating in the fast α and slow β regions. Immunoelectrophoretic analysis (IEA) of hemolymph revealed six distinct protein arcs. The antigenic components were further characterised with respect to lipid, saccharide and esterase content.

The hemolymph was found to possess strong hemagglutinating activity for a wide range of vertebrate erythrocytes. Cross absorption/

absorption studies indicated the presence of hemagglutinins with a degree of specificity for different erythrocytes, although a marked degree of cross reactivity was evident. No specificity with respect to human blood group antigens (ABO) was found.

Hemagglutinating activity was further characterised with regard to the characteristics of both some naturally occurring hemagglutinins which have been reported in other invertebrates, and of mammalian antibody. Experiments indicated that cockroach hemagglutinin is, or is bound to, a heat labile (56°C), non-dialysable protein which behaves as a euglobulin. It did not fix mammalian complement and hemagglutinating titres were not increased following prior 'immunisation' with sheep red cells. Overall, the hemagglutinating activity found in cockroach hemolymph appears to resemble that described in the hemolymphs of some other, non-insect, invertebrates. The extreme lability described for other insect hemagglutinins so far examined was not found with the cockroach.

It was established that this hemagglutinin was either itself antigenic, or associated with an antigenic protein, and attempts were made to establish its identity. Complete removal of activity by absorption did not result in the loss of any antigen arcs. It was found that nearly all the activity was precipitated with 25% ethanol, and was associated/

associated with the slowest moving antigenic arc, which contained lipid, saccharide and esterase activity.

Cockroach hemolymph was also found to agglutinate mouse sperms. Absorption with sheep red cells removed sperm agglutinating activity, implying the identity of sperm and red cell agglutinins.

Screening of hemolymph against various species of yeast and bacteria revealed an agglutinin directed against Aerobacter aerogenes. This activity was heat stable at 56°C and was considered to be different from the hemagglutinating activity.

2. Hemocyte Studies.

Cockroach phagocytic hemocytes (plasmatocytes) were studied during short term, in vitro culture in hemolymph-free and serum-free conditions. Sheep and chicken erythrocytes, and also fungal spores, were absorbed onto the surface of the hemocytes. Uptake of particles was not attributable to passive sticking, but was a temperature dependent process utilising metabolic energy. This adherence was interpreted as being preliminary to ingestion - intracellular particles were only occasionally observed. This marked uptake in the absence of humoral factors contrasts strikingly with the generally recognised serum dependence of most phagocytic activity in mammals. Most hemocytes were capable/

capable of taking up spores and erythrocytes simultaneously.

Hemocytes were treated with various enzymes and reactive chemicals in attempts to elucidate the nature of the 'receptor site' for red cells on the hemocyte surface. Trypsin treatment almost completely abolished red cell uptake, suggesting that a membrane protein was involved.

It was not possible to restore activity to trypsin-treated hemocytes by incubation in normal hemolymph. The site was also 2-mercaptoethanol sensitive.

Recent studies have shown that naturally occurring hemagglutinins in some invertebrates are opsonic for phagocytosis of red cells by hemocytes in vitro. Similar studies undertaken with cockroach hemocytes and cockroach hemagglutinin have shown no such activity.

Hemocytes incubated in a rabbit anti-sheep red cell serum and washed, showed a pronounced increase in red cell attachment. Metabolically-inhibited hemocytes showed decreased serum fixation. Pre-incubation of hemocytes with rabbit serum albumin did not reduce the amount of antibody fixed, suggesting the possibility of a specific uptake of γ globulin. However, hemocytes showed no preference for red cells presensitised with antibody, and it was concluded that the hemocyte surface does not possess receptor sites for γ globulin.

3/

3. Encapsulation Studies.

The encapsulation response to nylon monofilaments implanted in the cockroach hemocoele was studied and found to conform in general with encapsulation responses of other insects.

Attempts to dissociate these capsules into their component cells by various enzyme treatments were unsuccessful, as were attempts to demonstrate encapsulation in vitro.

Implantation of large doses of nylon showed that the encapsulation response is capable of dealing with a considerable challenge.

No encapsulation of nerve cords implanted between individual American cockroaches occurred. Interspecific implants between two species of cockroach were recognised as foreign and encapsulated, as were implants from an unrelated Dipteran, and from mice. One possibility was that homologous nerve cords contained a 'self' component which protected them from hemocyte attack. Attempts were made to render these cords susceptible to encapsulation by prior treatment with various enzymes. Both collagenase and lecithinase-c treated cords were encapsulated, these results being consistent with the idea that an intact neural lamella is required for avoidance of the hemocyte reaction.

The findings in this Thesis have been discussed in the light of other relevant invertebrate data. In particular, the/

the problem of what constitutes 'foreignness' to an invertebrate has been considered. Also, the possible mechanisms whereby the phagocytic hemocyte discriminates between 'self' and 'non-self' material have been considered, with special regard to the apparent lack of humoral factors involved in phagocytosis by cockroach hemocytes.

ACKNOWLEDGEMENTS

This work was carried out during the tenure of a Research Studentship from the Science Research Council. It was supported by a research grant from the Medical Research Council (No. 965/300/B) to Dr. J.G. Howard.

I should like to thank my Supervisor, Dr. H.S. Micklem, for his guidance and encouragement, and also for the many stimulating discussions during the course of the work.

Thanks are also due to Dr. J.G. Howard for his helpful advice, and to Norah F. Simpson for her amiable co-operation and excellent typing.

APPENDIX I

Cockroach Ringer (after BECHT et al., 1960)

9.32 gm. NaCl	dissolved in 1 litre distilled H_2O.
0.77 gm. KCl	
0.50 gm. $CaCl_2$	
0.18 gm. $NaHCO_3$	
0.01 gm. NaH_2PO_4	

APPENDIX II

STAINING TECHNIQUES.

a) Protein :

1 gm. Naphthalene Black/Ponceau Red was dissolved in 450 ml. sodium acetate. 60 ml. 1N acetic acid were added, followed by distilled H_2O to make up to 1000 ml. mark. Slides were immersed in Naphthalene Black or Ponceau Red stain for 3-4 hours or 10-20 minutes respectively.

1 gm. Lissamine Green was dissolved in a litre of distilled H_2O. Slides were immersed for 30 minutes.

b) Glycoprotein :

Reagents :- A. Periodic acid, 1% in 0.2M sodium acetate.

B. Phenylhydrazine-HCl, in acetate buffer pH 4.0 (mix 2M sodium acetate and 8M acetic acid in equal amounts).
Use this reagent within 24 hours.

C. Acetate buffer as in B.

D. Diazo Blue B (terazotised c-dianisidine), 0.1% in 1M sodium acetate. Prepare immediately before use.

E. Copper acetate saturated in 1M sodium acetate.

Method/

(Appendix II continued).

Methods :-

1a. For simple electrophoresis slides, fix in 50% ethanol containing 2% acetic acid.

1b. For immunoelectrophesis slides, wash thoroughly in saline.

1c. In either case dry under filter paper at 37°C.

2. Reagent A - 10 minutes.

3. Running water - 10 minutes.

4. Reagent B (heated to 60-70°C) 5 minutes.

5. Reagent C - 5 minutes.

6. Reagent D - 10 minutes.

7. Running water - 10 minutes.

8. Reagent E - 5 minutes.

9. Running water - 5 minutes.

A stable blue-violet colour indicates polysaccharide.

c) Lipoprotein :

Colourant :- Sudan Black saturated in 60% alcohol. Weigh out 300 mg./litre and leave 1-2 days at 37°C in a brown vessel. Before use add 0.1 ml. concentrated NaOH/50 ml.

Method/

(Appendix II continued).

Method :- Place two fragments of wood (match sticks) on a flat glass plate and put the dried slides face downwards upon them. Introduce the colourant underneath the slide by means of a pipette, tapping the surface to avoid bubble formation. Fill up periodically to compensate for evaporation. Rinse in two baths of 60% ethanol (few minutes each) after colouring for 2 hours.

d) Non specific esterase :

1. Dissolve 5 mg. β naphthyl acetate in 0.5 ml. acetone.
2. Add 25 ml. phosphate buffer (0.15M) at pH 7.5. This yields a cloudy solution.
3. Put in a flask containing 10 mg. diazo blue B and filter.
4. Incubate slide in colourant at room temperature until development is complete (less than 15 minutes).

N.B./

(Appendix II continued).

N.B. Immunoelectrophoresis slides are stained after drying. Simple electrophoresis slides are stained before fixing and drying. Prior to staining they are brought to the correct pH by a preliminary wash in buffer. After staining they are fixed as usual in 2% acetic acid.

APPENDIX III

Enzymes.

Hyaluronidase from ovine testes. Sigma type III.

Lecithinase-c from Cl. welchii. Sigma type I.

Collagenase from Cl. histolyticum. Sigma type I.

Trypsin from bovine pancreas. Sigma type III.

Lipase from wheat germ. Sigma type I.

Neuraminidase from Cl. perfringens. Sigma type VI.

APPENDIX IV

a) Formalinisation of sheep red cells.

1. x ml. red cells were washed in saline and made up to 8x ml. in buffered saline.
2. A dialysis bag was 2/3 filled with 2x ml. 40% formaldehyde. Air was removed and the bag was sealed.
3. The dialysis bag was added to the cell suspension and the suspension was shaken for 5 hours at room temperature.
4. After 5 hours the dialysis bag was punctured and the formalin allowed to escape into the cell suspension. The bag was removed.
5. The suspension was left for 12 hours with gentle stirring.
6. The cells were washed and resuspended in saline, and then stored in the refrigerator.

b) Tanning of formalinised sheep red cells.

0.15 ml. packed cells were washed twice in buffered saline (pH 7.2) and finally resuspended in 5 ml. buffered saline. 5 ml. Tannic acid (5 mg. in 50 ml. buffered saline) was added to the cell suspension and well mixed before incubation in a 37°C water bath for 15 minutes. Finally the cells were spun down and washed twice in saline, resuspended in buffered saline and stored in the refrigerator.

APPENDIX V

Uptake of I^{131} labelled hemolymph by tanned sheep cells.

(I am indebted to Mr. W.H. McBride for assistance and advice during these labelling experiments).

Hemolymph : Hemolymph in phosphate buffered saline (pH 7.2) was prepared in the same way as the saline extracts.

Radio-Iodine : Carrier free NaI^{131}. Radiochemical Centre, Amersham, Buckinghamshire.

Labelling : 1.5 mc. NaI^{131} was placed in a bijou bottle and 0.2 ml. buffered saline added with continuous stirring. 1.5 ml. hemolymph was then added and, immediately afterwards, 0.2 ml. Chloramine-T. After stirring for 1 minute another 1.0 ml. of buffered saline was added and the contents of the bottle transferred to a dialysis bag. The bottle was rinsed with a further 1.0 ml. buffered saline which was added to the contents of the bag. Dialysis against buffered saline was for 48 hours with four changes of dialysate. After dialysis, counts were made using a Nuclear Enterprises/

(Appendix V continued).

Enterprises Scintillation Counter with a 2" NaI well type crystal. The label was found to be 83% TCA precipitable and the specific activity of the hemolymph was calculated as 0.6 µc/mg.

Uptake : I^{131} hemolymph was absorbed twice with packed formalinised red cells to remove agglutinating activity. 0.5 ml. absorbed I^{131} hemolymph was incubated with 0.5 ml. tanned red cell suspension (5% in buffered saline) for 30 minutes at room temperature. Cells and supernatants were then counted during three washes with buffered saline - see over.

Series A/

(Appendix V continued).

	Wash	Counts/10 sec.	% label associated with cells
Series A	0	S - 370979 C - 18460	4.7
	1st	S - 6854 C - 5952	46.5
	2nd	S - 508 C - 4377	89.6
	3rd	S - 177 C - 4181	95.9
Series B	0	S - 371931 C - 18675	4.8
	1st	S - 5985 C - 7148	54.4
	2nd	S - 574 C - 4465	88.6
	3rd	S - 148 C - 4095	96.5

S = Supernatant

C = Cell pellet

APPENDIX VI

Ammonium sulphate precipitation of γ globulins.

One volume of saturated ammonium sulphate was added to two volumes of serum. Addition was drop wise at room temperature with constant stirring. The mixture was allowed to stand for 30 minutes at room temperature and then centrifuged. The pellet was washed twice with 40% saturated ammonium sulphate, it finally being redissolved in a volume of 0.85% saline equivalent to the original volume of the serum.

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